UNIVERSITÉ BORDEAUX SEGALEN

UNIVERSITÉ DE BORDEAUX

Année 2012

Thèse n° 1945

THÈSE

pour l'obtention du

DOCTORAT DE L'UNIVERSITÉ BORDEAUX 2

École doctorale Sciences de la Vie et de la Santé

Mention : Sciences, Technologie, Santé

Option : Génétique Moléculaire et Cellulaire

Les microARNs régulateurs de l'expression génique du Glypican-3 dans le Carcinome Hépatocellulaire

Présentée et soutenue publiquement

Le 21 Novembre 2012

Par Marion MAUREL

Née le 30/11/1986 à Clermont-Ferrand (63)

Membres du Jury

Mme Corinne CLAVÉ .....Présidente du jury
Mr Bernard MARI.....Rapporteur
Mr Éric OGIER-DENIS .....Rapporteur
Mr Jérôme CAVAILLÉ .....Examinateur
Mr Fabien DARFEUILLE .....Examinateur
Mr Christophe GROSSET.....Directeur de thèse
Mr Éric CHEVET.....Membre Invité

REMERCIEMENTS

Mes travaux de thèse ont été réalisés sous la direction de Christophe Grosset dans l'unité INSERM U1053 dirigée par Jean Rosenbaum que je remercie de m'avoir accueillie au sein de son laboratoire.

Je tiens à exprimer toute ma gratitude à l'ensemble des membres du jury, Corinne Clavé, Bernard Mari, Eric Ogier-Denis, Jérôme Cavaillé, Fabien Darfeuille, Christophe Grosset et Eric Chevet pour avoir accepté d'évaluer ce travail.

J'aimerai témoigner toute ma reconnaissance à Christophe, Eric, Hélène, Chantal et Sarah pour leur aide précieuse, leur soutien et leurs conseils pour la rédaction de ce manuscrit.

Mes pensées vont également vers Aude, Amélie, Nicolas, Arnaud, Stéphanie, Audrey, Aurélien, Sandra, Esther, Sandrine, Lisa, Daniela, Saïd, Violaine, Frédéric, Patrick, les 2 Nathalie, Véronique, Anne-Aurélie, Francis, Arisa, Valérie, Virginie, Lucie, Hélène, Franck, Paulette et Charles, pour l'ambiance sympathique, les bons moments partagés et la bonne humeur qui règne au sein de l'unité.

TABLE DES MATIERES

LISTE D'ABBREVATIONS

A
ADAM : a disintegrin and metalloprotease domain
AFP : alpha-fetoprotein
AFR : alpha-fetoprotein regulator
AGO : argonaute
AKT : v-akt murine thymoma viral oncogene homolog
APC : adenomatous polyposis coli
ATF6 : activating transcription factor 6
ARF : ADP-ribosylation factor
ARID1A : AT rich interactive domain 1A
ARN : acide ribonucléique
ARNA : activation ARN
ARNm : ARN messager
B
BCL2 : B-cell CLL/lymphoma 2
BIM : BH3-only group of Bcl-2 family members
BMP : bone morphogenetic protein
C
CAF1 : CCR4-associated factor 1
CDH1 : E-cadherin
CDK6 : cyclin-dependent kinase
CDKN1A : cyclin-dependent kinase inhibitor 2A
CK1 : casein kinase 1
C-KIT : v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CHC : carcinoma hépatocellulaire
CHOP : DNA-damage-inducible transcript
CHP1 : chromodomain protein 1
CCR4NOT : C -C chemokine receptor type 4
CLR : histone H3 methyltransferase
CCNB1 : cyclin B1
CSDC2 : cold shock domain containing C2
CTNNB1 : β-catenin
D
DALLY : division abnormally delayed
DCR1 : Dicer 1
DCP : decapping enzyme
DGCR8 : DiGeorge syndrome critical region gene 8
DLY : Dally-like
DND1 : dead end 1
DVL : disheveled
E
4EBP1 : Eukaryotic translation initiation factor 4E-binding protein 1
EF1A : eukaryotic elongation factor 1A
EGFP : enhanced green fluorescent protein
EIF : eukaryotic translation initiation factor
ERSE : ER Stress Response Element
EXT : exostosin
EZH2 : enhancer of zeste homolog 2
F
FGF : fibroblast growth factor

FUNREG : functional, integrated, and quantitative method to measure post-transcriptional regulations
FXR1A : fragile X mental retardation, autosomal
FZD : Frizzled

G

GADD : growth arrest and DNA damage
GAG : glucosamino-glycan
GPI : glycosylphosphatidylinositol
GPC3 : glypican-3
GRP78 : glucose-regulated protein 78kDa
GSK3 : Glycogen synthase kinase- 3
GW182 : glycine(G)-tryptophan(W) repeats 182

H

HBX : hepatitis B virus X protein
HH : Hedgehog
H3K9ME : méthylation des histones H3 sur la lysine 9
H3K27ME : méthylation des histones H3 sur la lysine 27
HME : Hereditary Multiple Exostoses
HP1 : heterochromatin protein 1
HUR : human antigen R
HSPG : heparan sulfate proteoglycans

I

IGF1R : insulin-like growth factor 1 receptor
IL : interleukin
IRE1α : inositol requiring enzyme-1 alpha

K

KBRAS2 : NFKB inhibitor interacting Ras-like protein 2
KLF4 : Kruppel-like factor 4
KRAS : v-Ki-ras2 Kirsten rat sarcoma viral

L

LIN28 : lin-28 homolog
LLC : leucémies lymphoïdes chroniques
LRRK2 : leucine-rich repeat kinase 2
LRP : low density lipoprotein receptor

M

MDM2 : Double minute 2 protein
MiARN : microARN
MIT1 : mitotic 15
MYC : v-myc myelocytomatosis viral oncogene homolog
MYT1 : myelin transcription factor 1

N

NADH : Nicotinamide adénine dinucléotide
NAFLD : Non-Alcoholic Fatty Liver Disease
NASH : Non-Alcoholic SteatoHepatitis
NFY : nuclear transcription factor Y
NT : non traduit

P

PABP : polyA-binding protein
PADPR : poly-ADP ribosilation
PERK : protein kinase RNA-like ER kinase
PIK3CA : phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PI3K : Phosphotidylinositol 3 kinase
POLR3D : polymerase (RNA) III (DNA directed) polypeptide D

PR : progesterone receptor
PTEN : phosphatase and tensin homolog

R

RAF : v-raf-1 murine leukemia viral oncogene homolog
RBP : rna binding protein
RDRC : RNA dependent RNA Polymerase Complex
RE : reticulum endoplasmique
RIDD : Regulated IRE1α Dependent Decay of mRNA
RISC : RNA Induced Silencing Complex
RITS : RNA Induced Transcriptional Silencing
RIP140 : receptor interacting protein 140

S

SGBS : syndrome de Simpson-Golabi-Behmel
SHREC : Snf2/Hdac-containing repressor complex
SiARN : small interfering RNA
SP1 : specificity protein 1
SRF : serum response factor
SULF2 : Sulfatase 2
SMAD : mothers against decapentaplegic homolog 2
SWI6 : SWItching deficient 6

T

TAS3 : RITS complex subunit 3
TCF/LEF : T-cell factor/lymphoid enhancer factor
TNFA : tumor necrosis factor a
TP53 : tumor protein 53
TRBP : TAR RNA binding protein
TNRC6 : trinucleotide repeat containing 6A

U

UPR : Unfolded protein response

V

VEGF : vascular endothelial growth factor
VHB : virus de l'hépatite B
VHC : virus de l'hépatite C

W

WNT : wingless-type MMTV integration site family

X

XBP1 : X-box Binding Protein 1
XBP1s : XBP1 spliced
XPO5 : exportin-5
XRN1 : 5'-3' exoribonuclease 1

Z

ZHX2 : zinc fingers and homeoboxes 2

INTRODUCTION

I. LES MICROARNS

Les microARNs (miARNs) sont des petits ARNs non codants d'environ 22 nucléotides jouant un rôle essentiel dans le contrôle de l'expression génique. Ils sont généralement définis comme des ARNs régulateurs qui agissent de manière post-transcriptionnelle en s'appariant avec la région 3' non traduite (NT) des ARNm, ce qui conduit à l'inhibition de l'efficacité de traduction et/ou de la stabilité de l'ARNm cible. Ils ont été identifiés pour la première fois en 1993 chez le ver *Caenorhabditis elegans* avec la découverte de lin-4, un miARN indispensable au développement du nématode (Lee et al., 1993; Wightman et al., 1993). Cependant, ce ne fut qu'à partir de 2001 que l'étude de ces ARNs régulateurs prit de l'ampleur, suite à l'identification de nombreux petits ARNs endogènes exprimés chez le ver, la mouche *Drosophila melanogaster*, et aussi dans les cellules humaines (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Ces 10 dernières années, l'étude des miARNs a attiré une attention remarquable, menant à de rapides avancées. Des miARNs ont depuis été identifiés chez la plupart des organismes vivants. On compte aujourd'hui plus de 2000 miARNs humains identifiés, séquencés et annotés dans le génome (Griffiths-Jones et al., 2008). Chez les mammifères, un miARN contrôle en moyenne l'expression de 300 ARNm. Ainsi, il a été estimé que 60% des gènes humains ont conservé au cours de l'évolution leurs sites d'appariement aux miARNs. Ces gènes semblent donc avoir été soumis à une pression de sélection pour maintenir ce niveau de régulation de l'expression génique (Friedman et al., 2009). La découverte des miARNs et de leur rôle de régulateurs de l'expression génique a remis en cause le dogme central de gènes régulateurs codant nécessairement pour des protéines. Elle a ouvert la voie à l'étude plus étendue des ARNs non-codants ayant une fonction de régulation (endo-siARNs, piwi-ARNs, long ARNs non-codants…) (Farazi et al., 2008) et qui semblent être indispensables à l'évolution et à l'apparition d'organismes de plus en plus complexes (Mattick, 2004). Parmi les classes variées de petits ARNs retrouvés chez les mammifères, les miARNs semblent jouer un rôle particulièrement important dans de nombreuses pathologies humaines dont le cancer. Dans ce travail, j'ai focalisé mon attention sur leurs fonctions, leur implication dans les cancers, et plus particulièrement dans le carcinome hépatocellulaire (CHC).

1. La biogénèse des miARNs

1.1. Origine et synthèse

Près de la moitié des gènes de miARNs de mammifères sont situés dans des introns de gènes codant pour des protéines (40%) ou pour des longs ARNs non codant (10%) (Griffiths-Jones et al., 2008; Rodriguez et al., 2004; Saini et al., 2007). La majorité de ces miARNs introniques seraient co-transcrits avec leur gène hôte par l'ARN polymérase II. Cependant, plusieurs études montrent qu'environ 30% d'entre eux peuvent aussi être transcrits à partir d'un promoteur interne, indépendant du gène hôte (Corcoran et al., 2009; Monteys et al., 2010; Ozsolak et al., 2008).

Les autres loci de miARNs sont organisés en unités de transcription indépendantes et environ un tiers sont regroupés en « clusters » (Lee et al., 2002; Lee et al., 2004). Du fait de cette organisation particulière, plusieurs miARNs matures vont être produits à partir d'un ARN primaire unique et polycistronique de plusieurs kilobases. Cet ARN est transcrit par l'ARN polymérase II et possède une coiffe m7G et une queue poly(A) (Cai et al., 2004; Gu et al., 2006; Saini et al., 2007).

La majorité des miARNs sont transcrits par l'ARN polymérase II, mais quelques miARNs associés avec des répétitions Alu sont transcrits par l'ARN polymérase III (Borchert et al., 2006).

1.2. La maturation des miARNs

Le transcrit primaire, appelé pri-miARN, contient des structures secondaires de type tige-boucle qui correspondent aux futurs précurseurs des miARNs. Chez les mammifères, la voie canonique de maturation du miARN **(Figure 1A)** débute par l'intervention du microprocesseur, un complexe protéique constitué de Drosha et « Di George syndrome critical region 8 » (DGCR8), qui génère un second précurseur d'environ 65 nucléotides, appelé pré-miARN. Le pré-miARN a une structure tige boucle avec une extrémité 5' sortante de 2 ou 3 nucléotides qui est reconnue par le complexe Exportine 5-RanGTP et permet son export nucléaire. Dans le cytoplasme, la RNase III Dicer avec ses partenaires « TAR RNA binding protein » (TRBP) et Argonaute 1-4 (AGO) catalysent le second clivage pour produire le miARN mature (Hutvagner et al., 2001; Ketting et al., 2001).

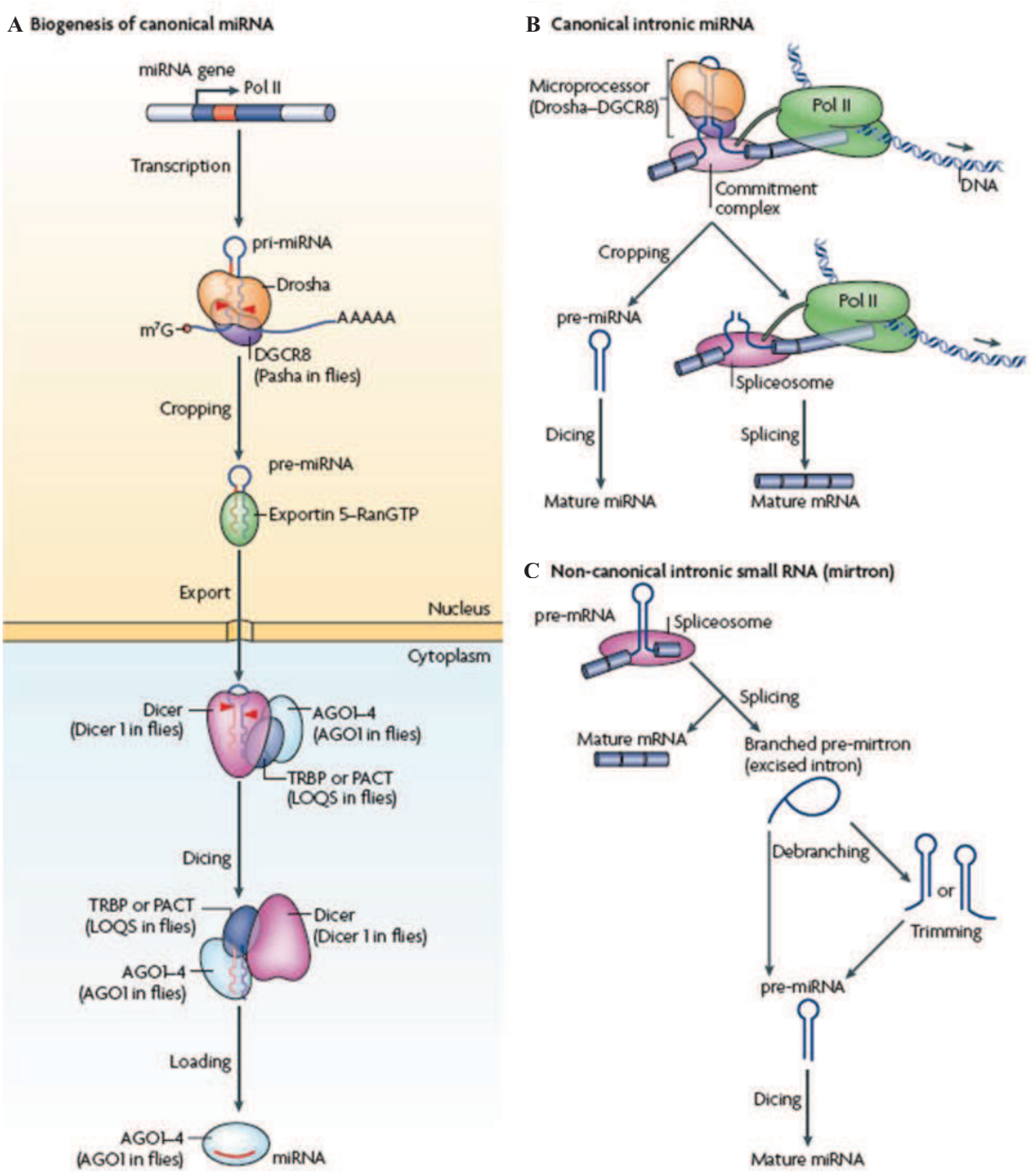


Figure 1 : La biogénèse des miARNs (Kim et al., 2009)

Cette étape génère le duplex miARN-miARN* final et permet la formation du « RNA Induced Silencing Complex » (RISC) (Chendrimada et al., 2005; Lee et al., 2006). Le duplex miARN-miARN* correspond à la partie inférieure de la tige du pré-miARN débarrassée de la boucle. L'un des deux brins du duplex, appelé brin guide (en rouge), reste associé à AGO (Hutvagner and Zamore, 2002). L'autre brin, miARN* ou brin passager (en bleu) est exclu du complexe RISC et dégradé. C'est la stabilité d'appariement nucléotidique des deux extrémités du duplex miARN-miARN* qui semble déterminer la sélection de l'un des deux brins. Ainsi,

le brin le plus faiblement apparié à son extrémité 5' est majoritairement incorporé (Khvorova et al., 2003; Schwarz et al., 2003). Cependant, certains pré-miARNs génèrent des miARNs matures provenant des deux brins à une fréquence comparable. De plus, il a été clairement démontré que certains miARN* sont fonctionnels et peuvent induire la répression de leurs gènes cibles *in vivo* (Okamura et al., 2008; Ro et al., 2007; Yang et al., 2011). Ils sont alors distingués par la notation miR-x-3p (brin en 3') et miR-x-5p (brin en 5') (Ro et al., 2007; Okamura et al., 2008; Kuchenbauer et al., 2011). La maturation des miARNs introniques **(Figure 1B)** débute avant la fin de l'épissage de l'ARNm. Le microprocesseur reconnaît et clive directement la structure tige-boucle dans l'intron pour générer le pré-miARN. Chez *D. melanogaster* et les mammifères, certains miARNs appelés mirtrons **(Figure 1C)** sont situés dans des introns courts et peuvent contourner l'étape faisant intervenir le microprocesseur. Après l'épissage de l'ARNm mature, l'intron excisé est directement reconnu comme un pré-miARN et exporté par le complexe Exportine 5-RanGTP.

1.3. Le complexe RISC

Le complexe RISC est un terme générique pour une famille hétérogène de complexes moléculaires programmés pour éteindre l'expression de gènes cibles. Il est caractérisé par la présence d'une protéine effectrice de la famille AGO et un petit ARN (siARN ou miARN) servant de guide au complexe en s'appariant avec un ARN cible. Chez les mammifères, il existe quatre protéines AGO (AGO1-4). Toutes les protéines AGO sont capables de se lier avec la même affinité à un duplex ARN-ARN (Liu et al., 2004; Meister et al., 2004), et sont redondantes pour l'utilisation des miARNs (Su et al., 2009). Pourtant seule AGO2 possède une activité catalytique de clivage d'un ARNm cible lorsqu'elle est associée à un siARN/miARN parfaitement complémentaire à la cible. Les protéines GW182 (TNRC6A, B et C chez les mammifères) sont également trouvées au sein des complexes RISC où elles jouent un rôle clé dans la répression de l'expression induite par les miARNs, en induisant la dégradation des ARNm cibles (voir section I.3) (Eulalio et al., 2008; Jakymiw et al., 2005; Liu et al., 2005; Rehwinkel et al., 2005). Les protéines AGO interagissent directement avec les protéines GW182 qui sont des composants de foci cytoplasmiques appelés « corps-P » qui contiennent des enzymes de dégradation des ARNm et sont impliqués dans le catabolisme et/ou le stockage des ARNm non traduits (Eulalio et al., 2008; Jakymiw et al., 2005; Liu et al., 2005; Rehwinkel et al., 2005).

2. L'interaction miARN-ARNm

La régulation de l'expression génique exercée par chaque miARN est spécifique et se fait *via* l'appariement d'un miARN mature associé au complexe RISC avec une séquence partiellement complémentaire située généralement dans la région 3' NT de l'ARNm cible. La mise en place et la stabilité de cet appariement sont primordiales pour la fonctionnalité du miARN et de nombreuses études sur ce sujet ont permis d'établir les critères moléculaires décrits ci-dessous.

2.1. Les différents types d'appariement

2.1.1. Les appariements conventionnels

La caractéristique la plus importante est un appariement parfait de type Watson et Crick entre l'ARNm cible et les nucléotides 2 à 7 en 5' du miARN qui constituent la séquence graine du miARN ou plus explicitement, la région de nucléation à l'origine de l'interaction entre le miARN et l'ARNm cible (Bartel, 2009).

Cinq types principaux de séquences graines conservées ont été identifiés (Lewis et al., 2005) **(Figure 2)** :

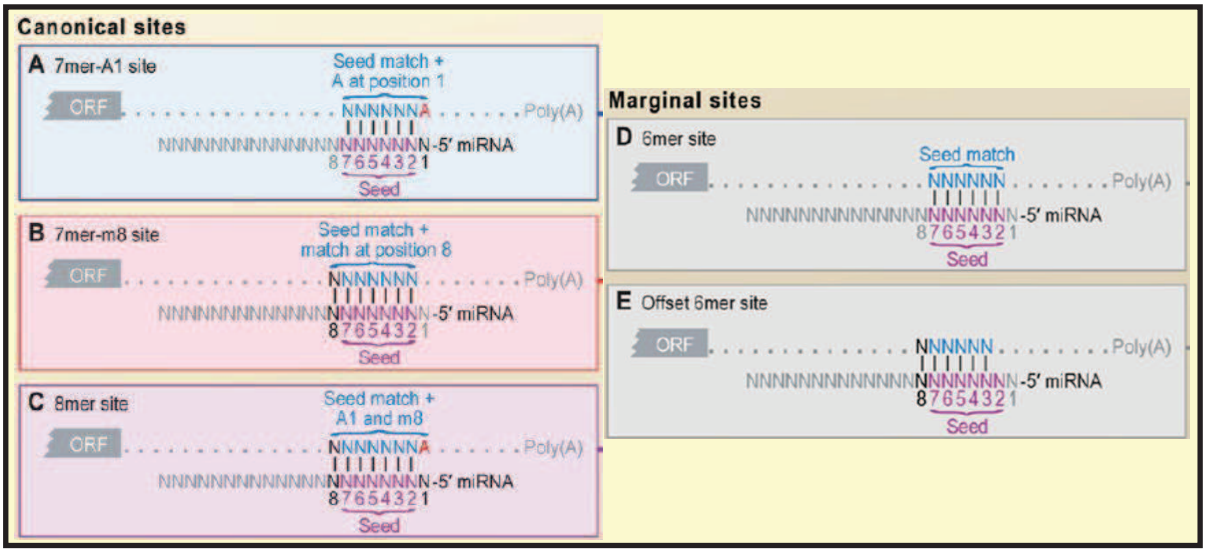


Figure 2 : Les appariements miARN/ARNm conventionnels (Bartel, 2009)

- Les sites canoniques :

    - Le site « 7mer-A1 » correspond à un appariement parfait de 6 nucléotides du nucléotide 2 à 7 et un A en face du nucléotide 1 du miARN

    - Le site « 7mer-m8 » possède un appariement de type Watson et Crick supplémentaire au niveau du nucléotide 8 du miARN, mais pas de A en face du nucléotide 1

    - Le site « 8mer » contient un appariement de type Watson et Crick supplémentaire au niveau du nucléotide 8 et un A en face du nucléotide 1 du miARN

- Les sites marginaux :

    - Le site « 6 mer » correspond à un appariement parfait de 6 nucléotides du nucléotide 2 à 7

    - Le site « 6 mer compensé » où l'appariement se situe du nucléotide 3 à 8

Les sites 8mer sont les moins conservés mais les plus efficaces, suivis des 7mer-m8 et 7mer-A1, puis les sites 6mer. Les sites 6mer présentent une probabilité plus forte d'être conservés car ils sont plus courts mais ils sont beaucoup moins efficaces pour générer une interaction miARN-ARNm stable (Bartel, 2009).

Des mésappariements sont généralement trouvés dans la région centrale des miARNs (nucléotides 9-12) empêchant le clivage endonucléolytique de l'ARNm cible dans le cas d'un complexe RISC contenant AGO2. Toutefois, la région 3' contient souvent quelques appariements qui pourraient permettre de stabiliser l'interaction du miARN avec sa cible, mais certains cas particuliers ont été identifiés.

2.1.2. Les appariements non-conventionnels

D'autres types de sites d'appariements de miARNs, plus rares, ont également été définis (Figure 3) :

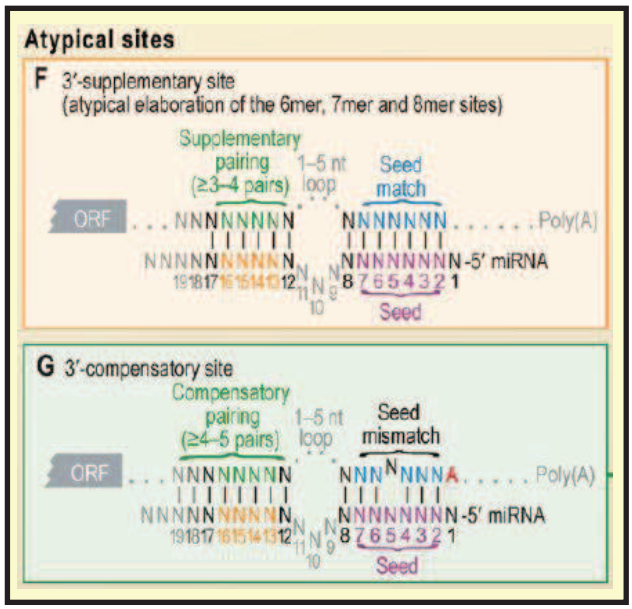


Figure 3 : Les appariements non-conventionnels (Bartel, 2009)

- Le site « 3' supplémentaire » possède un second appariement de type Watson et Crick en 3' du miARN au niveau des nucléotides 13 à 16. Ceci améliore la spécificité et la stabilité de l'interaction (Brennecke et al., 2005).

- Le site « 3' compensatoire » contient un mésappariement ou un appariement de type wooble (G-U) dans la région graine mais un appariement étendu en 3' qui compense l'appariement imparfait de la région graine (Grimson et al., 2007).

- Le site « central » (non représenté) qui correspond à un appariement parfait de onze nucléotides dans la région centrale du miARN pouvant entrainer un clivage de l'ARNm cible (Yekta et al., 2004; Davis et al., 2005; Shin et al., 2010).

2.2. Modèle de reconnaissance du miARN avec sa cible

Le miARN associé au complexe RISC se fixe directement sur la protéine AGO de manière à ce que la séquence graine soit positionnée pour favoriser un appariement efficace avec la cible **(Figure 4A)**.

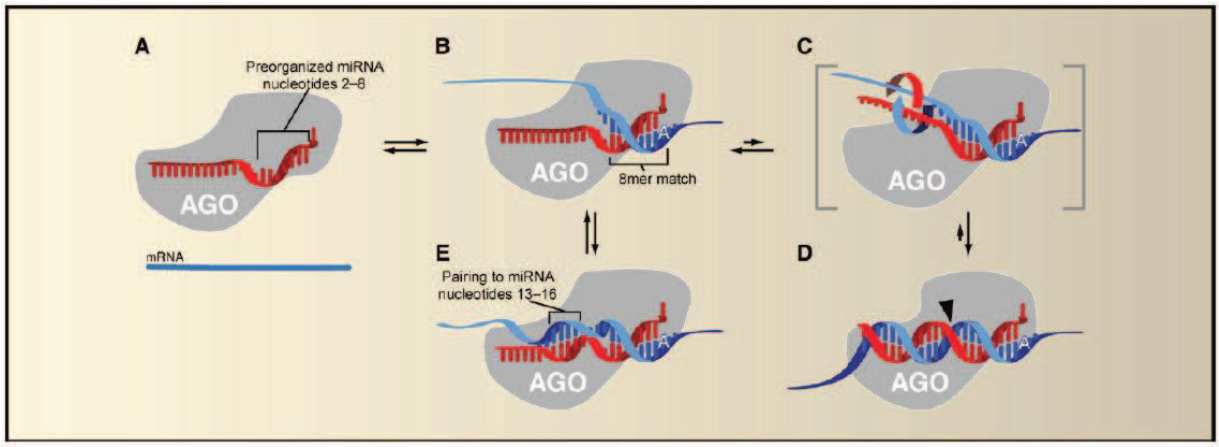


Figure 4 : Modèle de reconnaissance du miARN avec sa cible (Bartel, 2009)

Le premier nucléotide du miARN, tourné à l'opposé de l'hélice, et les nucléotides 9 à 11 opposés à l'ARNm cible ne sont pas accessibles pour la liaison. Le reste du miARN est lié dans une configuration qui n'a pas été organisée pour un appariement efficace. Un A en position 1 du site serait reconnu directement par AGO ou une autre protéine du complexe RISC **(Figure 4B)**. Les rares sites ayant une séquence graine qui s'étend dans la région centrale du miARN libèreraient la partie 3' du miARN de l'emprise d'AGO **(Figure 4C)**. Ceci permettrait la formation d'un duplex miARN-ARNm plus long et l'apparition d'un site de clivage pour AGO, similaire à celui des siARNs **(Figure 4D)**. Les sites présentant en 3' un appariement supplémentaire du nucléotide 13 à 16 peuvent entraîner la formation d'une seconde courte hélice sans perturber la protéine AGO. La présence d'une telle boucle préviendrait le clivage endonucléolytique catalysé par AGO, et de ce fait distinguerait le mécanisme de répression induit par les miARNs chez les plantes et les métazoaires **(Figure 4E)**.

D'après l'ensemble des données expérimentales, les miARNs ciblent préférentiellement la région 3'NT des ARNm. Plus rarement, les miARNs ont aussi été décrits comme ciblant la région 5'NT (Lytle et al., 2007), la région codante (Tay et al., 2008) et même certains promoteurs (Huang et al., 2011; Kim et al., 2008; Place et al., 2008; Younger and Corey, 2011). Une hypothèse pour cette préférence d'interaction serait que le complexe d'initiation de la traduction limiterait l'association du complexe RISC avec la région 5'NT et la séquence codante (Gu et al., 2006). Mais la prévalence d'interaction avec la région 3'NT pourrait résulter d'un biais dû à la focalisation des études sur cette région particulière de l'ARNm et le manque d'outils bioinformatiques de prédiction prenant en compte les interactions entre miARNs et les autres régions cibles.

2.3. Les outils bioinformatiques de prédiction

Aujourd'hui il existe une dizaine de logiciels bioinformatiques de prédiction des cibles géniques des miARNs **(Figure 5)**. Ils sont basés sur les règles d'appariement décrites précédemment entre un miARN et l'ARNm cible (John et al., 2004; Krek et al., 2005; Lewis et al., 2005 ; Min and Yoon, 2010) :

- la présence d'un appariement parfait entre la séquence graine du miARN et la cible
- le type et le nombre de sites
- la conservation des sites de fixation du miARN, ce qui favorise l'identification de sites fonctionnels sélectionnés au cours de l'évolution.

Name	Target species[a]	Algorithms	Performance	Distinguishing feature
DIANA-microT	Any	Thermodynamics	Precision: 66%[b]	Target structure comes before seed complementarity
EIMMo	Humans, mice, fishes, flies, worms	Bayesian method	Sensitivity: 0.8; specificity: 0.95[c]	Infers the phylogenetic distribution of functional target sites for each miRNA
miRanda	Flies, vertebrates	Complementarity	FPR: 24-39%(Fly)	Also provides the expression profile of miRNA in various tissues.
MirTarget2	Humans, mice, rats, dogs, chickens	SVM classifier	FPR: 22-31%; precision rate is 80% when the recall rate is below 20%	Microarray transcriptional profiling dataset is used for algorithm training
miTarget	Any	SVM classifier	An area under the ROC curve of 88.7% with the complete feature set	Training data is derived from vali dated miRNA targets from literature survey
PicTar	Vertebrates, flies, worms	Thermodynamics	FPR: 30%	Uses cross-species comparisons to filter out false positives
rna22	Any	Pattern recognition	FPR: 19-25.7% Sensitivity: 83%	Eliminates the use of cross-species conservation filtering, and leads to putative targets sites in 5' UTRs and ORF
RNAhybrid	Any	Thermodynamics, statistical model	SNR: 2.9:1 (vs 3.2:1[d]); run-time: 13-181 times faster than RNAfold[e]	An extension of the classical RNA secondary structure prediction algorithm[f]
TargetScan	Vertebrates	Seed complementarity	FPR: 31% (human, mouse, rat), 22% (pufferfish, mammal)	Mainly searches for the presence of conserved 8- and 7-nt seed matches
TargetScanS	Vertebrates	Seed complementarity	FPR: 22% (mammal);	Requires 6-nt seed match and conserved Adenosine

Figure 5 : Les outils bioinformatiques de prédiction des gènes cibles des miARNs (Min and Yoon, 2010)

En effet chez les métazoaires, la région 5' des miARNs contenant la séquence graine est la séquence la plus conservée (Lim et al., 2003). Chez les mammifères, la majorité des gènes codant pour des protéines ont évolué pour maintenir spécifiquement des séquences complémentaires aux séquences graines d'un ou plusieurs miARNs (Friedman et al., 2009; Lewis et al., 2005). Parallèlement, de nombreux gènes ont évolué pour éviter spécifiquement de contenir des séquences complémentaires à la graine de miARNs fortement co-exprimés

dans les mêmes cellules (Farh et al., 2005; Stark et al., 2005). Un appariement de 6 nucléotides au niveau de la séquence graine en 5' sans appariement en 3' peut être suffisant pour inhiber un gène cible (Brennecke et al., 2005). De plus, des substitutions dans la séquence graine, donnant lieu à des mésappariements miARN-ARNm, empêchent la répression de l'expression de la cible (Brennecke et al., 2005; Doench and Sharp, 2004; Kloosterman et al., 2004).

D'autres facteurs que l'appariement lui-même interviennent dans la reconnaissance d'une séquence cible par un miARN et dans sa fonctionnalité. L'accessibilité du site de fixation peut être influencée par la structure secondaire de l'ARNm. La position du site de fixation à plus de 15 nucléotides du codon stop et la présence de sites de fixation pour d'autres miARNs, ainsi que de régions riches en A/U à proximité, semblent favoriser la répression de la cible par le miARN (Didiano and Hobert, 2006; Grimson et al., 2007; Nielsen et al., 2007; Sun et al., 2010; Vella et al., 2004).

La simple prédiction de la présence d'un site de fixation d'un miARN dans un ARNm est insuffisante pour présumer de sa réalité biologique. Des validations expérimentales sont nécessaires dans chaque cas pour démontrer que la cible prédite est bien une cible fonctionnelle du miARN dans des conditions se rapprochant le plus possible du contexte cellulaire où s'exprime le miARN endogène. Chaque logiciel de prédiction utilise des paramètres et des algorithmes mathématiques quelque peu différents (Witkos et al., 2011), d'où l'intérêt de croiser les prédictions obtenues par chacun d'entre eux. Au cours de ma thèse, j'ai préférentiellement utilisé le logiciel miRWalk (Dweep et al., 2011) car il croise ses résultats de prédiction avec ceux de 9 algorithmes différents et prend en considération les interactions entre un miARN et les différentes régions possibles (5'NT, séquences codantes, 3'NT et séquences promotrices).

3. Les mécanismes d'action des miARNs

Classiquement, les miARNs sont considérés comme des régulateurs négatifs de l'expression génique ; ils agissent dans le cytoplasme au niveau post-transcriptionnel en ciblant principalement les régions 3'NT de leurs ARNm cibles. Cependant au cours de ces dernières années, différentes études ont révélé que les miARNs pouvaient aussi (i) être des régulateurs positifs de l'expression génique, (ii) cibler les régions codantes, les régions 5'NT et les promoteurs (iii) et agir dans le noyau comme régulateurs transcriptionnels.

3.1. Les mécanismes d'action post-transcriptionnels

Le miARN mature associé au complexe RISC s'apparie à l'ARNm cible et inhibe son expression par l'intermédiaire de différentes protéines du complexe RISC. Malgré les différences de reconnaissance d'une cible par un miARN, il est maintenant clairement établi que les miARNs contrôlent l'expression génique en réprimant la traduction des ARNm cibles et/ou en induisant leur dégradation *via* un mécanisme dépendant de la déadénylation. Toutefois, les mécanismes moléculaires mis en jeu sont encore sujet à de fortes controverses. Les facteurs associés au complexe RISC qui sont indispensables à la régulation de l'expression génique au niveau post-transcriptionnel par les miARNs sont les suivants :

- AGO 1-4
- GW182
- « poly(A) binding protein » (PABP)
- les composants du complexe de déadénylation (CAF1, CCR4-NOT)
- les facteurs intervenant dans l'enlèvement de la coiffe (DCP1, DCP2)

3.1.1. Inhibition de l'expression génique

3.1.1.1. Inhibition de la traduction

L'initiation de la traduction requiert de nombreux facteurs qui sont recrutés au niveau de l'ARNm grâce à la coiffe et à la queue poly(A). Ces facteurs coopèrent pour promouvoir la formation du complexe d'initiation de la traduction :

Dans le cytoplasme, le complexe d'initiation eIF4F **(Figure 6)** contient eIF4E, la protéine de liaison à la coiffe, eIF4G une protéine d'échafaudage et eIF4A, une hélicase ARN-dépendante (Kahvejian et al., 2005; Tarun et al., 1997). EIF4G interagit avec le facteur d'initiation eIF3 qui recrute la sous-unité ribosomale 40S. La PABP associée à la queue poly(A) interagit avec eIF4G, ce qui permet le rapprochement des 2 extrémités de l'ARNm. Cette interaction permet au transcrit de prendre une conformation circulaire qui favorise le recyclage de la grande

sous-unité 60S du ribosome, augmente l'efficacité de traduction et protège l'ARNm de la dégradation.

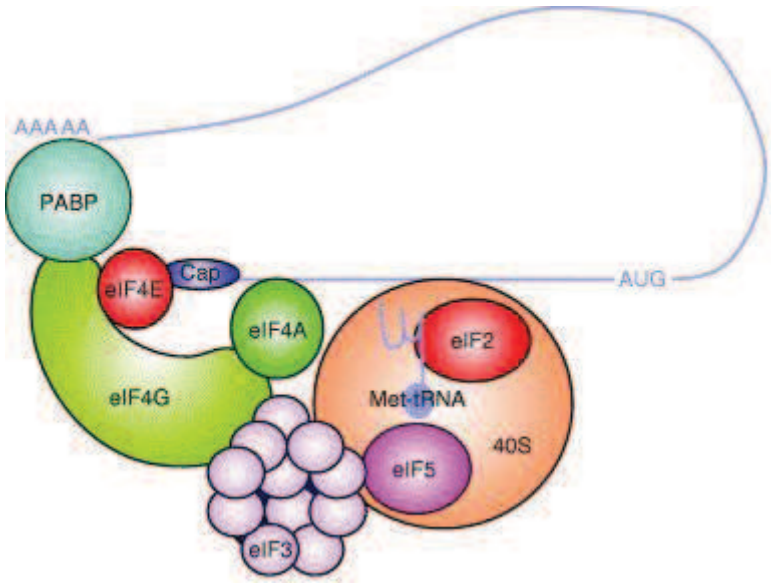


Figure 6 : Le complexe d'initiation de la traduction chez les eucaryotes (Robaglia and Caranta, 2006)

Différentes études ont révélés que les miARNs peuvent inhiber la traduction pendant et après l'initiation de la traduction et plusieurs modèles de mécanisme d'action ont été proposés :

- Blocage de l'initiation de la traduction **(Figure 7A)** :

AGO2 se fixe à la coiffe *via* un motif ressemblant à celui d'eIF4E (Kiriakidou et al., 2007), ce qui empêcherait la reconnaissance de la coiffe par eIF4F et inhiberait l'association de la sous-unité 40S avec l'ARNm (Mathonnet et al., 2007; Thermann and Hentze, 2007). Les miARNs pourraient aussi empêcher l'ARNm de prendre une structure circulaire suite à la déadénylation de l'ARNm ou grâce à GW182 par une interaction avec les PABP (Izaurralde and Zamore, 2009; Zipprich et al., 2009). De plus, eIF6, une protéine qui empêche l'association prématurée des sous-unités 40S et 60S du ribosome, a été retrouvée associée au complexe RISC (Chendrimada et al., 2007). Les miARNs pourraient donc inhiber le recrutement de la sous-unité 60S sur le transcrit et de ce fait empêcher l'initiation de la traduction.

- Blocage après initiation de la traduction **(Figure 7B)** :

Certains auteurs ont proposé que la chaîne naissante polypeptidique pourrait être dégradée au cours de la traduction (Nottrott et al., 2006) ou encore que l'élongation pourrait être bloquée ce qui dissocierait prématurément le ribosome (Petersen et al., 2006).

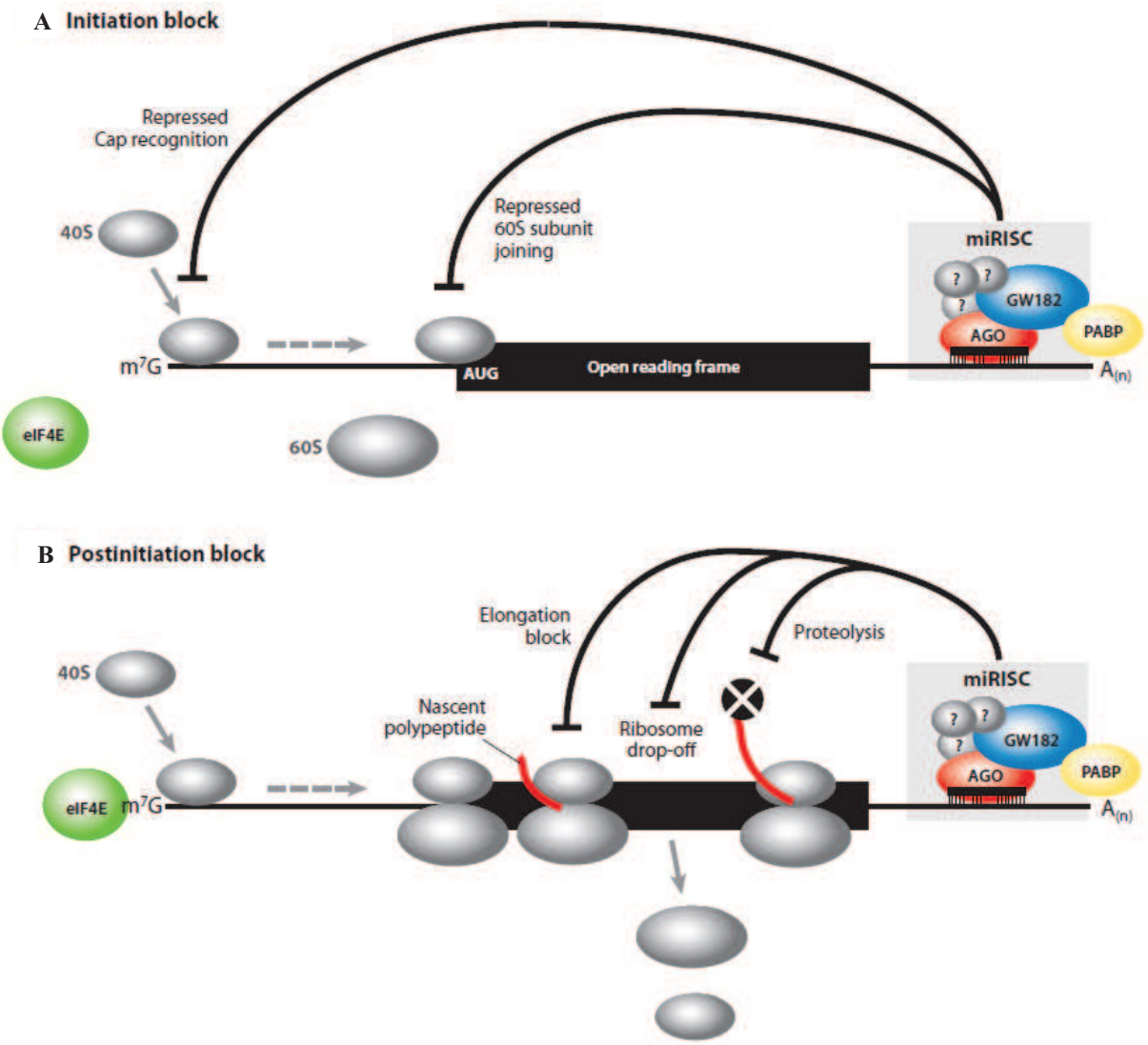


Figure 7 : L'inhibition de la traduction par les miARNs (Fabian et al., 2010)

3.1.1.2. Augmentation de la dégradation des ARNm

Alors que les études initiales sur le rôle des miARNs montraient que le niveau d'expression des ARNm réprimés restait inchangé et suggérait que la répression dépendante des miARNs n'affecte pas la stabilité des ARNm (Olsen and Ambros, 1999; Seggerson et al., 2002; Wightman et al., 1993), de plus en plus d'études ont montré depuis, que la répression

de l'expression de nombreuses cibles de miARNs s'accompagne de leur déadénylation et de leur dégradation (Eulalio et al., 2009; Giraldez et al., 2006; Lim et al., 2005; Wakiyama et al., 2007; Wu et al., 2006). Lorsque les miARNs ont un long appariement complémentaire avec leur ARNm cible, ils peuvent entrainer le clivage endonucléolytique de celle-ci. Mais ce cas reste rare dans les cellules animales et la grande majorité des appariements sont partiellement complémentaires. Différentes études montrent que les miARNs entrainent deux réactions aboutissant à la dégradation de l'ARNm ciblé **(Figure 8)** (Fabian et al., 2010).

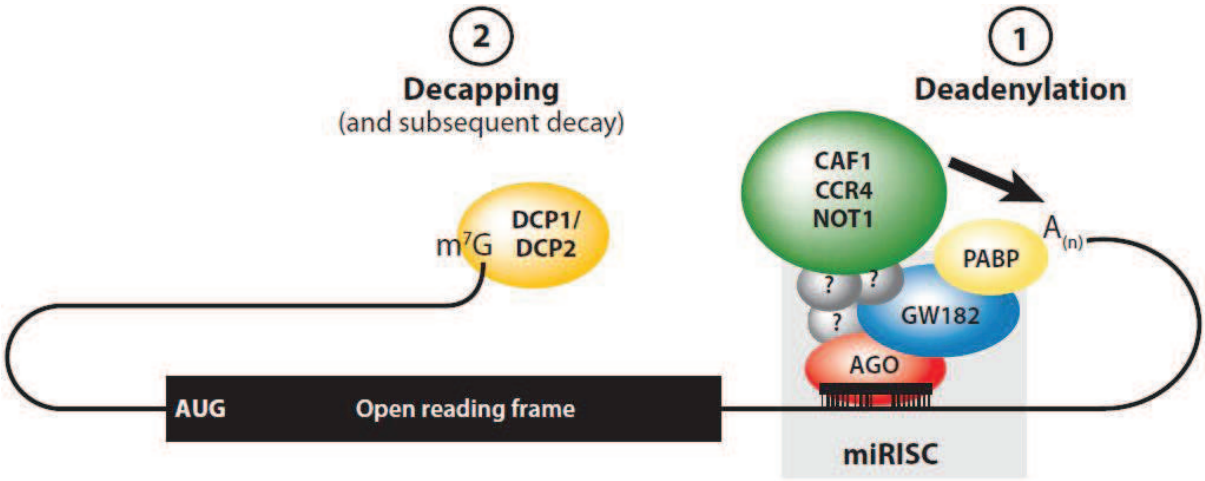


Figure 8 : L'activation de la dégradation des ARNm par les miARNs (Fabian et al., 2010)

- Première étape : La déadénylation

GW182 recrute le complexe de déadénylation CCR4-NOT et lorsqu'un nombre minimum de résidus adénosine est éliminé, l'ARNm peut être dégradé par l'exosome, un complexe protéique contenant différentes activités exonucléase 3'-5'.

- Deuxième étape : L'enlèvement de la coiffe

L'ARNm est engagé dans une dégradation rapide, le complexe enzymatique dipeptidyl carboxypeptidase 1 et 2 (DCP1, DCP2) hydrolyse la coiffe. L'ARNm non protégé en 5' est rapidement dégradé par XRN1, une exonucléase 5'-3' (Behm-Ansmant et al., 2006; Chen et al., 2009; Eulalio et al., 2009; Eulalio et al., 2007; Rehwinkel et al., 2005).

3.1.1.3. Traduction et/ou dégradation ?

Si les mécanismes exacts permettant la répression traductionnelle sont encore sujets à controverse, il paraît clair que les miARNs peuvent inhiber l'expression de leurs cibles aussi bien par des mécanismes d'inhibition de la traduction que par la dégradation des ARNm Récemment, des approches globales protéomique et transcriptomique, ont montré que la grande majorité des cibles réprimées par un miARN présentent une diminution du niveau de l'expression de leur ARNm qui reflète la diminution en protéine (Baek et al., 2008; Guo et al., 2010; Hendrickson et al., 2009; Selbach et al., 2008). On peut émettre l'hypothèse que les deux types de mécanismes sont en fait couplés. La dégradation des ARNm pourrait finalement être une conséquence du blocage de la traduction, et inversement, la déadénylation pourrait conduire indirectement à une inhibition de la traduction. En effet, GW182 interagit avec PABP et entraine la déadénylation de l'ARNm en recrutant le complexe CCR4-NOT (Zekri et al., 2009). Cette interaction pourrait intervenir de différentes manières dans la répression de l'expression génique dépendante des miARNs (Wakiyama et al., 2007). Tout d'abord, la déadénylation provoque une inhibition de la traduction, en empêchant l'ARNm de prendre une conformation circulaire (Fabian et al., 2009). Par ailleurs, elle induit une augmentation de la dégradation en entrainant une dégradation par l'exosome, qui précède la dégradation de l'ARNm grâce à l'enlèvement de la coiffe par DCP1 et DCP2 et la digestion par XRN1 (Huntzinger et al., 2010). Les incertitudes concernant l'ordre et le lien éventuel entre les évènements restent à élucider, mais des études récentes indiquent que l'inhibition traductionnelle constituerait la première étape, suivie de la déadénylation, puis de la dégradation de l'ARNm (Bazzini et al., 2012; Djuranovic et al., 2012). Les différentes stratégies expérimentales utilisées peuvent justifier les données contradictoires obtenues sur les mécanismes d'action des miARNs. Par exemple, les méthodes de transfection cellulaire (Lytle et al., 2007) ou encore les promoteurs des gènes rapporteurs utilisés peuvent favoriser un mode de régulation par rapport à un autre (Kong et al., 2008).

3.1.2. Activation de l'expression génique

Dans des situations spécifiques, les miARNs peuvent activer plutôt que réprimer l'expression génique au niveau post-transcriptionnel. Ces mécanismes peuvent faire intervenir une interaction directe ou non entre le miARN et sa cible, en collaboration ou non avec des « RNA Binding Proteins » (RBP).

3.1.2.1. Exemples de miARNs augmentant directement l'expression génique

- Augmentation de la traduction en fonction de la physiologie cellulaire **(Figure 9)** :

En fonction de la physiologie cellulaire, les miARNs peuvent inhiber ou activer l'expression de certains gènes en ciblant directement leurs régions 3'NT. La présence de GW182 (dans les cellules proliférantes) ou de FXR1a (dans les cellules quiescentes) dans le complexe RISC conduit respectivement à la répression ou à l'activation de la traduction de TNFα et KLF4 par miR-369-3p et miR-206/miR-344. Le même mécanisme a été identifié chez *Xenopus Levis* où l'ARNm Myt1 est activé par xl-miR16 (Lin et al., 2011; Mortensen et al., 2011; Vasudevan et al., 2007).

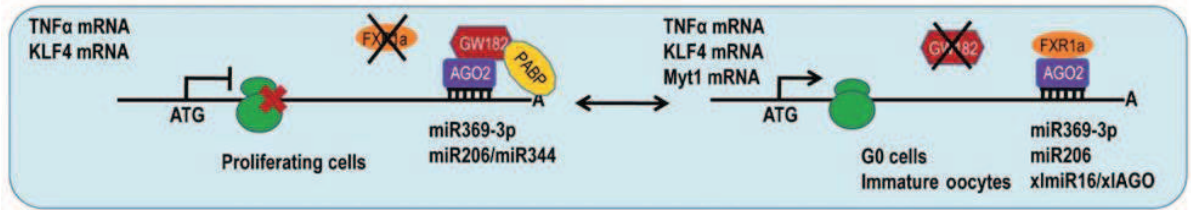


Figure 9 : Augmentation de la traduction en fonction de la physiologie cellulaire (Vasudevan, 2012)

- Augmentation de la traduction par ciblage des régions 5' ou 3'NT **(Figure 10)** :

MiR-122, un miARN spécifique du foie, stimule la traduction de l'ARN du VHC en reconnaissant deux sites cible dans la région 5'NT. MiR-346 interagit avec la région 5'NT de RIP140 et augmente sa traduction dans le tissu cérébral de souris. MiR-145 cible la région 3'NT de l'ARNm de la myocardine et augmente son expression au cours du développement du muscle lisse. Mmu-miR34a/34b-5p reconnaît la région 3'NT de la β-actine et augmente sa traduction dans les cellules neuronales murines (Cordes et al., 2009; Ghosh et al., 2008; Henke et al., 2008; Tsai et al., 2009a).

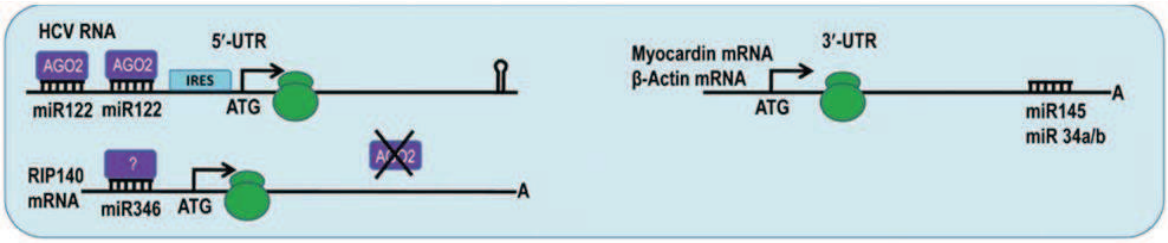


Figure 10 : Augmentation de la traduction par ciblage des régions 5' ou 3'NT (Vasudevan, 2012)

3.1.2.2. Exemples de miARNs induisant indirectement l'expression génique

- Inhibition de la répression de l'expression génique induite par les miARNs grâce aux RBP **(Figure 11)** :

Certaines RBP, tels que HuR (Bhattacharyya et al., 2006) et DND1, peuvent se lier aux éléments riche en A/U dans les régions 3'NT d'ARNm cibles et empêcher l'effet régulateur du miARN en modifiant la structure secondaire de l'ARNm cible et en limitant l'accès au site de fixation du miARN (Kedde et al., 2007).

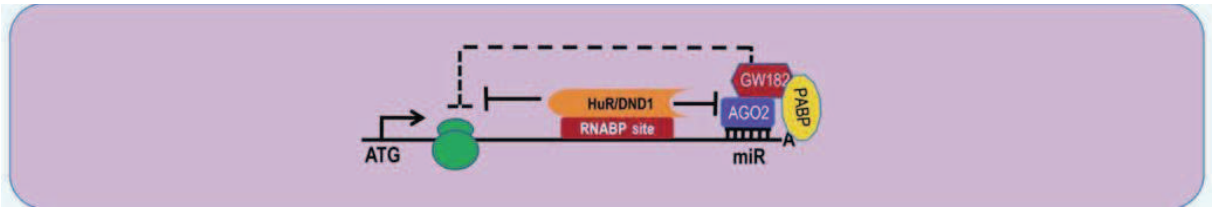


Figure 11 : Inhibition de la répression de l'expression génique induite par les miARNs grâce aux RBP (Vasudevan, 2012)

- Inhibition de la répression de l'expression génique induite par les miARNs grâce aux pseudogènes et à AGO10 **(Figure 12)** :

Les pseudogènes de PTEN et KRAS possèdent les mêmes sites de fixation des miARNs que leurs gènes respectifs. Par un mécanisme de compétition, miR-19b et miR-20a sont piégés par l'ARN codés par les pseudogènes, ce qui libère les ARNm cibles et augmente leur expression. Chez *Arabidopsis thaliana*, AGO10 capture miR-165 et miR-166, ce qui les empêche de se lier à AGO1 et de réprimer leurs cibles (Poliseno et al., 2010; Zhu et al., 2011).

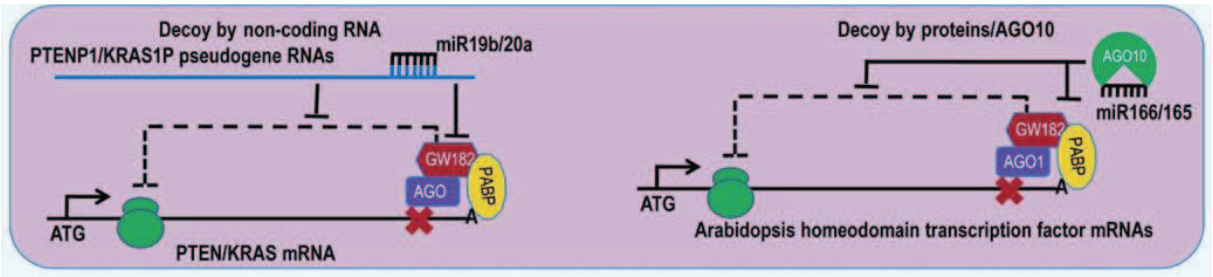


Figure 12 : Inhibition de la répression de l'expression génique induite par les miARNs grâce aux pseudogènes et à AGO10 (Vasudevan, 2012)

- Inhibition de la répression de l'expression génique induite par les miARNs par modification d'AGO ou du miARN **(Figure 13)** :

Les protéines AGO peuvent être modifiées par poly-ADP ribosilation (pADPr) en réponse à des conditions de stress comme la privation en acides aminés ou en glucose, empêchant son association aux ARNm cibles. Dans la maladie de Parkinson, le mutant LRRK2 phosphoryle 4E-BP1 (p4EBP1), qui s'associe à AGO et abroge la répression par Let-7 et miR-184*. Les miARNs peuvent également être modifiés par urydilation à leur extrémité 3 '. C'est le cas de miR-26a et miR-26b qui inhibent l'expression d'IL6. Leurs urydilation diminue leurs stabilité et donc limite leur effet répresseur (Gehrke et al., 2010; Jones et al., 2009).

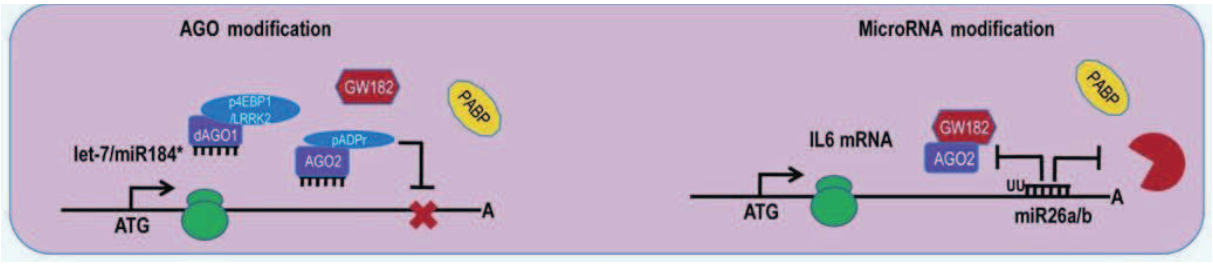


Figure 13 : Inhibition de la répression de l'expression génique par les miARNs par modification d'AGO ou du miARN (Vasudevan, 2012)

3.2. Les mécanismes d'action transcriptionnels

Les miARNs sont prédits pour cibler avec une forte complémentarité de nombreuses séquences promotrices dans le génome entier (Portnoy et al., 2011; Younger and Corey, 2011). De plus, différents facteurs appartenant au complexe RISC ont été détectés dans le noyau et les mitochondries (Bandiera et al., 2011; Cernilogar et al., 2011; Robb et al., 2005; Tan et al., 2009), ce qui suggère que les miARNs régulent également l'expression des gènes dans des compartiments cellulaires autres que le cytoplasme. Dans les cellules humaines, les miARNs arrivés à maturation dans le cytoplasme peuvent être importés dans le noyau (Liao et al., 2010) grâce à l'Importine 8 (Weinmann et al., 2009) pour réguler l'expression des gènes au niveau transcriptionnel.

Les miARNs peuvent cibler les séquences promotrices par deux mécanismes d'action : le « RNA Induced Transcriptional Silencing » (RITS) et l'activation ARN (ARNa) qui respectivement diminue ou augmente l'activité transcriptionnelle par des mécanismes moléculaires qui restent encore à élucider.

3.2.1. L'inhibition par RITS

Le RITS a initialement été décrit chez la levure et les plantes, dans la mise en place de l'hétérochromatine (Verdel et al., 2009) **(Figure 14)**.

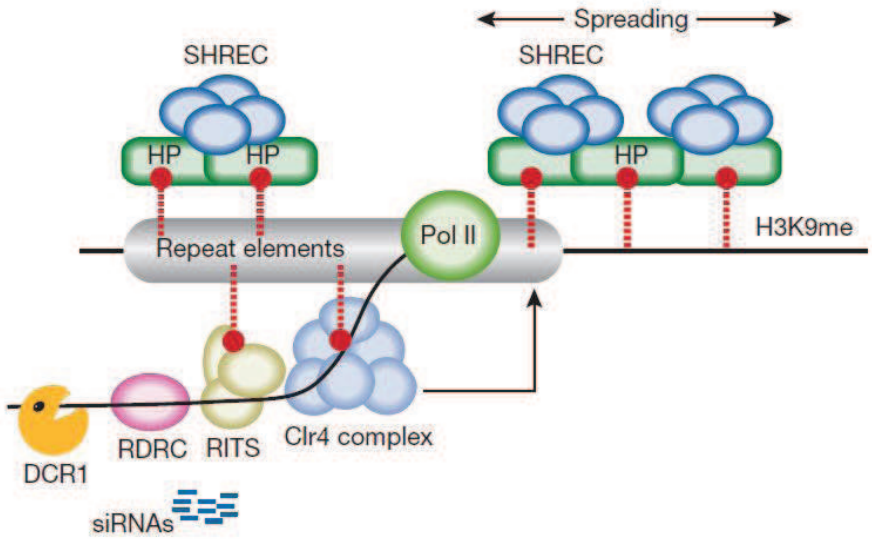


Figure 14 : La formation de l'hétérochromatine chez la levure par RITS (Verdel et al., 2009)

Chez la levure, l'ARN polymérase II transcrit un ARN non codant à partir des éléments répétés du génome. Le « RNA dependent RNA Polymerase Complex » (RDRC), génère un ARN antisens au transcrit issu des éléments répétés. Cette étape aboutit à la formation d'un duplex ARN-ARN qui va être reconnu et utilisé par Dicer (DCR1 sur la Figure 14) comme matrice pour produire des siARNs. Ces petits ARNs s'associent à AGO1 dans le complexe RITS, contenant aussi les protéines Tas3 et Chip1, et permettent son adressage sur le transcrit primaire par complémentarité des bases. Ensuite, le complexe RITS localisé au niveau des régions hétérochromatiques induit la méthylation des histones H3 sur la lysine 9 (H3K9me) catalysé par le complexe Clr4, ce qui permet le recrutement des protéines de liaison à l'hétérochromatine tels que Swi6/HP1. La liaison de la protéine HP crée une plateforme qui permet la propagation du complexe « Snf2/Hdac-containing repressor complex » (SHREC), composé de Clr1, Clr2, Clr3 et Mit1, qui permet la propagation de la répression de l'expression génique par méthylation (Sugiyama et al, 2007).

Très récemment, Younger et al. (Younger and Corey, 2011) ont identifié plusieurs analogues de miARNs (miR-423-5p, miR-372, miR-373, miR-520c-3p) qui inhibent l'expression de récepteurs de la progestérone (PR). Un ARN non codant est transcrit à partir du promoteur de PR. Cet ARN est reconnu de manière parfaitement complémentaire par les

miARNs associés à AGO2, ce qui induit la méthylation des H3K9me au niveau du promoteur PR. Ces résultats suggèrent que les changements épigénétiques ont été induits par RITS.

Par analyse informatique, différents miARNs présentant une complémentarité parfaite avec des séquences promotrices ont été identifiés. Parmi eux, miR-320 est transcrit à partir du promoteur du gène POLR3D, dans une orientation antisens. Il induit une diminution de l'expression de POLR3D en ciblant son promoteur. Associé aux protéines AGO1, H3K27me3 et EZH2, une méthyltransférase d'histone, miR-320 inhiberait l'expression de POLR3D en modifiant la structure de la chromatine *via* le complexe RITS (Kim et al., 2008).

3.2.2. L'activation par ARNa

Un petit ARN double brin peut cibler de manière partiellement complémentaire (Li et al., 2006; Matsui et al., 2010) les promoteurs et activer l'expression génique par l'intermédiaire d'un processus appelé activation ARN (ARNa) (Portnoy et al., 2011) **(Figure 15)**. Deux mécanismes moléculaires ont été décrits jusqu'à présent. Le petit ARN associé à AGO peut cibler le promoteur directement en formant un duplex ARN-ADN **(Figure 15A)** ou par l'intermédiaire d'un ARN non-codant transcrit à partir du promoteur **(Figure 15B)**.

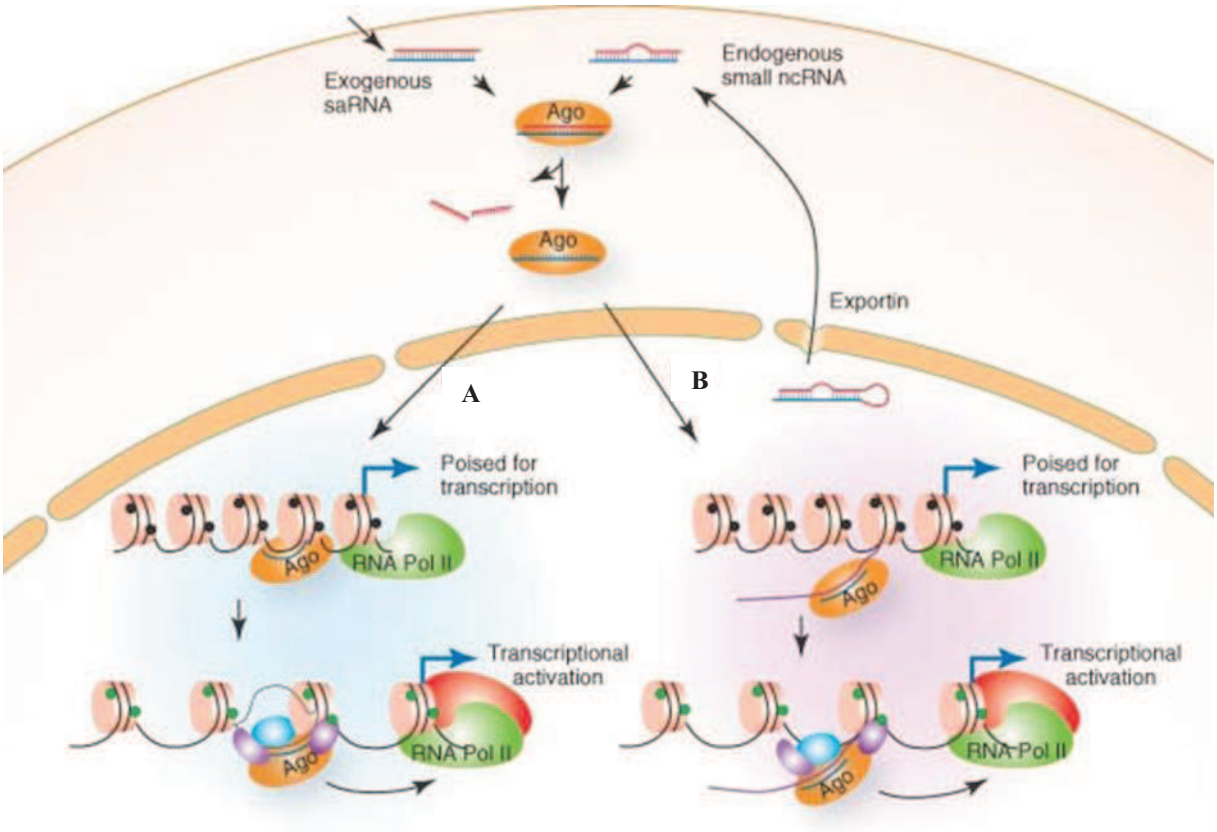


Figure 15 : L'activation de la transcription par ARNa (Portnoy et al., 2011)

Ces dernières années, plusieurs travaux ont montré que certains miARNs nucléaires activent l'expression génique en s'appariant avec une forte complémentarité à des séquences promotrices de gènes cibles (Huang et al., 2011; Kim et al., 2008; Place et al., 2008; Younger and Corey, 2011). Cette observation a conduit à l'hypothèse que ces miARNs pourraient également déclencher la transcription par ARNa. MiR-373 est capable d'activer l'expression de l'E-cadhérine (CDH1) et de la « Cold-shock domain-containing protein C2 » (CSDC2) grâce à un appariement complémentaire de plus de 80% avec le promoteur de ces gènes. Par ailleurs, miR-744 présente une complémentarité de plus de 90 % avec le promoteur de la cycline B1 (Ccnb1) chez la souris. Il augmente l'expression de la Ccnb1 par un mécanisme qui implique le recrutement d'AGO1, de l'ARN Polymérase II, ainsi qu'une augmentation de H3K4me3 au niveau du promoteur de la Ccnb1 (Huang et al., 2011).

Ces travaux fournissent les premiers exemples d'ARNa pertinent physiologiquement et démontrent que les miARNs ont une fonction nucléaire régulant positivement la transcription des gènes.

4. MicroARNs et cancers

Les cancers sont des pathologies complexes caractérisées par la transformation d'une cellule somatique normale en cellule maligne. La transformation est causée par une dérégulation du programme génétique cellulaire qui a pour conséquence la prolifération incontrôlée des cellules anormales avec envahissement local ou à distance du tissu hôte. L'initiation, la promotion et la progression du cancer résultent de la dérégulation de nombreuses voies de signalisation qui vont permettre aux cellules cancéreuses d'acquérir différentes capacités (Hanahan and Weinberg, 2011):

- un signal de prolifération soutenu
- l'échappement aux signaux inhibiteurs de prolifération
- une résistance à la mort cellulaire
- une immortalisation cellulaire
- une induction de l'angiogénèse
- une activation de l'invasion
- l'échappement au système immunitaire
- une induction de l'inflammation
- une instabilité génomique
- une dérégulation du métabolisme énergétique

Les mécanismes moléculaires responsables de l'acquisition de ces caractéristiques ont longtemps été associés à des gains et/ou des pertes d'expression de gènes codant pour des protéines (activation d'oncogènes et/ou inactivation de gènes suppresseurs de tumeurs), liés à des modifications génétiques ou épigénétiques. D'autres facteurs impliqués dans la régulation post-transcriptionnelle sont de plus en plus étudiés dans les cancers. Ces facteurs régulateurs peuvent par exemple être des protéines se liant à l'ARN « RNA binding proteins » (RBP) ou des miARNs. Il existe peu de miARNs identifiés comme oncogènes ou suppresseurs de tumeur, mais les recherches menées sur les miARNs ont révélé leur rôle important dans la régulation de l'expression génique et leur implication dans la plupart des capacités biologiques acquises par les cellules cancéreuses.

4.1. L'expression des miARNs dans les cancers

Ces dernières années, de nombreuses approches globales permettant d'étudier le miRNome (ensemble des miARNs exprimés par un génome dans une condition physiologique donnée) ont été développées. Ces études ont permis d'identifier des signatures spécifiques à l'expression des miARNs qui reflètent l'état physiologique des cellules cancéreuses, l'origine de la tumeur, les différents stades de différenciation, l'agressivité des cellules tumorales ou encore la présence de métastases. Elles ont également permis d'établir de nouvelles classifications moléculaires des tumeurs. Les miARNs sont faciles à extraire à partir de petites biopsies tissulaires congelées ou incluses en paraffine et sont même détectables dans le sérum des patients. Leur détection dans les fluides biologiques représente un intérêt majeur en clinique car ils constituent de nouveaux marqueurs biologiques non invasifs pour établir un diagnostic et un pronostic chez les patients atteints de cancer (Lu et al., 2005).

4.2. La dérégulation de l'expression des miARNs dans le cancer

L'expression des miARNs augmente au cours de la différenciation des cellules normales. Plusieurs études montrent que l'expression des miARNs est globalement diminuée dans les tissus tumoraux par rapport aux tissus normaux (Gaur et al., 2007 ; Lu et al., 2005 ; Ozen et al., 2008). La sous-expression globale des miARNs dans le cancer pourrait donc être une conséquence du retour à un état peu différencié des cellules tumorales. Néanmoins, parmi les miARNs sous-exprimés dans le cancer, certains agissent comme des gènes suppresseurs de

tumeurs. D'autres miARNs, appelés oncomiRs, sont en revanche surexprimés dans certains types tumoraux et ont une activité d'oncogène.

4.2.1. Exemples de miARNs suppresseurs de tumeurs

MiR-15a et miR-16-1 sont les deux premiers miARNs définis comme des gènes suppresseurs de tumeurs. Un ensemble de données expérimentales ont permis de le démontrer *in vivo*. Leurs gènes sont regroupés dans le cluster miR-15a~16-1 qui est localisé au niveau du chromosome 13q14, une région délétée dans plus de la moitié des leucémies lymphoïdes chroniques B (LLC). L'expression de ces deux miARNs est diminuée chez 68% des patients (Calin et al., 2002). La délétion de ce cluster dans un modèle murin conduit à l'apparition d'une LLC (Klein et al., 2010). Les deux miARNs possèdent la même séquence graine et ciblent plusieurs gènes impliqués dans le contrôle du cycle cellulaire (cycline D1, cycline D3, cycline E1, CDK6…) et l'apoptose (BCL2). La restitution de l'expression de miR-15a et miR-16-1 dans des lignées de LLC entraine un arrêt du cycle cellulaire, l'apoptose et la régression des tumeurs *in vivo* (Klein et al., 2010).

4.2.2. Exemples d'oncomiRs

Les premiers miARNs identifiés comme oncomiRs sont localisés dans le cluster miR-17-92. Ce cluster code pour un pri-miARN polycistronique de six miARNs (miR-17, miR-20a, miR-20b, miR-106a, miR-106b et miR-92a). Il est situé dans le chromosome 13q31-q32 dans le 3ème intron d'un transcrit primaire non-codant, C13orf25. La région 13q31-q32 est fréquemment amplifiée dans le lymphome de type B (Ota et al., 2004) et les miARNs de ce cluster sont surexprimés dans 65% des échantillons tumoraux. Dans un modèle murin, ils activent la prolifération cellulaire, inhibent l'apoptose et augmentent la lymphomatogénèse (He et al., 2005). Plusieurs miARNs du cluster miR-17-92 ciblent différents gènes suppresseurs de tumeurs (Xiao et al., 2008), comme (i) « phosphatase and tensin homolog deleted on chromosome 10 » (PTEN) qui inhibe la voie PI3K/Akt impliquée dans la prolifération et la survie (Cantley and Neel, 1999), (ii) Bim qui active l'apoptose (O'Connor et al., 1998) et (iii) CDKN1A (Fontana et al., 2008), un inhibiteur de complexes cycline-CDK qui bloque la transition G1-S du cycle cellulaire (Harper et al., 1993).

4.3. Les mécanismes de dérégulation de l'expression des miARNs

Trois mécanismes principaux sont responsables de la dérégulation des miARNs dans le cancer : la modification de mécanismes épigénétiques, la variation de l'activité des facteurs de transcription contrôlant leur expression et l'altération de leur voie de biosynthèse (ou maturation).

4.3.1. La modification des mécanismes épigénétiques

Les miARNs sont fréquemment localisés dans des sites chromosomiques fragiles et des régions génomiques impliqués dans le cancer (Calin et al., 2004). Leur expression peut être réprimée par une hyperméthylation aberrante des îlots CpG, une modification des histones, une mutation ponctuelle, ou encore une délétion du cluster ou gène du miARN (Kunej et al., 2011 ; Lehmann et al., 2008 ; Sevignani et al., 2006). Les gènes des miARNs peuvent plus rarement être surexprimés par amplification, par translocation d'un proto-oncogène près du promoteur d'un gène de miARN ou par translocation d'un miARN près d'un promoteur d'oncogène (Metzler et al., 2004 ; Sevignani et al., 2006).

4.3.2. La variation de l'activité de facteurs de transcription

Certains facteurs de transcriptions contrôlant l'expression de miARNs peuvent parfois être dérégulés dans les cancers. MYC et p53, qui sont des facteurs de transcription clés de la tumorigénèse, contrôlent également l'expression de nombreux miARNs et participent à leur sous-expression dans les tumeurs.

MYC est un oncogène surexprimé dans de nombreux cancers qui peut réprimer directement ou indirectement l'expression de miARNs suppresseurs de tumeur ayant des activités anti-prolifératives, anti-tumorigènes et pro-apoptotiques **(Figure 16)**, tels que Let-7, miR15a/16-1 (voir section I 4.2.1), miR-26 et la famille miR-34 (Bui and Mendell, 2010; Chang et al., 2008).

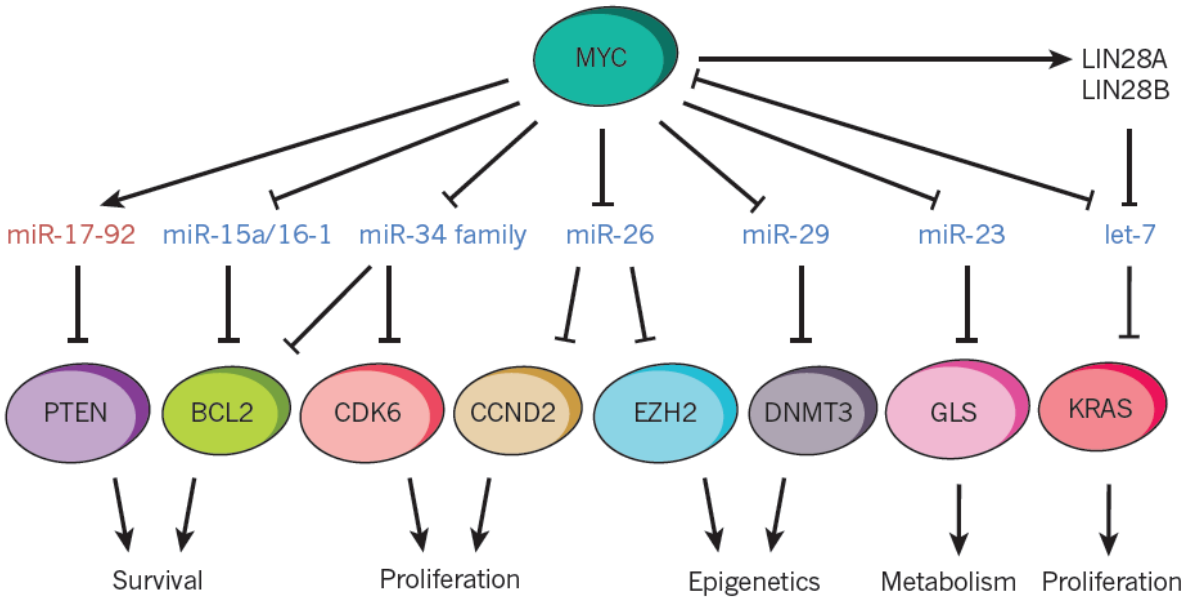


Figure 16 : Myc est un oncogène régulant l'expression de différents miARNs (Lujambio and Lowe, 2012)

MYC peut aussi activer l'expression d'oncomiRs comme ceux du cluster miR-17-92 (voir section I 4.2.2) (O'Donnell et al., 2005). De plus, MYC réprime l'expression de la famille Let-7 au niveau post-transcriptionnel en activant l'expression de deux RBP, LIN28A et LIN28B. Or certains membres de la famille Let-7 inhibent MYC constituant ainsi une boucle de régulation rétroactive d'activation de Myc (Bueno et al., 2011).

P53 (voir section II 2.2.2) est un gène suppresseur de tumeur muté dans de multiples cancers qui active l'expression de nombreux miARNs suppresseurs de tumeur **(Figure 17)**, comme la famille miR-34, miR-107, miR-200 et miR-192 qui induisent l'apoptose, inhibent la prolifération cellulaire, l'angiogénèse et la transition épithélio-mésenchymateuse. P53 augmente l'expression de miR-192, miR-194, miR-215 et miR-605 qui inhibent l'expression de MDM2, un inhibiteur de p53. Cette boucle d'activation de p53 est souvent réprimée par des mécanismes épigénétiques dans le cancer (Pichiorri et al., 2010). De plus, p53 est impliqué dans la biogénèse des miARNs. D'après certaines études, il s'associe à Drosha et p68 pour favoriser la maturation de certains pri-miARNs (Bates et al., 2005; Suzuki et al., 2009).

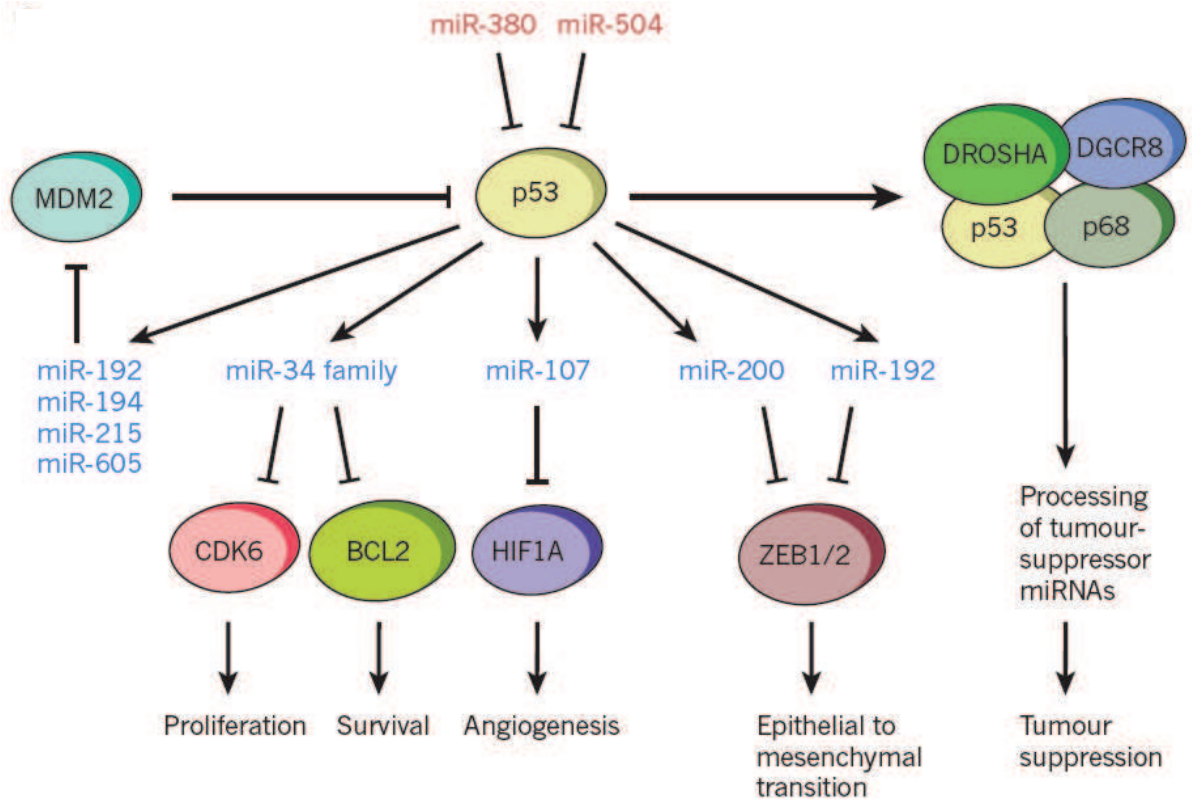


Figure 17 : p53 est un suppresseur de tumeur régulant l'expression de nombreux miARNs (Lujambio and Lowe, 2012)

4.3.3. L'altération de la biogénèse des miARNs

Dans les tissus normaux, il existe une corrélation entre l'expression des pri-miARNs et celle des miARNs matures. En revanche dans les tissus tumoraux, l'expression des pri-miARNs ne reflète pas toujours celle des miARNs matures. L'inhibition de la maturation des miARNs semble donc être un mécanisme important qui pourrait expliquer leur diminution dans les cancers **(Figure 18)** (Lujambio and Lowe, 2012).

Les enzymes clés de la biogénèse des miARNs sont des gènes suppresseurs de tumeur haploinsuffisants. La perte d'un allèle de Dicer1 ou de Drosha accélère la transformation et la tumorigénèse (Kumar et al., 2007), alors que leur délétion complète est létale car les miARNs sont indispensables aux fonctions cellulaires (Kumar et al., 2009). La sous-expression de Drosha diminue la production de pré-miARNs. Des mutations ponctuelles dans l'exportin-5 (XPO5 dans la Figure 18) ont été identifiées dans certains cancers et bloquent l'export des pré-miARNs vers le cytoplasme (Melo et al., 2010; Melo et al., 2009). Des mutations dans TRBP (TARBP2 dans la Figure 19) ou la sous-expression de Dicer1 réduisent la quantité de miARNs matures (Melo et al., 2010; Melo et al., 2009).

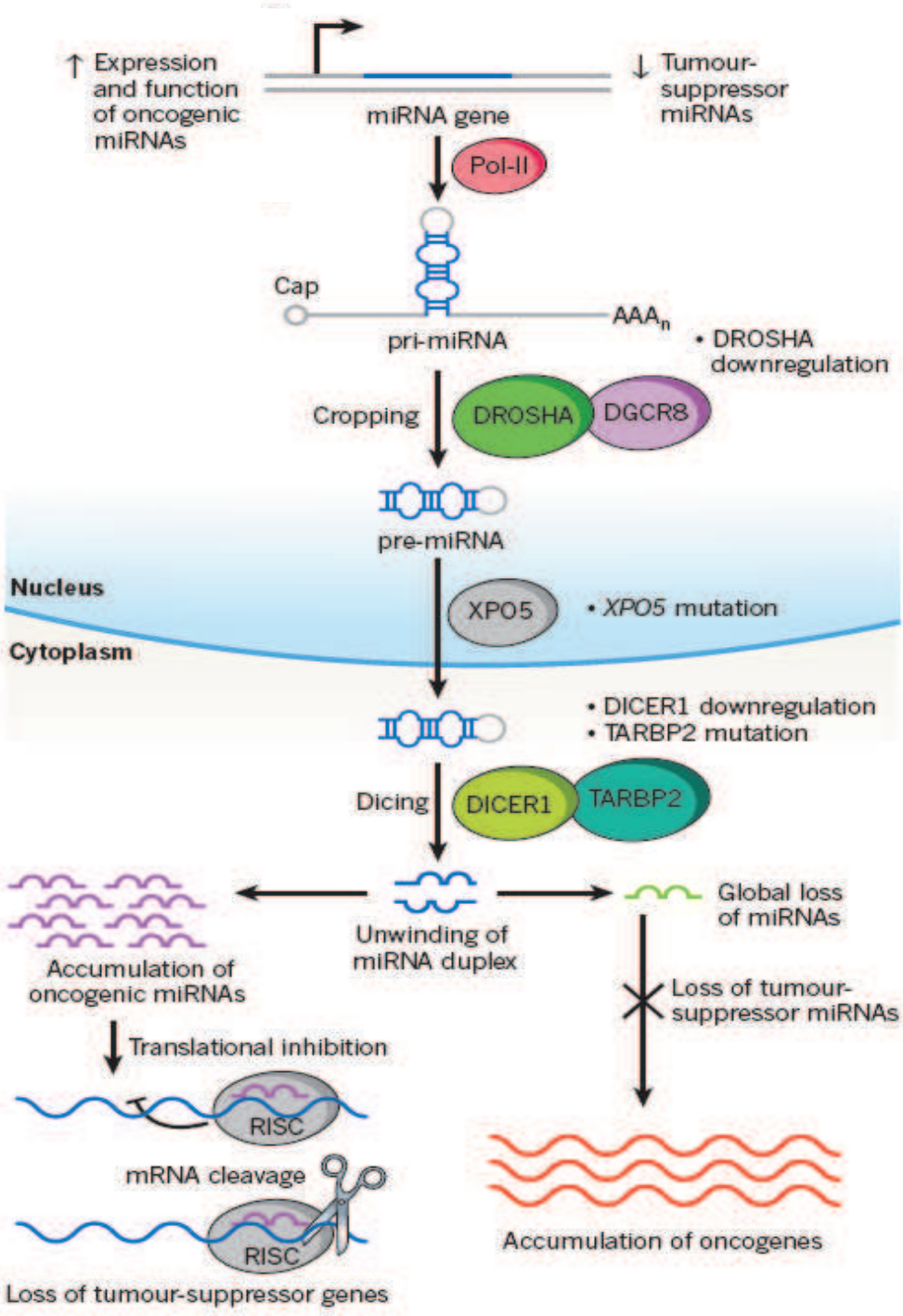


Figure 18 : Mécanismes d'altération de la biogénèse des miARNs (Lujambio and Lowe, 2012)

D'autres régulateurs peuvent aussi être importants, comme LIN28A et LIN28B qui répriment la maturation de la famille des Let-7 (Piskounova et al., 2008), des miARNs suppresseurs de tumeur. Par ailleurs, p68, SMAD1 et SMAD5 contribuent au développement du cancer en altérant la biogénèse des miARNs (Newman and Hammond, 2010a, b).

4.4. Conclusion

Les miARNs sont des régulateurs clés de l'expression génique. L'altération de leur expression est retrouvée dans tous les cancers et affecte tous les processus biologiques liés au phénotype tumoral. Il n'y a pas d'approches réellement efficaces pour identifier les cibles d'un miARN, car le mode de reconnaissance de leurs cibles n'est pas entièrement compris et leur nombre en perpétuel augmentation. De plus, un miARN régule plusieurs cibles ARNm et une cible peut être contrôlée par plusieurs miARNs. Des miARNs paralogues (miARNs divergents suite à la duplication du gène ancestral et possédant la même séquence graine) existent souvent en plusieurs copies dans le génome. Ils ont beaucoup de cibles communes et constituent ainsi des familles. Chez les mammifères, chaque famille de miARNs conservés au cours de l'évolution cible en moyenne 300 ARNm (Friedman et al., 2009). L'ensemble forme un réseau redondant qui augmente la complexité des processus intervenant dans la régulation de l'expression génique et qui dépend de facteurs environnementaux comme le contexte cellulaire et la quantité respective de miARNs et de transcrits cibles dans chaque cellule. La compréhension de la fonctionnalité des miARNs et des mécanismes par lesquels ils contribuent à la tumorigénèse pourraient permettre de développer de nouvelles stratégies thérapeutiques anticancéreuses et de définir de nouveaux marqueurs diagnostiques et pronostiques utilisables en clinique.

II. LE CARCINOME HÉPATOCELLULAIRE

1. Généralités

Le carcinome hépatocellulaire (CHC) est le principal cancer primitif du foie (85 à 90%) qui se développe après transformation maligne des hépatocytes (Bosch et al., 1999). Le foie remplit de nombreuses fonctions vitales pour l'organisme. En effet il est l'organe principal du métabolisme des protéines, lipides, glucides, vitamines, ainsi que du fer. Il est également responsable de la détoxication et de la biotransformation des substances qui peuvent être dangereuses pour les autres tissus (produits chimiques, médicaments, polluants de l'environnement) **(Annexe 1)**. D'après le Centre International de Recherche sur le Cancer, près de 750 000 nouveaux cas de CHC sont diagnostiqués dans le monde chaque année. Le CHC est le sixième cancer le plus fréquent dans le monde (Forner et al., 2012), c'est un problème majeur de santé publique car, en raison d'un diagnostic trop tardif, il est de très mauvais pronostic et correspond à la troisième cause de décès par cancer dans le monde. La majorité des CHC (80%) se développe sur des foies déjà malades suite à des altérations chroniques provenant de différentes étiologies. Ces altérations du foie conduisent au développement d'une fibrose, puis d'une cirrhose, terrain privilégié pour le développement du CHC (Farazi and DePinho, 2006). Dans des cas plus rares (20%), le CHC peut se développer sur des foies sains, non fibreux, mais les mécanismes sous-jacents restent encore mal caractérisés.

1.1. Les facteurs de risque

- Le Virus de l'Hépatite B :

L'insertion de l'ADN viral dans le génome des hépatocytes peut provoquer une instabilité génomique et une mutagénèse insertionnelle altérant l'expression de certains gènes (Brechot et al., 2001; Chen et al., 2010). De plus, la protéine virale transactivatrice HBx est capable d'activer la transcription de gènes liés à la prolifération cellulaire (Ueda et al., 1995).

- Le Virus de l'Hépatite C :

Le VHC est un virus à ARN qui ne s'intègre pas dans le génome. Les mécanismes induisant la transformation maligne des hépatocytes seraient liés à la protéine structurale du VHC « HCV core », qui agit sur la différenciation, la prolifération cellulaire et l'apoptose (Tsai and Chung, 2010). De plus, 90% des individus infectés par le VHC développent une hépatite chronique, et 10 à 30% d'entre eux évoluent vers une cirrhose 25 à 30 ans après l'infection. Une fois la cirrhose établie, le risque de CHC augmente de 1 à 3% par an (Bruno et al., 2007; El-Serag and Rudolph, 2007).

- L'alcool :

Une consommation importante et régulière d'alcool peut induire l'apparition d'une fibrose hépatique qui, au cours du temps, se transforme en cirrhose (Fattovich et al., 2004). De plus, la métabolisation de l'éthanol dans le foie se traduit par la formation de molécules toxiques pour le tissus hépatique et très réactives comme l'acétaldéhyde et les radicaux libres qui sont capables, entre autres, de changer le potentiel redox des hépatocytes. La conséquence est une augmentation du rapport NADH/NAD+ dans le cytosol et les mitochondries des hépatocytes, qui perturbe ainsi de nombreuses réactions métaboliques et qui entraîne à terme une altération du métabolisme des lipides, des carbohydrates, des protéines, du lactate et de l'acide urique (Zakhari and Li, 2007).

- Les maladies métaboliques :

L'obésité et le diabète entraînent une production élevée de nombreuses cytokines pro-inflammatoires qui aboutissent à l'accumulation d'acides gras libres dans les hépatocytes (Starley et al., 2010). Cette accumulation de lipides dans les hépatocytes, appelée stéatose hépatique est définie sous le nom de « Non-Alcoholic Fatty Liver Disease » (NAFLD). Cette pathologie peut se compliquer et induire une inflammation chronique, « Non-Alcoholic SteatoHepatitis » (NASH), puis une cirrhose et un CHC. De son côté, l'hémochromatose est une maladie héréditaire caractérisée par une absorption excessive de fer dans les hépatocytes. L'augmentation du fer dans le foie entraine la production de radicaux libres causant des altérations de l'ADN génomique (Asare et al., 2006).

- L’aflatoxine B1 :

Cette mycotoxine est produite par des champignons (*Aspergillus flavus* et *Aspergillus parasiticus*) qui contaminent les graines d’arachide et diverses céréales (maïs, riz, blé) stockés dans de mauvaises conditions. Après ingestion, elle est assimilée par le foie qui la convertit en un métabolite secondaire toxique responsable de lésions de l’ADN génomique (Qian et al., 1994).

1.2. Dépistage, diagnostics et traitements actuels

L’échographie et le dosage de l’alpha-foetoprotéine (AFP) sont les examens de référence pour le dépistage du CHC. Seule la population à risque (hépatites virales, cirrhose, maladies métaboliques) bénéficie d’un dépistage annuel. Néanmoins, la sensibilité de l'échographie pour le dépistage d’un CHC reste globalement moyenne (71 % pour un nodule de 1 cm de diamètre) (Bolondi et al., 2001; Caturelli et al., 2002). De plus, le dosage de l’AFP sérique ne doit pas être utilisé seul en raison de sa mauvaise valeur prédictive positive comprise entre 9 et 30 % (Sherman, 2001). La valeur de l’AFP est normale dans 80 % des cas lors de CHC de petite taille. Le principal intérêt de son dosage réside dans le suivi thérapeutique de patients traités qui présentaient un marqueur élevé avant prise en charge.

Le diagnostic est ensuite confirmé par différents examens radiologiques tels que l’imagerie par résonnance magnétique, la tomodensitométrie et l’échographie de contraste. En cas de tumeur atypique, une biopsie suivie d’une analyse histologique doivent être effectuées.

L’évaluation personnalisée de chaque patient permet de choisir une prise en charge adaptée en fonction du stade de la maladie. Les patients présentant un CHC précoce ont accès à des thérapies curatives telles que la résection chirurgicale, la transplantation hépatique et le traitement percutané (induction d’une nécrose des hépatocytes tumoraux par injection d’agents anticancéreux ou par thermothérapie localisée) (Forner et al., 2012). Cependant, le CHC précoce est asymptomatique et la sensibilité du dépistage est insuffisante, donc seulement 10% des cas sont diagnostiqués à un stade suffisamment précoce pour bénéficier d’un traitement curatif. En général, la transplantation hépatique reste la meilleure chance de guérison, mais elle n’est accessible qu'à un très petit nombre de patients en raison de la pénurie de greffons.

Il n'existe pas de traitement curatif aux stades plus avancés du CHC. La détérioration des fonctions hépatiques et en particulier la diminution des capacités de détoxification de l'organisme rendent les traitements anticancéreux inefficaces. Les patients avec un CHC intermédiaire bénéficient d'un traitement palliatif, la chimio-embolisation transartérielle (introduction d'agents thérapeutiques emboliques par voie endovasculaire). Pour les stades avancés, seule l'administration de Sorafénib, un inhibiteur de protéine-kinases (Raf, VEGF et c-Kit) qui inhibe la prolifération cellulaire et l'angiogenèse (Wilhelm et al., 2004), a montré un effet bénéfique sur la survie de 3 mois (Llovet et al., 2008). Au stade final, il est conseillé de donner des traitements symptomatiques uniquement.

2. La carcinogénèse hépatique

2.1. Progression histopathologique du CHC

Les CHC sont des tumeurs hétérogènes d'étiologies variées. La carcinogénèse hépatique est un processus complexe se développant dans 80% des cas sur un foie lésé depuis de nombreuses années (El-Serag and Rudolph, 2007; Sherman, 2010). Les différents facteurs de risques (voir section 1.1) créent des lésions hépatiques qui entrainent une nécrose suivie par la prolifération des hépatocytes **(Figure 19)**.

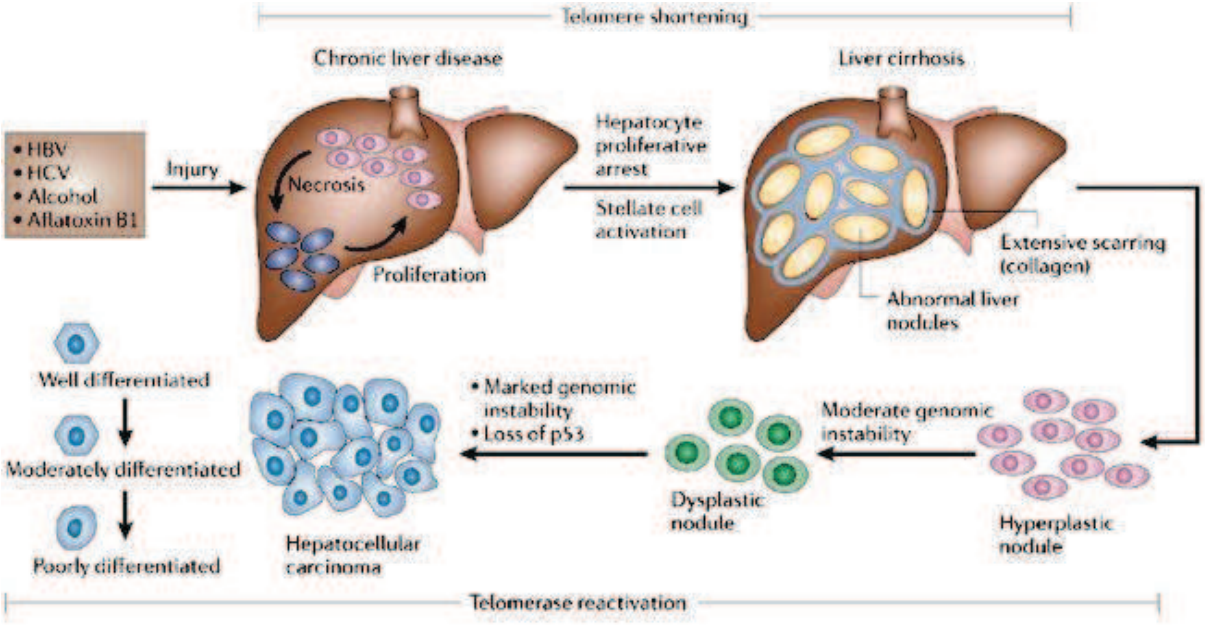


Figure 19 : Progression histopathologique du CHC(Farazi and DePinho, 2006)

Les cycles de nécrose/régénération des hépatocytes sont à l'origine d'une maladie chronique du foie, appelée cirrhose. Lors de la cicatrisation, les cellules inflammatoires (les cellules de Kupffer) envahissent le parenchyme hépatique. Les cellules étoilées du foie synthétisent du collagène et remplacent le parenchyme par du tissu fibreux, ce qui altère la structure de la matrice extracellulaire et le fonctionnement du microenvironnement hépatique. Des foyers d'hépatocytes anormaux apparaissent, puis des nodules dysplasiques se forment et peuvent dans certains cas dégénérer en CHC (Farazi and DePinho, 2006).

2.2. Les mécanismes moléculaires de la carcinogénèse hépatique

La survenue du CHC est le résultat de l'accumulation progressive de remaniements chromosomiques, d'altérations génétiques ou épigénétiques conduisant à la dérégulation de voies cruciales au fonctionnement de la cellule et qui aboutissent à la transformation maligne d'un hépatocyte. Les principales mutations sont retrouvées dans le gène p53 (environ 25-40% des cas), le gène de la β-caténine (CTNNB1) (environ 25% des cas) et le gène ARID1A (environ 12-17% des cas) (Guichard et al., 2012). Des amplifications (1q, 6p, 8q, 17q, 20q) et des délétions chromosomiques (4q, 8p, 11q, 13q, 16q, 17p) sont fréquentes. Les modifications épigénétiques sont mal définies mais l'inhibition de suppresseurs de tumeurs (comme l'E-cadhérine (CDH1)) ou la réactivation d'oncogènes (comme C-MYC) ont été décrites (Farazi and DePinho, 2006; Villanueva et al., 2007). Enfin, la modification du profil d'expression des miARNs peut moduler l'expression d'oncogènes et de suppresseurs de tumeurs importants (Zucman-Rossi, 2010).

2.2.1. La classification moléculaire des CHC

Ces dernières années, des analyses transcriptomiques d'un grand nombre de CHC ont permis de déterminer des groupes de tumeurs (Imbeaud et al., 2010) mais il n'existe pas encore de consensus international. En 2007, Boyault et al. ont proposé une classification des CHC en 6 groupes **(Figure 20)** qui corrèlent avec les altérations génétiques et les données cliniques de chaque tumeur (Boyault et al., 2007). Au cours de ma thèse, les échantillons de CHC que nous avons analysés avaient été classés selon cette étude.

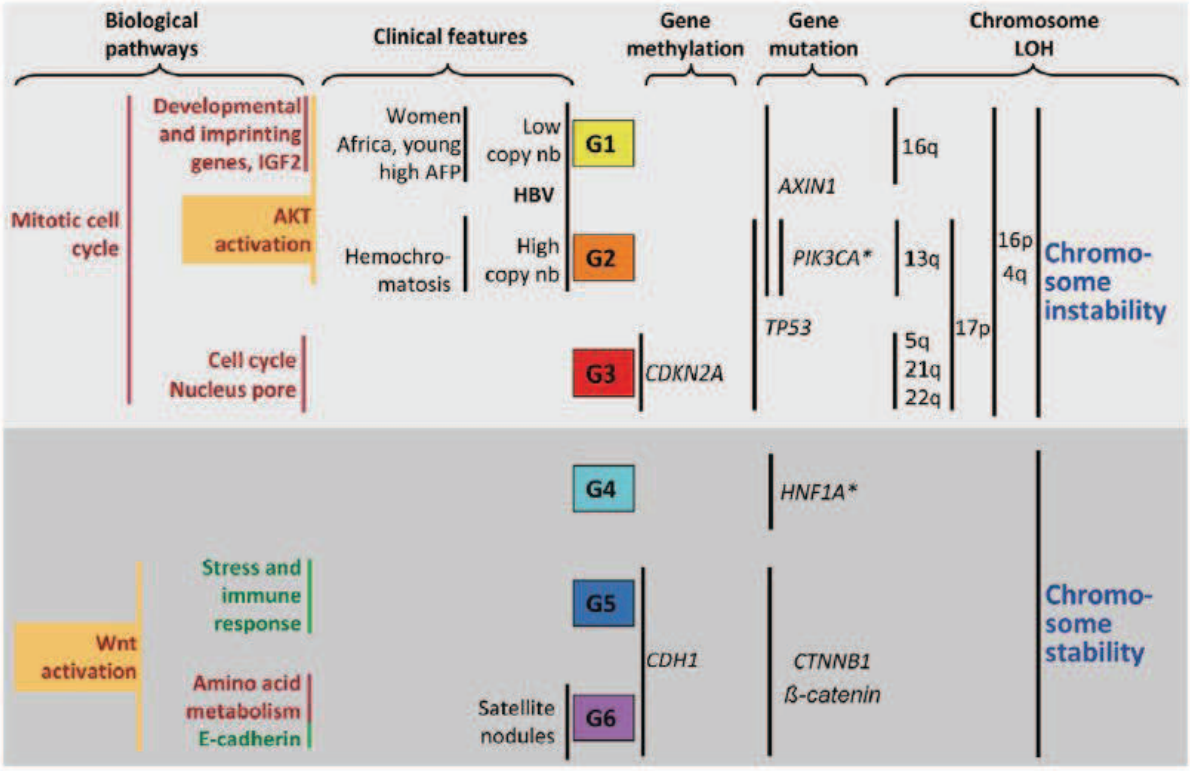


Figure 20 : Les différents groupes de CHC (Boyault et al., 2007)

Les groupes G1 à G3 sont les CHC de plus mauvais pronostic. Ils sont associés à une forte instabilité chromosomique, à des mutations de « tumor protein 53 » (p53 ou TP53 dans la Figure 20) et de l'Axine1, et à l'infection par le VHB. Le groupe G1 est associé avec un faible nombre de copies du VHB et à une surexpression de l'AFP. Le groupe G2 inclut des tumeurs avec un nombre élevé de copies de VHB et des mutations dans PIK3CA et p53. Les groupes G1 et G2 présentent aussi une activation spécifique de la voie AKT. Le groupe G3 est caractérisé par des mutations de p53 et une surexpression de gènes contrôlant le cycle cellulaire. Les groupes G4 à G6 sont associés à une relative stabilité chromosomique et à des mutations activatrices de la β-caténine. Le groupe G4 est hétérogène : il est représenté par des tumeurs mutées pour TCF1. Les groupes G5 et G6 contiennent les tumeurs mutées pour la β-caténine. Le groupe G6 est caractérisé par la présence de nodules satellites, une forte activation de la voie Wnt et une sous-expression de CDH1.

À la suite de ces modifications, plusieurs cascades de signalisation liées à la survie et à la prolifération cellulaire sont altérées. Les principales mutations sont retrouvées dans (i) la voie p53 qui joue un rôle primordial dans le maintien de l'intégrité du génome en induisant soit l'arrêt de la croissance cellulaire, soit l'apoptose en cas d'altérations importantes de

l'ADN, et (ii) la voie Wnt/β-caténine, qui est impliquée dans l'adhésion cellulaire, la différenciation, la prolifération cellulaire et la régénération hépatique.

2.2.2. La voie p53

P53 est le gène suppresseur de tumeur le plus fréquemment muté dans les CHC. La principale mutation est une substitution R249S qui est associée aux populations exposées à l'aflatoxine B1 et à l'infection par le VHB (Bressac et al., 1990; Hsu et al., 1991). C'est un facteur de transcription à l'intersection d'un réseau de voies de signalisation essentielles à la régulation de la croissance cellulaire et de l'apoptose induite par des stresses génotoxiques et non génotoxiques **(Figure 21)** (Vogelstein et al., 2000; Vousden, 2002).

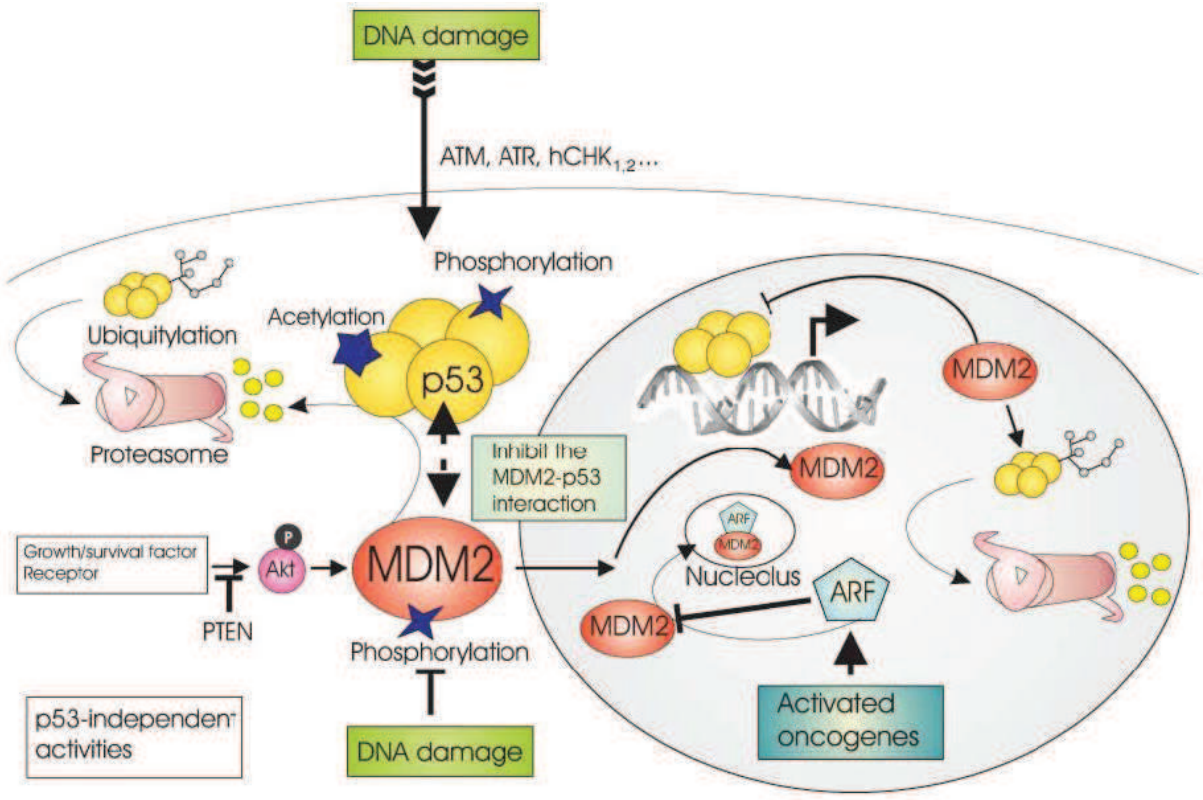


Figure 21 : La voie p53 (Moll and Petrenko, 2003)

Dans des cellules normales, MDM2 inhibe p53 en bloquant son activité transcriptionnelle, en favorisant son transport nucléo-cytoplasmique et en induisant son ubiquitinylation, puis sa dégradation par le protéasome. P53 peut être activée en réponse à différents agents causant des dommages à l'ADN ou en réponse à l'expression anormale d'oncogènes : (i) Les signaux de survie, tel que l'activation d'AKT, induisent l'import de

MDM2 dans le noyau. (ii) Les dommages à l'ADN entrainent une phosphorylation de p53 qui empêche son interaction avec MDM2. (iii) La surexpression d'oncogènes induit la protéine ARF qui séquestre MDM2 dans le noyau. Dans tous les cas, la protéine p53 activée s'accumule puis active l'expression de ses gènes cibles qui vont induire soit l'arrêt du cycle cellulaire et la réparation de l'ADN, soit la mort cellulaire par apoptose (Levine et al., 2006; Moll and Petrenko, 2003).

2.2.3. La voie Wnt/β-caténine

Wnt dérive du nom de ses deux orthologues « wingless », identifié chez la drosophile et « int-1 » chez la souris (Rijsewijk et al., 1987). Les protéines de la famille WNT sont des ligands sécrétés dans le milieu extracellulaire qui vont se fixer à différents récepteurs à la surface membranaire tels que « Frizzled » (Fzd) et « low-density lipoprotein receptor-related protein » (LRP). La reconnaissance du ligand par son récepteur active la voie de signalisation Wnt/β-caténine en contrôlant la β-caténine. La β-caténine est un facteur de transcription retrouvé au niveau des jonctions cellulaires adhérentes où il interagit avec CDH1 (symbolisé par E-CAD sur la Figure 22) dans le cytoplasme et le noyau.

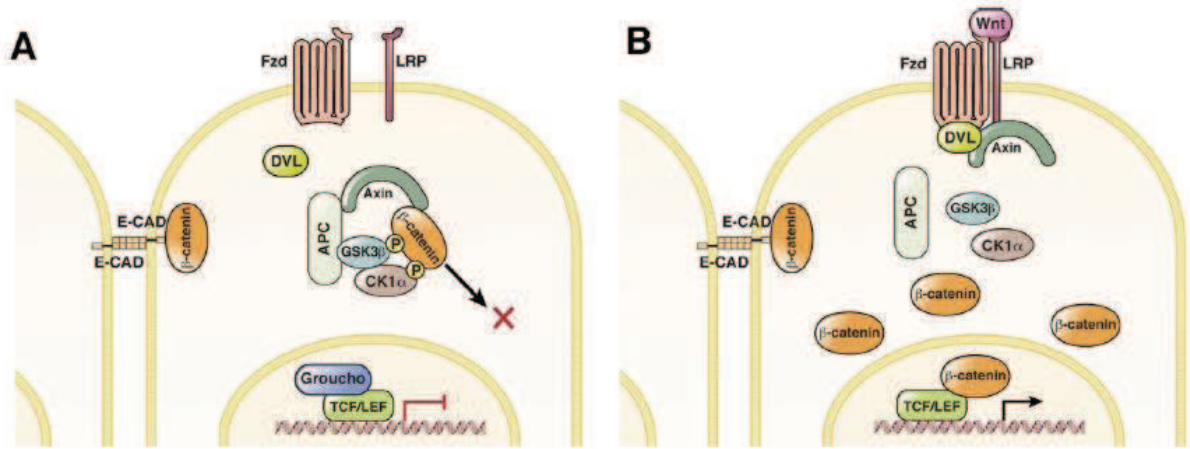


Figure 22 : La voie Wnt/β-caténine (White et al., 2012)

En absence d'activation de la voie de signalisation (**Figure 22A)**, la β-caténine interagit avec CDH1 ou est phosphorylée par le complexe de destruction composé de l'Axine, de « adenomatous polyposis coli » (APC), de la « glycogen synthase kinase 3 » (GSK3) et de la « casein kinase 1 » (CK1). La phosphorylation de la β-caténine dans son extrémité N-terminale provoque son ubiquitinylation, ce qui induit son adressage au protéasome et sa

dégradation. Dans cette configuration, l'expression des gènes cibles de la β-caténine est inhibée par Groucho, un répresseur transcriptionnel.

La liaison de Wnt aux récepteurs Fzd et LRP recrute « Disheveled » (DVL) et l'Axine à la membrane, ce qui dissocie le complexe de destruction **(Figure 22B)**. La β-caténine n'est plus phosphorylée, ce qui lui permet d'échapper à la dégradation par le protéasome, de s'accumuler dans le cytoplasme et d'être importée dans le noyau. Dans ce compartiment, la β-caténine interagit avec les membres de la famille de facteurs de transcription « T-cell factor/lymphoid enhancer factor » (TCF/LEF) et active la transcription de gènes cibles impliqués dans la différenciation, la prolifération, la migration et l'adhésion cellulaire.

La voie Wnt est constitutivement activée dans un tiers des CHC à cause (i) de mutations activatrices de la β-caténine (abolition de la phosphorylation du domaine N-terminal), (ii) des mutations perte de fonction d'APC, (iii) de l'inactivation de CDH1 ou (iv) de la surexpression de Fzd ou de WNT (White et al., 2012). Le Glypican-3, un corécepteur de Wnt qui est surexprimé dans 72% des CHC, stimule la voie Wnt/β-caténine et augmente la croissance tumorale en facilitant l'interaction de Wnt avec ses récepteurs (Capurro et al., 2005).

2.2.4. Les miARNs dans le CHC

2.2.4.1. Les signatures d'expression des miARNs

Dans la littérature plus de 400 articles ont pour sujet l'étude des miARNs dans le CHC. De nombreuses études ont comparé les profils d'expression des miARNs entre des échantillons de foie sain et pathologique. Mais en raison des différentes méthodologies utilisées, de l'origine variée des échantillons tissulaires et de l'importante hétérogénéité des tumeurs, il y a peu de résultats communs d'une étude à l'autre. De plus, les données ne sont pas forcément à jour. On compte aujourd'hui près de 2000 miARNs humains, alors que les analyses de miRNome ont été réalisées sur seulement quelques centaines de miARNs (Wang et al., 2012).

Globalement, les données obtenues révèlent que chaque facteur de risque est caractérisé par la dérégulation de plusieurs miARNs. La **Figure 23** récapitule les principaux miARNs qui ont été identifiés comme dérégulés dans plusieurs études indépendantes et qui sont impliqués dans différentes étapes de la carcinogénèse hépatique (Wang et al., 2012). L'étude du miRNome a donc permis d'établir différentes signatures d'expression à chaque étape de la

progression tumorale (foie sain ou lésé par les hépatites virales, l'alcool, les maladies métaboliques, foie fibrotique ou cirrhotique et CHC).

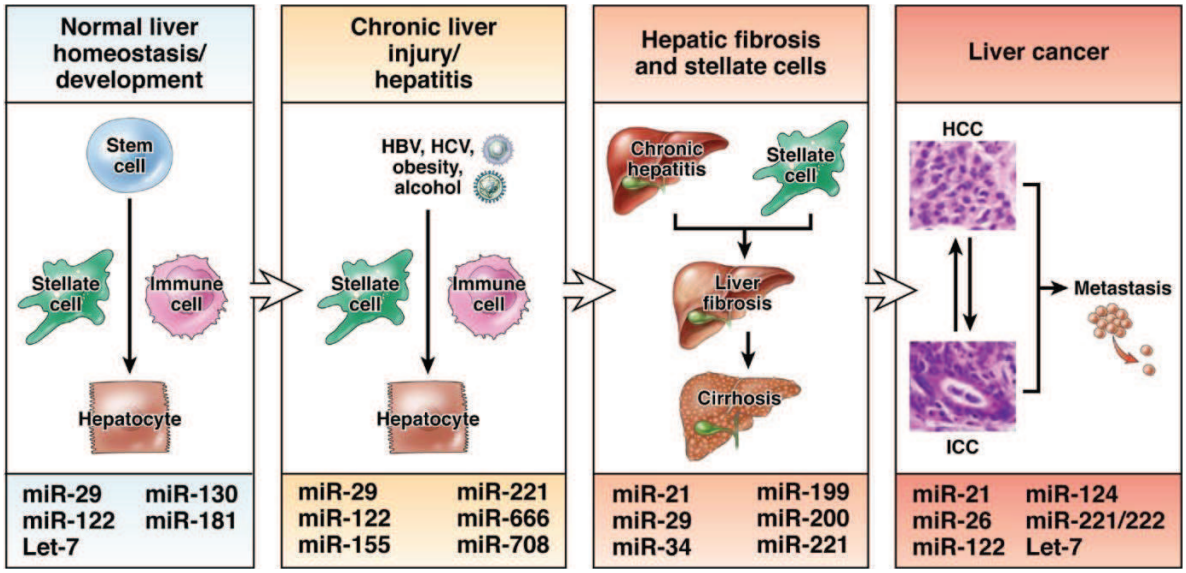


Figure 23 : Le profil d'expression des miARNs à chaque étape de la carcinogénèse hépatique (Wang et al., 2012)

2.2.4.2. Le réseau de régulation des miARNs dans le CHC

Les miARNs dérégulés dans le CHC et leurs gènes cibles forment un réseau complexe qui contrôle la progression du CHC et contribue à la croissance tumorale. De plus, ce réseau miARN-gènes cibles participe à l'inhibition de l'apoptose dans les cellules tumorales et à l'apparition de métastases **(Figure 24)**. La Figure 24 représente les principaux miARNs retrouvés dérégulés dans le CHC avec leurs cibles associées. Au cours de ma thèse, j'ai utilisé miR-21 et miR-122 comme contrôles.

MiR-21 est l'oncomiR le plus fréquemment surexprimé dans les cancers. Il réprime l'expression du gène suppresseur de tumeur PTEN, ce qui contribue à la progression tumorale dans le CHC (Meng et al., 2007). L'inhibition de miR-21 diminue la prolifération cellulaire, la migration et l'invasion (Meng et al., 2006). Son expression est également associée aux stades avancés des tumeurs hépatiques et à un mauvais pronostic de survie des patients (Yan et al., 2008).

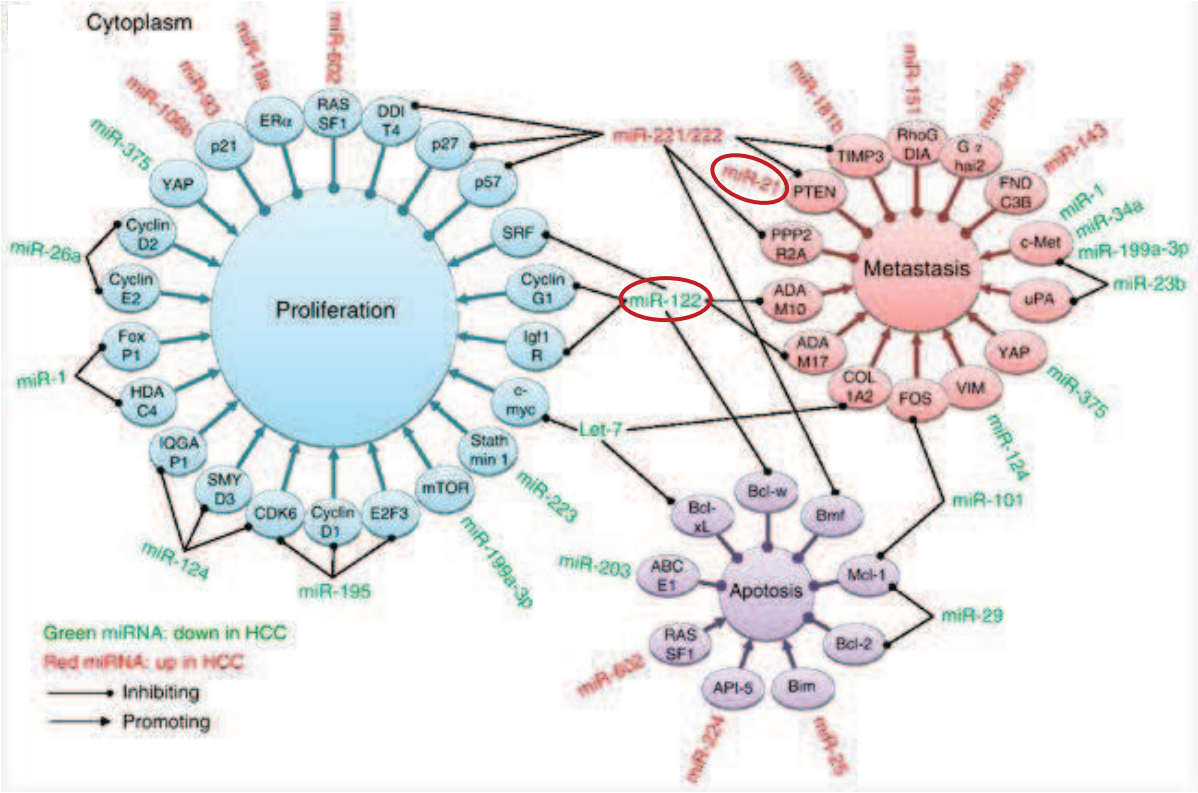


Figure 24 : Réseau de miARNs impliqués dans la progression du CHC (Huang and He, 2011)

MiR-122 est un miARN suppresseur de tumeur qui est spécifique du foie. Il représente 70% des miARNs exprimés dans les hépatocytes et son expression est réduite dans le CHC. Il peut contrôler de multiples cibles et affecter plusieurs processus biologiques. Il module notamment le métabolisme lipidique, le développement tissulaire du foie et la différenciation hépatocytaire. Chez les patients infectés par le VHC, miR-122 potentialise la fixation des ribosomes à l'ARN viral du VHC et stimule la traduction des protéines virales. Cette découverte a mené au premier essai clinique concernant un miARN. Le traitement utilise un oligonucléotide inhibiteur ou antimiR dirigé spécifiquement contre miR-122 (Jopling et al., 2005). L'administration de l'antimiR-122 bloque la réplication virale hépatique chez le singe (Elmen et al., 2008 ; Lanford et al., 2010). Chez l'Homme, cette molécule est en essai clinique de phase II. Les résultats actuels montrent qu'après 4 semaines de traitement, la quantité d'ARN viral est diminuée de 2 à 3 log et ce, sans toxicité apparente (Mendell and Olson, 2012).

Dans le CHC, miR-122 inhibe l'expression de la Cycline G1, d'ADAM10, d'ADAM17, de SRF, d'Igf1R et de Bcl-w (Huang and He, 2011). La diminution de miR-122 semble corréler avec l'apparition de métastases et la perte des principales fonctions hépatiques. A l'opposé, sa surexpression réduit la tumorigénèse des cellules cancéreuses, l'angiogénèse et

l'apparition de métastases dans un modèle orthotopique murin de CHC (Tsai et al., 2009b). Contrairement à l'hépatite virale C, la stratégie thérapeutique à adopter pour le CHC serait donc une surexpression d'un analogue synthétique de miR-122. En raison des effets multiples de miR-122 et de son implication dans différentes pathologies hépatiques (Huang and He, 2011), l'utilisation d'une stratégie thérapeutique basée sur ce miARN est à prendre avec beaucoup de précautions.

3. Le Glypican-3

3.1. La structure des glypicans

Le Glypican-3 (GPC3) est une protéine appartenant à la famille des « heparan sulfate proteoglycans » (HSPG). Cette famille de HSPGs est composée de six membres chez les mammifères (GPC1-GPC6). Les glypicans sont constitués d'un squelette peptidique ou protéine « core » (60-70 kDa) possédant quatorze cystéines dont la position est conservée entre les espèces et qui forment une région globulaire *via* des ponts disulfures **(Figure 25)**.

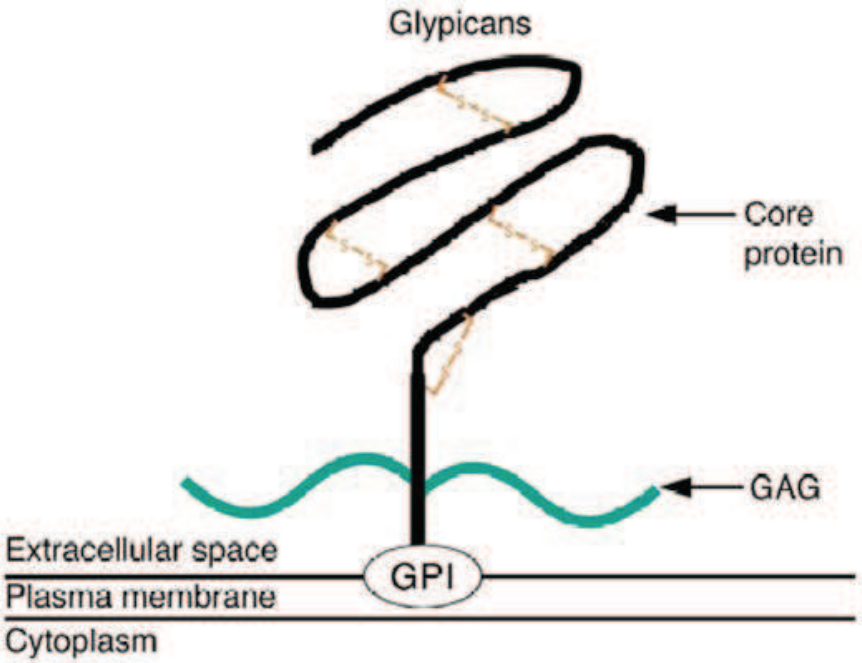


Figure 25 : Structure des glypicans (Filmus and Selleck, 2001)

La protéine "core" est située sur la face externe de la membrane plasmique de la cellule, liée à celle-ci grâce à une ancre « glycosyl-phosphatidylinositol » (GPI) (David, 1993), mais les glypicans peuvent également exister sous forme soluble. La région globulaire comporte trois à

quatre sites d'attachement exclusifs des chaînes d'héparanes-sulfates, le tout formant un large domaine extracellulaire (Filmus and Selleck, 2001). Le domaine N-terminal contient une séquence peptide signal permettant la translocation de la chaîne peptidique vers le lumen du réticulum endoplasmique et possède des sites de N-glycosylation pour l'ajout glycosaminoglycanes (GAG). La séquence consensus DSGSGSG pour l'attachement des chaînes d'héparanes-sulfates est située entre le domaine central et l'extrémité C-terminale. Cette portion C-terminale possède la séquence signal nécessaire à l'insertion des glypicans au niveau de la membrane plasmique des cellules *via* l'ancre GPI.

3.2. Les fonctions biologiques des glypicans

Les fonctions du GPC3 ne sont pas entièrement élucidées. Les chaînes d'héparanes-sulfates ont une forte charge négative. Grâce à elles, les glypicans sont capables, par des interactions électrostatiques, de stabiliser l'interaction du ligand avec son récepteur et de jouer un rôle de co-récepteur de faible affinité. En effet, il a été montré que les chaînes d'héparanes-sulfates interagissent avec des cytokines et différents facteurs de croissance tels que le « Fibroblast Growth Factor 2 » (FGF2) et la « Bone Morphogenic Proteins-7 » (BMP7) (Midorikawa et al., 2003; Song et al., 1997). Les glypicans peuvent être clivés et libérés sous forme soluble dans le milieu extracellulaire sous l'action de la lipase Notum (Filmus et al., 2008; Traister et al., 2008). Ils peuvent en piégeant des ligands extracellulaires en contrôler la diffusion et la répartition tissulaire. Ainsi, ces molécules sont capables de moduler la transduction du signal associé à des processus physiologiques tels que le développement, la prolifération cellulaire et l'angiogenèse (Whitelock and Iozzo, 2005).

Chez *D. melanogaster,* deux membres de la famille Glypican ont été identifiés : « division abnormally delayed » (DALLY) qui est l'orthologue du GPC3 et 5 ; dally-like (DLY) qui est l'orthologue du GPC1, 2, 4 et 6 (Filmus et al., 2008). Des formes mutantes de DALLY ou DLY génèrent de nombreuses anomalies développementales (Nakato et al., 1995) et affectent la voie Wingless au cours du développement embryonnaire (Baeg et al., 2001).

Chez les mammifères, le GPC3 régule (i) la signalisation de nombreux facteurs de croissance par l'intermédiaire d'une interaction avec les chaînes d'héparanes-sulfates (Midorikawa et al., 2003) et (ii) les voies de signalisation Wnt et « Hedgehog » (Hh) grâce à une interaction qui fait intervenir le « core » de la protéine (Capurro et al., 2008; Ho and Kim, 2011) **(Figure 26)**.

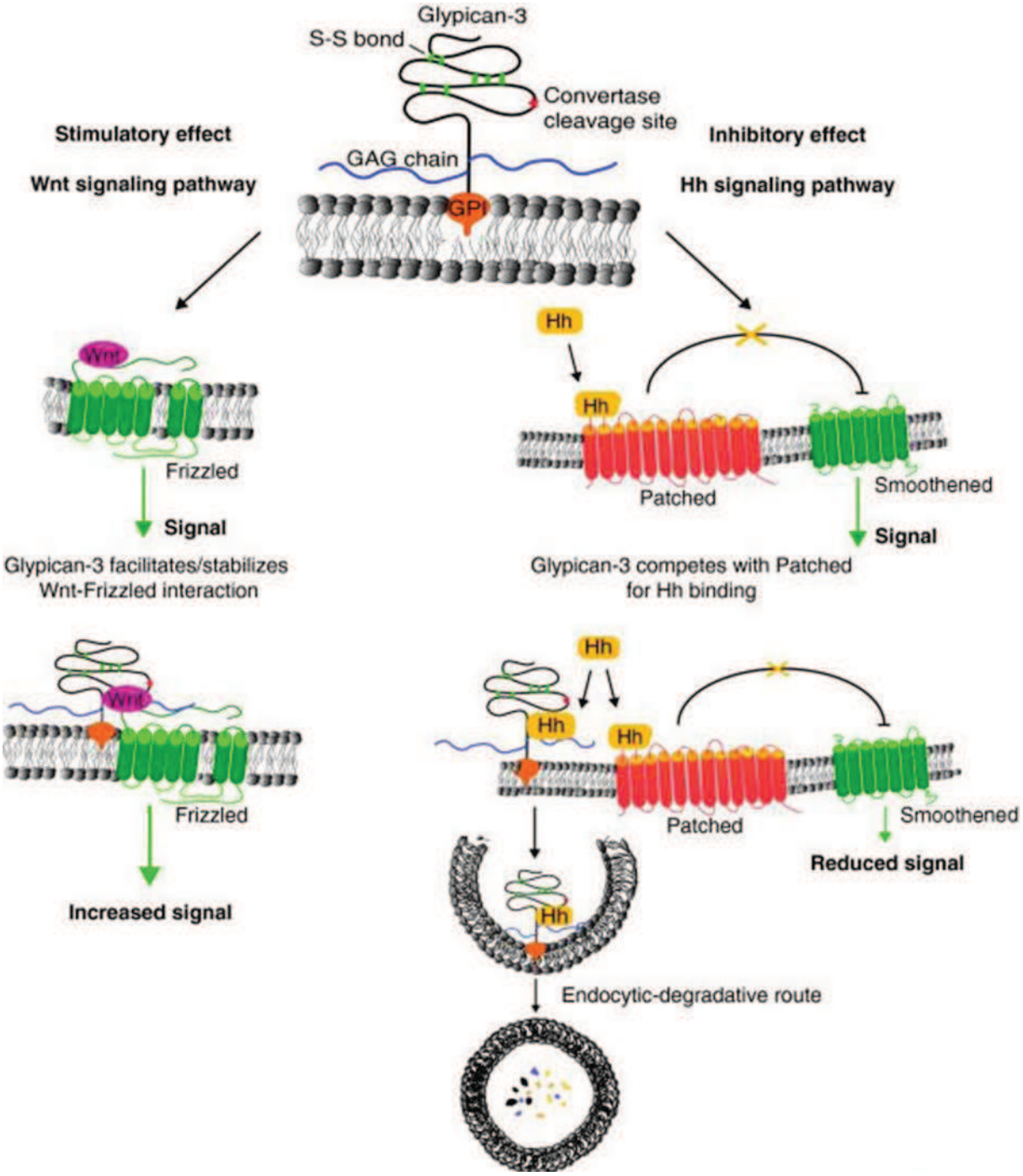


Figure 26 : Les effets positifs et négatifs du GPC3 sur la signalisation cellulaire (Filmus and Selleck, 2001)

Dans la voie de signalisation Wnt **(Figure 26 à gauche)**, le GPC3 exerce un effet positif. Wnt se lie au récepteur de Fzd pour induire la voie de signalisation Wnt/β-caténine. Le GPC3 concentre le ligand Wnt à proximité de son récepteur Fzd, ce qui facilite ou stabilise leur interaction et augmente l'activation du signal (Capurro et al., 2005). Dans la voie de signalisation Hedgehog (Hh) **(Figure 26 à droite)**, le GPC3 exerce un effet inhibiteur. En absence d'activation, Patched (PTC), le récepteur de Hh, inhibe Smoothened et bloque le signal de transduction. La liaison de Hh à PTC réprime l'inhibition de Smoothened et

déclenche la voie de signalisation. Le GPC3 est un récepteur de Hh en compétition avec PTC. L'interaction de Hh avec le GPC3 déclenche l'endocytose et leur dégradation, ce qui réduit la quantité de protéine Hh et diminue la transduction du signal (Capurro et al., 2008).

Selon le contexte biologique, le GPC3 peut stimuler ou réprimer la prolifération cellulaire en modulant les voies de signalisation Wnt et Hh. De plus, le GPC3 est une protéine oncofœtale, son profil d'expression varie au cours de l'embryogénèse, disparaît chez l'adulte, mais réapparaît dans certains types de cancer, ce qui souligne son importance dans le contrôle de la croissance cellulaire durant la morphogénèse et dans les mécanismes de cancérisation (Li et al., 1997) **(Figure 27)**.

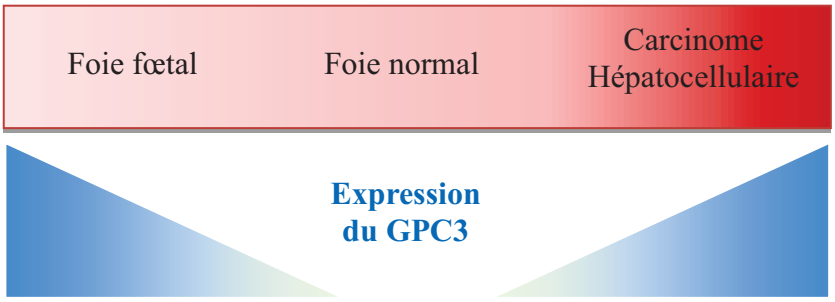


Figure 27 : Le profil d'expression du GPC3 dans le foie

3.3. Les fonctions physiopathologiques du GPC3

3.3.1. Le syndrome de Simpson-Golabi-Behmel

Chez l'homme, la mutation ponctuelle du gène codant pour le GPC3 est responsable du syndrome de Simpson-Golabi-Behmel (SGBS) (Filmus and Selleck, 2001 ; Jakubovic and Jothy, 2007). Le tableau clinique de ce syndrome est très lourd. Il est caractérisé par une macromégalie faciale, une surcroissance prénatale et postnatale, une prédisposition tumorale, un défaut cardiaque congénital, une syndactylie, une polydactylie et une dysplasie rénale. Les mutations retrouvées produisent des GPC3 dont le rôle dans la signalisation des facteurs de croissance au cours du développement est altéré (Pilia et al., 1996). Dans ce contexte, les anomalies développementales du SGBS montrent que le GPC3 a un rôle inhibiteur de la prolifération cellulaire et la prédisposition tumorale indiquerait qu'il a un rôle suppresseur de tumeur (Gonzalez et al., 1998).

3.3.2. L'ostéochondromatose

L'ostéochondromatose ou « Hereditary Multiple Exostoses » (HME) est une affection rare où de multiples tumeurs bénignes, appelées ostéochondromes, se développent à la surface des os. Cette maladie est causée par la mutation des « Exostosin-1 et 2 » (EXT1 et EXT2) codant pour des glycosyltransférases localisées dans la membrane du réticulum endoplasmique et impliquées dans la biosynthèse des chaînes d'héparanes-sulfates (Lind et al., 1998). La production de chaînes d'héparanes-sulfates anormales induit une croissance cellulaire aberrante qui peut se transformer en tumeur maligne (chondrosarcome et ostéosarcome) dans 0,5 à 2% des cas (Wicklund et al., 1995).

3.3.3. Le CHC

Les découvertes réalisées chez *D. melanogaster* ainsi que les phénotypes obtenus dans le SGBS et le HME ont clairement indiqué que le GPC3 a un rôle dans la croissance cellulaire et le développement. La transformation maligne peut se produire par l'intermédiaire des glypicans soit en modifiant leur niveau d'expression à la membrane cellulaire, soit en changeant la structure des chaînes d'héparanes-sulfates (Sasisekharan et al., 2002). Le GPC3 est surexprimé dans de nombreux cancers dont le CHC, l'hépatoblastome, les tumeurs de Wilm's, le mélanome, le neuroblastome, certains carcinomes ovariens et tumeurs testiculaires. Mais il est aussi retrouvé sous-exprimé dans le cancer du sein, le mésothéliome, le cancer ovarien épithélial et l'adénocarcinome pulmonaire (Ho and Kim, 2011).

Dans le CHC, le GPC3 est surexprimé dans 72% des cas. Il n'est pas détecté dans le foie normal ou les tumeurs bénignes et son niveau d'expression corrèle négativement avec la survie du patient, ce qui en fait un nouveau marqueur diagnostic et pronostic prometteur (Capurro et al., 2003; Hsu et al., 1997 ; Zhu et al., 2001). En effet, la forme soluble du GPC3 est détectable dans le sérum des patients atteints d'un CHC et la combinaison de l'AFP et du GPC3 comme marqueurs moléculaires permet d'augmenter la spécificité du diagnostic du CHC à 82% (Capurro et al., 2003). Le GPC3 peut aussi permettre la recherche de micrométastases dans la moelle osseuse des patients (Sutcliffe et al., 2005).

Dans le CHC, le GPC3 participe au processus oncogénique. D'une part, la « Sulfatase 2 » (SULF2), une endosulfatase des chaînes d'héparanes-sulfates, élimine les groupements sulfates du GPC3, ce qui augmente la fixation de FGF2 et amplifie l'activation du signal FGF.

D'autre part, SULF2 augmente l'expression du GPC3 qui stabilise l'interaction WNT-FZD (voir section II 3.2). WNT, GPC3 et SULF2 formeraient un complexe qui amplifierait l'activation de la voie Wnt et stimulerait la croissance, la différenciation et la migration des cellules tumorales (Gao and Ho, 2011 ; Lai et al., 2010; Lai et al., 2008).

De plus, le GPC3 est une cible thérapeutique intéressante contre le CHC car il correspond à un antigène fortement et spécifiquement exprimé par les cellules cancéreuses (Ho and Kim, 2011). L'anticorps anti-GPC3 « GC33 » réprime la croissance tumorale chez la souris (Ishiguro et al., 2008 ; Nakano et al., 2009). Actuellement, la version humaine de l'anticorps hGC33 est en cours d'essai clinique sur des patients atteints d'un CHC avancé ou métastatique et en combinaison avec du Sorafenib ou non (ClinicalTrials.gov NCT00746317 et NCT00976170).

3.4. La régulation de l'expression génique du GPC3

Dans la section précédente, nous avons souligné qu'en fonction du tissu et de l'état de différenciation cellulaire, le GPC3 peut jouer un rôle pro ou anti-oncogénique. Ceci suggère que le contrôle de l'expression du GPC3 a un rôle fondamental dans la complétion de ses fonctions. Le profil d'expression du GPC3 semble être finement régulé au cours du développement embryonnaire et lors de la tumorigénèse, cependant les mécanismes régulant son expression génique sont peu connus.

- Régulation épigénétique :

Le GPC3 est localisé sur le chromosome Xq26 et il peut être soumis à une régulation dépendant de l'inactivation du chromosome X. Dans des conditions physiologiques, sa région promotrice n'est pas méthylée chez les hommes, mais est partiellement méthylée chez les femmes. L'hyperméthylation de son promoteur associée à une inhibition de son expression a déjà été observée dans des cancers ovariens et mammaires (Lin et al., 1999 ; Xiang et al., 2001). À l'opposé, une perte de méthylation de la région promotrice du GPC3 a été observée chez les femmes atteintes de neuroblastome (Boily et al., 2004). Ces données coïncident avec une autre étude qui révèle que le GPC3 est plus fréquemment surexprimé chez les femmes atteintes de CHC (95% vs 67% chez les hommes) (Hsu et al., 1997).

- Régulation transcriptionnelle :

Les facteurs de transcription Sp1 et NFY peuvent se fixer sur le promoteur du GPC3 et activer la transcription de ce gène (Boily et al., 2007; Huber et al., 1998). Le GPC3 et l'AFP ont des profils d'expression similaires dans le foie. Morford et collègues ont donc émis l'hypothèse que ces deux gènes pourraient avoir des facteurs régulateurs en commun. En effet, « zinc fingers and homeoboxes 2 » (ZHX2) et « alpha-foetoprotein regulator 2 » (AFR2) sont deux facteurs de transcription contrôlant l'expression du GPC3 et de l'AFP. ZHX2 réprime l'expression du GPC3 dans le foie adulte et AFR2 active son expression dans le foie en régénération (Morford et al., 2007)). Dans le CHC, SULF2 (voir II 3.3.3.) augmente l'expression du GPC3 par un mécanisme encore inconnu (Lai et al., 2008). Récemment, le GPC3 a été identifié comme une cible transcriptionnelle de c-MYC et la surexpression de cet oncogène corrèle avec la surexpression du GPC3 dans le CHC (Li et al., 2012). C-MYC active la transcription du GPC3 et réciproquement le GPC3 augmente l'expression de c-MYC, ce qui forme une boucle de régulation positive.

OBJECTIFS

La compréhension des mécanismes altérant l'expression du GPC3 dans le CHC permettrait de mieux appréhender sa fonction dans les mécanismes pro-oncogéniques. L'ensemble des régulateurs connus actuellement ne suffit pas pour expliquer le profil d'expression complexe du GPC3 dans ce cancer, ce qui suggère qu'il existerait d'autres régulateurs.

À mon arrivée dans le laboratoire, Laloo et collaborateurs avaient développé la méthode « **Fun**ctional integrated and quantitative analyse to measure post-transcriptional **REG**ulation » (FunREG) ((Laloo et al., 2009) et **Annexe 2**). À titre indicatif, la méthode FunREG utilise deux populations cellulaires transduites par un transgène lentiviral différent **(Figure 28)** contenant (i) une région 3'NT de référence ou (ii) la région 3'NT du GPC3. Ces deux transgènes contiennent la séquence codante de l'eGFP sous le contrôle transcriptionnel du promoteur constitutif et ubiquitaire EF1α.

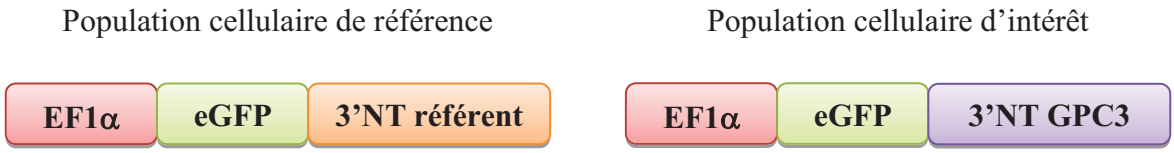


Figure 28 : Schéma des transgènes utilisés dans la méthode FunREG

Les deux populations ont le même taux de transcription de l'eGFP, car elles sont issues du même type cellulaire et expriment l'eGFP à partir du même promoteur. Par conséquent, les variations d'expression de l'eGFP entre les deux populations sont dues à des mécanismes de régulation post-transcriptionnelle spécifique de la région 3'NT d'intérêt. La méthode FunREG permet de mesurer de manière quantitative et comparative la régulation post-transcriptionnelle entre des cellules normales et des lignées tumorales.

Grâce à cette méthode, l'équipe a montré que le GPC3 est surexprimé dans les lignées de CHC par rapport aux hépatocytes normaux, en partie par un mécanisme de régulation post-transcriptionnelle dépendant de sa région 3'NT (Maurel et al., 2012). Ce résultat indique qu'il y a des facteurs régulateurs agissant en *trans* au niveau de la région 3'NT du GPC3 qui sont dérégulés entre les lignées de CHC et les hépatocytes normaux.

Comme les miARNs sont des régulateurs importants de l'expression génique et qu'ils sont largement décrits comme dérégulés dans le cancer, nous avons émis l'hypothèse que **certains miARNs dérégulés dans le CHC altèrent l'expression du GPC3 par un mécanisme dépendant de sa région 3'NT**.

C'est dans ce cadre que s'inscrit l'objectif principal de ma thèse qui est d'identifier les miARNs régulateurs du GPC3 qui participent à sa surexpression dans le CHC. Le premier objectif de mon travail de thèse a été de mettre au point un crible fonctionnel, basé sur le principe du FunREG, pour identifier de manière systématique l'ensemble des miARNs régulant l'expression du GPC3 *via* sa région 3'NT. Le second objectif de ce travail visait à valider et à caractériser le mode d'action de miARNs candidats identifiés dans le crible. J'ai plus particulièrement focalisé mon attention sur :

- miR-1271 qui est globalement sous-exprimé dans le CHC et inhibe l'expression du GPC3 (Manuscrit 1).

- miR-1291 qui augmente l'expression du GPC3 et est surexprimé dans certains groupes de CHC (Manuscrit 2).

RÉSULTATS

I. Manuscrit 1

1. Introduction

A mon arrivée au laboratoire, j'ai eu l'opportunité de participer techniquement à un projet dont l'objectif principal visait à identifier des miARNs régulateurs de l'expression du GPC3 sur la base exclusive de prédictions réalisées à partir d'outils bioinformatiques ((Jalvy-Delvaille et al., 2012) en **Annexe 3**). Les prédictions réalisées par miRWalk indiquent que deux cents miARNs peuvent s'apparier à la région 3'NT du GPC3. MiR-96 et miR-182 sont les deux miARNs ayant la plus forte probabilité de cibler cette région (6/10 et 5/10 algorithmes testés). Ces deux miARNs appartiennent au cluster miR-96/182/183 et sont des paralogues, car ils possèdent la même séquence graine. À l'aide de la méthode FunREG, nous avons montré que miR-96 inhibe l'expression du GPC3 alors que miR-182 en est incapable. Nos résultats montrent que ces deux paralogues ne régulent pas les mêmes gènes, car ils diffèrent par leur type d'appariement avec leur cible. L'appariement prédit entre le GPC3 et miR-96 est de type 8mer alors qu'il est de type 7mer-1A avec miR-182 (voir section I. 2.1.1).

Ces données montrent que la complexité et la subtilité des mécanismes d'interaction miARN : ARNm a un impact important sur l'expression des gènes cibles. Même si les outils de prédiction permettent de rechercher de manière globale les interactions possibles entre l'ensemble des miARNs et une cible donnée, cependant ils fournissent une liste de plusieurs centaines de candidats qui ne peuvent pas être tous testés expérimentalement de manière individuelle.

Pour résoudre ce problème, le premier objectif de ma thèse a visé à développer un crible fonctionnel pour identifier l'ensemble des miARNs régulateurs du GPC3. Pour cela, j'ai modifié la méthode FunREG en utilisant la fluorescence de deux gènes rapporteurs (eGFP et tdTomato) coexprimés dans la même lignée. J'ai criblé une bibliothèque de 876 miARNs humains et validé cinq miARNs régulateurs du GPC3. Deux d'entre eux sont dérégulés dans le CHC, miR-1271 et miR-1291. Dans ce manuscrit, je me suis d'abord focalisé sur le rôle de miR-1271 dans la régulation de l'expression du GPC3.

Functional screening identifies five microRNAs controlling Glypican-3: Role of miR-1271 down-regulation in hepatocellular carcinoma

Functional Screening Identifies Five MicroRNAs Controlling Glypican-3: Role of miR-1271 Down-regulation in Hepatocellular Carcinoma

Marion Maurel,[1,2] Sandra Jalvy,[1,2]* Yannick Ladeiro,[3,4]* Chantal Combe,[1,2] Laetitia Vachet,[1,2] Francis Sagliocco,[1,2] Paulette Bioulac-Sage,[1,2] Vincent Pitard,[5,6] Hélène Jacquemin-Sablon,[1,2] Jessica Zucman-Rossi,[3,4] Benoît Laloo,[1,2] and Christophe F. Grosset[1,2]

Hepatocellular carcinoma (HCC) is the major primary liver cancer. Glypican-3 (*GPC3*), one of the most abnormally expressed genes in HCC, participates in liver carcinogenesis. Based on data showing that *GPC3* expression is posttranscriptionally altered in HCC cells compared to primary hepatocytes, we investigated the implication of microRNAs (miRNAs) in *GPC3* overexpression and HCC. To identify *GPC3*-regulating miRNAs, we developed a dual-fluorescence FunREG (functional, integrated, and quantitative method to measure posttranscriptional regulations) system that allowed us to screen a library of 876 individual miRNAs. Expression of candidate miRNAs and that of *GPC3* messenger RNA (mRNA) was measured in 21 nontumoral liver and 112 HCC samples. We then characterized the phenotypic consequences of modulating expression of one candidate miRNA in HuH7 cells and deciphered the molecular mechanism by which this miRNA controls the posttranscriptional regulation of *GPC3*. We identified five miRNAs targeting *GPC3* 3′-untranslated region (UTR) and regulating its expression about the 876 tested. Whereas miR-96 and its paralog miR-1271 repressed *GPC3* expression, miR-129-1-3p, miR-1291, and miR-1303 had an inducible effect. We report that miR-1271 expression is down-regulated in HCC tumor samples and inversely correlates with *GPC3* mRNA expression in a particular subgroup of HCC. We also report that miR-1271 inhibits the growth of HCC cells in a *GPC3*-dependent manner and induces cell death. *Conclusion:* Using a functional screening, we found that miR-96, miR-129-1-3p, miR-1271, miR-1291, and miR-1303 differentially control *GPC3* expression in HCC cells. In a subgroup of HCC, the up-regulation of *GPC3* was associated with a concomitant down-regulation of its repressor miR-1271. Therefore, we propose that *GPC3* overexpression and its associated oncogenic effects are linked to the down-regulation of miR-1271 in HCC. (HEPATOLOGY 2012;00:000-000)

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer.[1] It usually develops on an affected liver with cirrhosis due to viral infection (hepatitis B virus, HBV; hepatitis C virus, HCV), alcohol abuse, metabolic disorders, or carcinogenic agent.[1-3] HCC is a very heterogeneous class of tumors characterized by multiple genomic damage associated with its various etiologies.[2-4] This tumor diversity arises from multistep hepatocarcinogenic processes requiring sequential genetic

Abbreviations: AFP, alpha-fetoprotein; AM1271, antimiR-1271; ATP, adenosine triphosphate; CT, control 3′-UTR; DF-FunREG, dual fluorescence-FunREG; FACS, fluorescence-activated cell sorting; FunREG, functional, integrated, and quantitative method to measure posttranscriptional regulations; GFP, enhanced green fluorescent protein; GLO, β-globin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; M, GFP mRNA; miRNA, microRNA; m.o.i., multiplicity(ies) of infection; mRNA, messenger RNA; NTL, nontumoral liver; P, GFP protein; PCR, polymerase chain reaction; qPCR, quantitative PCR; qRT-PCR, quantitative reverse transcription PCR; siRNA, small interfering RNA; TCN, transgene copy number; TOM, tdTomato; UTR, untranslated region.

From the [1]Univ. Bordeaux, Physiopathologie du cancer du foie, Bordeaux, France; [2]INSERM, Physiopathologie du cancer du foie, Bordeaux, France; [3]Université Paris Descartes, Labex Immuno-oncology, Sorbonne Paris Cité, Faculté de Médecine, Paris, France; [4]INSERM, Altération génétique des tumeurs hépatiques, Paris, France; [5]University Bordeaux, CIRID, Bordeaux, France; and [6]CNRS, CIRID, Bordeaux, France.

Received February 16, 2012; accepted July 25, 2012.

Supported by the Agence Nationale pour la Recherche (ANR)-Programme Jeunes Chercheurs (Paris, France) (Grant no: JC07_184264 to C.G.), by La Ligue Nationale Contre le Cancer, by the Biointelligence consortium (OSEO) and INCa (PAIR-CHC). M.M and Y.L. were recipients of fellowships from the Ministère de l'Enseignement Supérieur et de la Recherche (MESR) and ANRS, respectively.

and epigenetic alterations including gene mutations and/or chromosome instability.[1-5]

Glypican-3 (*GPC3*), one of the numerous genes abnormally expressed in HCC, participates in hepatocarcinogenesis.[4,6,7] The GPC3 protein belongs to the family of heparan-sulfate proteoglycans. Glypicans act as coreceptors and control signaling pathways by regulating growth factor/cell-surface receptor interactions.[6,7] In adult liver, *GPC3* is generally not detectable. Nevertheless, this oncofetal protein plays a critical role in liver regeneration and hepatic growth during embryogenesis.[6,7] As shown in the X-linked genetic Simpson-Golabi-Behmel syndrome, loss-of-function mutations of *GPC3* cause postnatal overgrowth with multiple congenital anomalies, highlighting its implication in control of cell proliferation and tissue growth.[7] *GPC3* is strongly expressed in most tumoral hepatic tissues in which it potentiates the malignancy of hepatic cells through the canonical Wnt/β-catenin pathway.[6,7] In HCC, its expression is associated with a poor histological tumor differentiation and a high-proliferative state of cancerous hepatic cells,[3,8] as well as with poor prognosis and short overall survival.[9] These observations suggest that *GPC3* expression is strongly linked to aggressive behavior of the tumors and are in agreement with its increased expression in several embryonic cancers.[6,7] Therefore, *GPC3* clearly represents a relevant molecular target in several cancers, including HCC.[6,7]

Besides deregulations occurring as a result of gene amplification, gene mutation, or transcriptional variations during carcinogenesis, there is now clear evidence that microRNAs (miRNAs), actively participate in gene misexpression.[3,5,10,11] MiRNAs are small noncoding RNAs that control gene expression by modulating stability and/or translation of messenger RNA (mRNA)[12,13] through interactions with specific sequences located in either the coding or the untranslated regions (UTR).[14] MiRNAs are intricately involved in human diseases and actively participate in carcinogenesis as oncogenes or tumor suppressor genes.[10,11] Although the effects mediated by miRNAs on any particular target are modest, the simultaneous regulation of a broad array of targets by one miRNA can lead to a profound gene reprogramming and cell-phenotype changes.[10,11,15] In the cancerous context, restoring high levels of a tumor suppressor miRNA or inhibiting the biological activity of an oncogenic miRNA constitutes a very promising avenue of investigation in anticancer therapy.[15]

We previously showed that posttranscriptional dysfunctions associated with 3′-UTRs of genes are deregulated in two examples of human pathological disorders.[13,16,17] Here we report that the 3′-UTR-mediated posttranscriptional regulation of *GPC3* is altered in HCC cells and that the 3′-UTR favors *GPC3* expression in HCC. In an attempt to identify miRNAs involved in this dysfunction, we developed a methodology, called dual-fluorescence functional, integrated, and quantitative method to measure posttranscriptional regulations (DF-FunREG), which allows the functional screening of miRNA libraries and the systematic identification of miRNAs controlling one gene. We report the regulation of *GPC3* expression by five miRNAs. Among them, two *in silico*-predicted miRNAs acted negatively on *GPC3* expression,[18] whereas three acted positively. Finally and importantly, we report that expression of one of these miRNAs is broadly decreased in HCC tumors and show that its down-regulation contributes to *GPC3* overexpression and HCC-cell growth.

Materials and Methods

Plasmids. Plasmids are as described in the Supporting Information.

Cell Lines and Primary Hepatocytes. Fresh primary human hepatocytes (Biopredic, Rennes, France), as well as HCC-derived HuH7 and SNU398 cell lines, were grown as described.[13,18] TGG and TG HuH7 cells expressing the pL-Tomato+pL-GFP-GPC3 and the pL-Tomato+pL-GFP transgenes, respectively, were developed by lentiviral transduction (multiplicity(ies) of infection [m.o.i.] = 3) and cell sorting.

Liver Samples and Clinical Data. Liver tissues were immediately frozen in liquid nitrogen and stored at −80°C until used for molecular studies. All patients were recruited in accordance with French law and institutional ethical guidelines. Liver samples were clinically, histologically, and genetically characterized. A first series of 133 liver samples (112 HCC and 21 nontumoral liver [NTL] samples) was collected from

Address reprint requests to: Christophe F. Grosset, Christophe F. Grosset, Université Bordeaux Segalen, Physiopathologie du cancer du foie, INSERM U1053, 146 Rue Léo Saignat, F-33076 Bordeaux cedex, France. E-mail: christophe.grosset@u-bordeaux2.fr; fax: (33) 556514077.

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.25994
Potential conflict of interest: Nothing to report.
Additional Supporting Information may be found in the online version of this article.

118 patients surgically treated at French University Hospitals (Supporting Table S1). These HCCs were classified according to the clinical, pathological, and genetic features as described.[4] A second series of 38 liver paired samples (19 HCC and their corresponding NTL samples) was collected from 19 patients surgically treated at French University Hospitals (Supporting Table S2).

Lentiviral Production and Cell Transduction. Production and titration of infectious lentiviral particles, as well as cell transduction, were as described elsewhere.[13,18]

Small RNAs, miRNA Library, Cell Transfection, miRNA Quantification, FunREG and DF-FunREG Analyses, Western Blotting, Cellular Assays, and Statistical Analyses. All materials and methods are as described in the Supporting Information.

Results

GPC3 Expression Is Posttranscriptionally and Differentially Regulated in HCC Cells Compared to Primary Hepatocytes. Using the FunREG method,[13] we investigated whether *GPC3* expression is posttranscriptionally regulated in hepatic cells and whether this regulation is altered in HCC compared to normal cells. Infectious lentiviral particles were used to deliver a GFP-reporter transgene bearing a control 3′-UTR (CT) fusioned to either the *GPC3* or the rabbit *β*-globin (*GLO*) 3′-UTR (Fig. 1A) into HCC-deriving HuH7 and SNU398 cells, and primary hepatocytes. One week later, the P/TCN (transgene copy number) ratio, which is indicative of a posttranscriptional regulation, was calculated.[13] Compared to the referent GFP-3′-UTR GLO transgene, *GPC3* 3′-UTR increased GFP expression by 25% in HuH7 and SNU398 cells (Fig. 1B). In contrast, its presence strongly reduced GFP expression in normal hepatocytes. Consequently, normalized expression of the GFP-*GPC3* transgene was 4-fold higher in HCC cells than in primary hepatocytes (Fig. 1B). These results showed that the *GPC3* 3′-UTR-mediated regulation is differentially controlled in normal and tumoral hepatic cells and that the 3′-UTR promotes *GPC3* expression in the HCC context.

Systematic Identification of MiRNAs Targeting the GPC3 3′-UTR in HCC Cells by DF-FunREG. Given the major role of miRNAs in gene regulation and liver carcinogenesis,[11] we hypothesized that these small RNAs could be responsible for the posttranscriptional dysfunction associated with *GPC3* 3′-UTR in HCC cells. As *in silico* miRNA target predictions sometimes

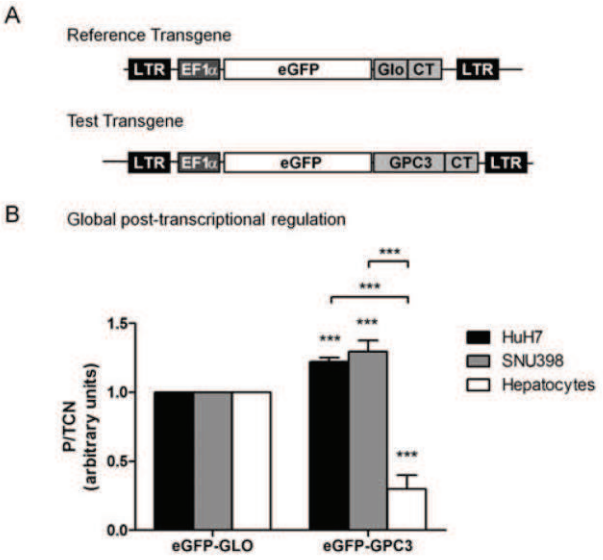


Fig. 1. The posttranscriptional regulation mediated by *GPC3* 3′-UTR is altered in HCC cells. (A) Schematic representations of the *GFP*-GLO (top) and *GFP-GPC3* (bottom) transgenes. (B) HuH7, SNU398 cells, or hepatocytes were transduced once with lentiviruses expressing the indicated transgene and then analyzed by FunREG.[13] After 1 week the *GFP* protein expression (P) and transgene copy number (TCN) were determined by FACS and quantitative polymerase chain reaction (qPCR), respectively. The P/TCN ratio is indicative of global posttranscriptional regulation (analysis of variance [ANOVA]: $P < 0.0001$; n = 3).[13] Otherwise stated, in this figure and following ones, bars represent means, error bars represent standard deviations (SD) and the ANOVA test was followed by a Bonferroni's multiple comparison posttest. ***$P < 0.001$.

lack accuracy,[18-20] we used a systematic approach in order to identify miRNAs controlling *GPC3* expression through its 3′-UTR. Based on FunREG technology,[13,16] we developed an *in cellulo* system that allowed us to functionally screen a library of 876 miRNAs. This system, called DF-FunREG, is based on the dual expression of the green fluorescent protein (GFP) and the tdTomato (TOM). Using infectious lentiviruses, we established two HuH7 cell lines. The TGG cell line expressed the reference TOM transgene that bears the CT 3′-UTR and the test GFP transgene bearing the *GPC3* 3′-UTR upstream of the CT 3′-UTR (see Fig. 2A; Primary Screen box), whereas the TG cell line expressed TOM and a GFP transgene only bearing the CT 3′-UTR (see Fig. 2A, Secondary Screen box). According to our pipeline strategy (Fig. 2A), we performed two successive *in cellulo* screens. First, TGG cells were transfected by a control miRNA, miR-96 as a positive control for cell transfection (not shown) or each of the 876 human miRNAs from the library. Three days later, expressions of GFP and TOM were measured by fluorescence-activated cell sorting (FACS) and the GFP/TOM ratio was calculated in each case.

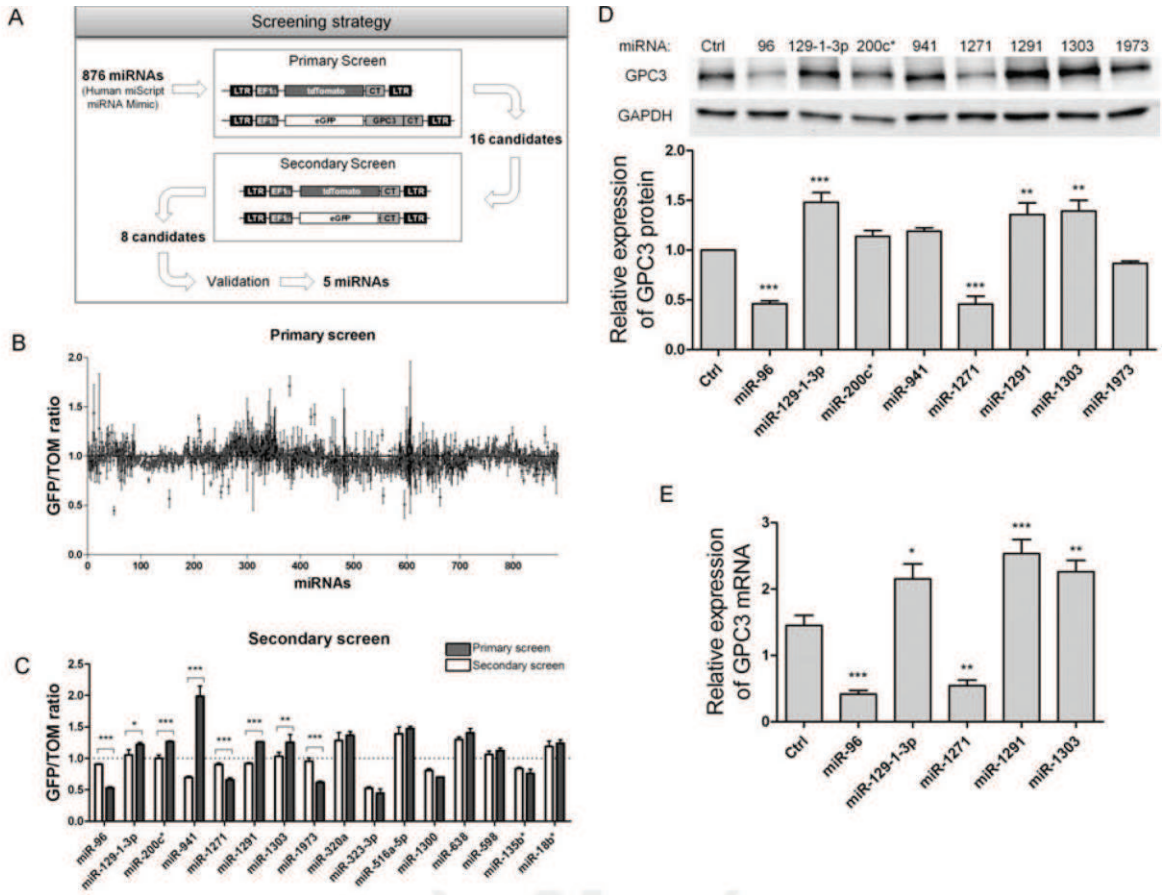


Fig. 2. Five miRNAs regulate *GPC3* expression through its 3′-UTR. (A) Screening strategy. Primary screen: schematic representation of the pL-GFP-*GPC3* and pL-Tomato transgenes expressed in TGG HuH7 cell line. Secondary screen: schematic representation of the pL-GFP and pL-Tomato transgenes expressed in TG HuH7 cell line. Validation: The ability of eight miRNA candidates to control *GPC3* expression was assessed by molecular approaches. (B) Primary screen using the TGG cell line and the library of 876 miRNAs (ANOVA: $P < 0.0001$; n = 3). (C) Secondary screen using the TG cell line and the 16 selected miRNA candidates, and comparison with data from the primary screen (ANOVA: $P < 0.0001$; n = 3). B,C: The negative small noncoding RNA control (not shown) was normalized to 1. (D,E) Validation experiments. Relative expression of *GPC3* protein (D) and mRNA (E) in cells transfected by the indicated RNAs (ANOVA: $P < 0.0001$; n = 7). A representative western blot experiment is shown on top of (D). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Hits corresponding to miRNAs of interest were selected on the base of a significant change of GFP/TOM ratio compared to the negative-control (three independent experiments). As shown in Fig. 2A,B, the primary screen led to the selection of 16 miRNAs. Second, TG cells (lacking the *GPC3* 3′-UTR) were transfected with the 16 selected miRNAs and analyzed by FACS. The GFP/TOM ratio was calculated for each miRNA and compared to the one obtained with TGG cells (Fig. 2C). We reasoned that if an miRNA specifically targets *GPC3* 3′-UTR, the GFP/TOM ratio will differ between TG and TGG cells. Conversely, if the GFP/TOM ratio does not differ, this means that the miRNA recognizes the reporter transgenes outside the sequence of interest. As shown in Fig. 2A,C, eight miRNAs fulfilled this criterion and significantly changed the GFP/TOM ratio. MiR-96, already known as a *GPC3* regulator,[18] was among them, supporting the validity of our screening approach. Finally, these eight miRNAs were further characterized for their ability to control expression of the endogenous *GPC3* in HuH7 cells. As shown in Fig. 2D, five miRNAs were validated as true modulators of *GPC3* protein expression. Although miR-96 and its paralog miR-1271 significantly down-regulated *GPC3* protein expression, miR-129-1-3p, miR-1291, and miR-1303 had a stimulatory effect. The functional effect of these five miRNAs on *GPC3* expression was also demonstrated at the level of mRNA (Fig. 2E), suggesting mechanisms acting on mRNA stability. Presently, it is not clear why miR-200c*, miR-941, and miR-1973 had no effect on expression of endogenous *GPC3*. This

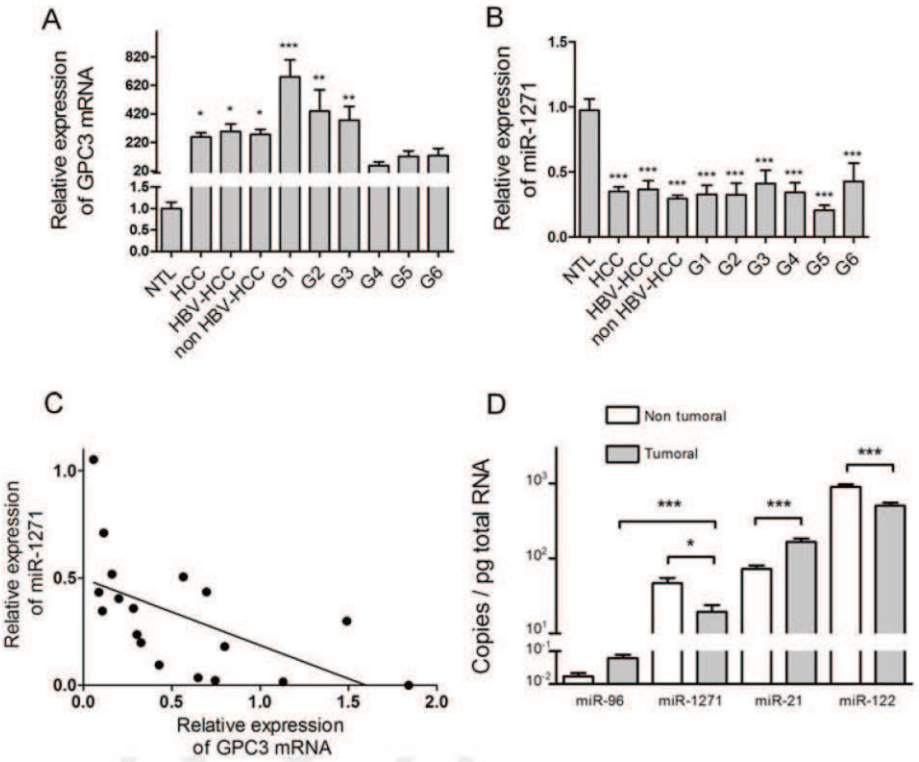


Fig. 3. miR-1271 expression is down-regulated in HCC and negatively correlated with *GPC3* expression in HBV-HCC tumors. (A,B): Relative expression of *GPC3* mRNA (A) and miR-1271 (B) in 112 HCC and 21 NTL samples (ANOVA: $P < 0.0001$; mean + standard error of the mean). Expressions in HCC subgroups are as indicated.[4] (C) Correlative analysis of miR-1271 and *GPC3* mRNA expression in the HBV-HCC subgroup (Spearman $r = -0.72$; ***P [two-tailed] $= 0.0008$). (D) Absolute expression of miR-96, miR-1271, miR-21, and miR-122 in 19 HCC and their corresponding adjacent NTL samples (ANOVA: $P < 0.0001$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

could be due to the presence of other *cis*-regulatory sequences into the *GPC3* mRNA located outside the 3′-UTR, or to differential processing of *GPC3* and GFP transcripts along steps of mRNA maturation, export, or cytoplasmic localization.

Although bioinformatics yield important insights into miRNA biology, the different available algorithms predicted that a large number of miRNAs should target *GPC3* 3′-UTR.[18] Using miRWalk (a computational approach that compares its own miRNA:target predictions with those of nine established algorithms[21]), 200 miRNAs were predicted to target *GPC3* through its 3′-UTR. Among them, 24 showed a higher probability of targeting with 4 to 6 positive predictions over 10 (Supporting Table S3). MiR-96 and miR-1271 paralogs appeared among the top ranked, with 6 and 4 positive predictions over 10, respectively. The three miRNAs having a positive effect on *GPC3* expression (miR-129-1-3p, miR-1291, and miR-1303) were not predicted by any algorithms.

MiR-1271 Expression Is Decreased in HCC and Correlated with GPC3 mRNA in HBV-HCC Subgroup. To investigate the relevance of the five *GPC3*-regulating miRNAs that we identified in HuH7 cells, we measured their relative expression as well as that of *GPC3* mRNA in 112 HCC tumors and 21 NTL. As shown in Fig. 3A, *GPC3* mRNA was highly and significantly overexpressed in all HCC including those from patients infected (HBV-HCC) or not (non-HBV-HCC) by HBV, as well as in the more aggressive tumors classified in G_1 to G_3 subgroups as defined by a transcriptome analysis.[4] Concerning the miRNAs, whereas miR-129-1-3p was not detected in liver tissues, miR-1303 expression remained unchanged in HCC compared to NTL (Supporting Fig. S1A). Expression of miR-1291 significantly increased (3-fold, $P < 0.05$) in the G_3 subgroup (Supporting Fig. S1B), whereas that of miR-96 was increased (201-fold, $P < 0.05$) in all HCC and more specifically in the G_1 and G_3 subgroups (Supporting Fig. S1C).[22] Finally, compared to NTL, miR-1271 was found significantly decreased (−2.4-fold; $P < 0.001$) in all HCC groups (Fig. 3B,D). Importantly, miR-1271 expression inversely and strongly correlated with that of *GPC3* mRNA in the HBV-HCC subgroup (Fig. 3C; $P = 0.0008$), whereas no correlation was found in any groups with any other miRNAs.

As reported,[23] miR-96 and miR-1271 are paralogs with functional similarities *in cellulo*. However, they are not expressed in the same tissues. Although miR-96 is expressed in sensory organ, miR-1271 is expressed in a variety of human tissues including liver, suggesting distinctive functions *in vivo*.[23] To obtain insight into the opposite deregulation of miR-96 and

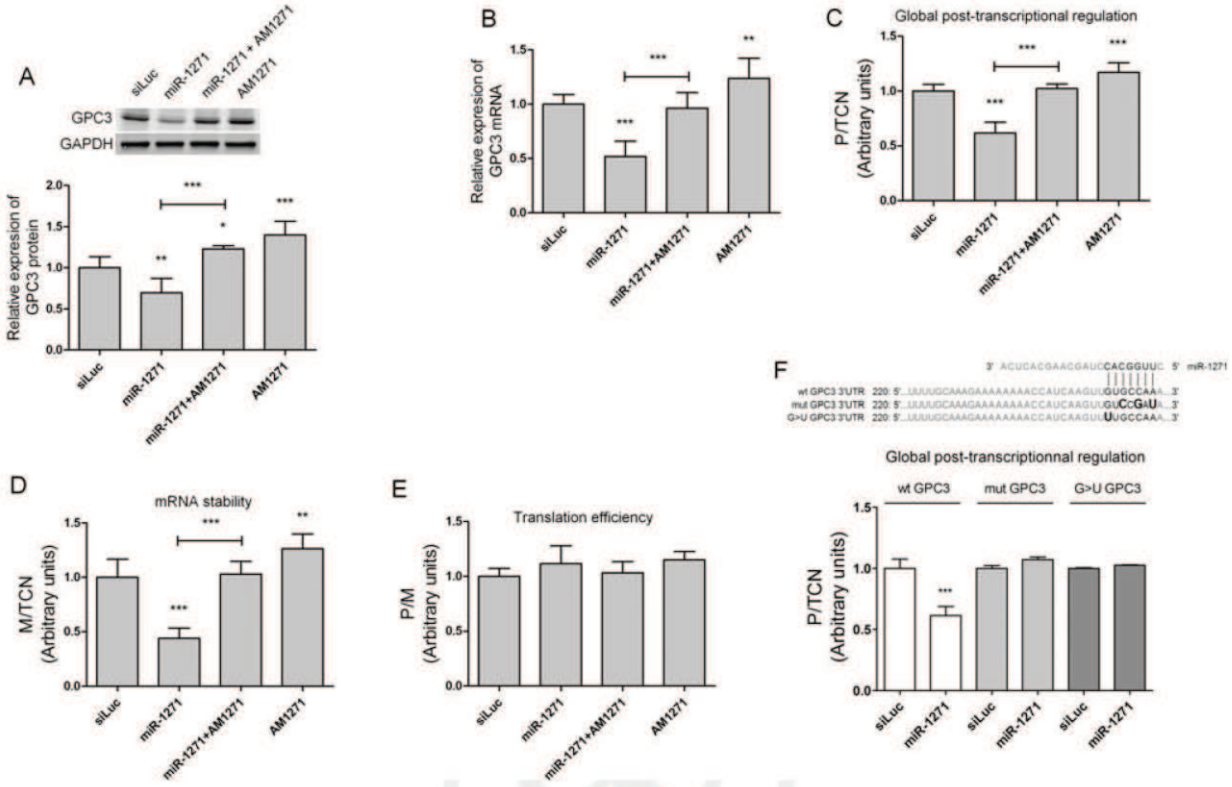


Fig. 4. Molecular basis of the posttranscriptional regulation mediated by miR-1271 on *GPC3*. (A,B) Relative expression of *GPC3* protein (A) and mRNA (B) in cells transfected with the indicated RNAs (ANOVA: $P < 0.0001$; n = 5). A representative western blot experiment is shown on top in (A). (C-E) *GFP-GPC3*-expressing HuH7 cells (Fig. 1A; TCN value known) were transfected with the indicated RNAs. Then the *GFP* protein (P) and mRNA (M) expressions were analyzed following the FunREG method.[13] (C) Global posttranscriptional regulation (ANOVA: $P < 0.0001$; n = 5). (D) mRNA stability (ANOVA: $P < 0.0001$; n = 5). (E) Translation efficiency (ANOVA: P = NS; n = 5). (F) HuH7 cells expressing the indicated transgene (TCN value measured) were transfected with the indicated RNAs and analyzed as described in Fig. 1B (ANOVA: $P < 0.0001$; n = 3). Top panel shows miR-1271 pairing with the *GPC3* 3′-UTR sequence in its wildtype or mutated versions. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

miR-1271 in HCC and into the lack of correlation between expression of *GPC3* mRNA and that of miR-96 in any subgroups, we measured the absolute copy number of each miRNA in 19 HCC and the corresponding NTL samples. As a comparison, we concomitantly measured the absolute copy number of two liver miRNAs, miR-122 and miR-21. As previously reported,[11] miR-122 was highly expressed in liver and its expression significantly decreased in HCC, whereas that of miR-21 increased. Moreover, miR-1271 expression was similar to that of miR-21 in NTL, suggesting that miR-1271 is rather abundant in hepatic tissues (Fig. 3D). Finally, miR-1271 was 2,770-fold and 319-fold more expressed than miR-96 in the NTL and HCC tissues, respectively (Fig. 3D). Therefore, we concluded that, compared to miR-1271, the much lower expression of miR-96 in both NTL and HCC prevents drawing conclusions concerning its regulatory role in *GPC3* overexpression. Altogether, our data suggest that the overexpression of *GPC3* mediated by the posttranscriptional up-regulation of its 3′-UTR (Fig. 1B) results at least partly from the marked underexpression of miR-1271 in HCC cells compared to NTL.

MiR-1271 Directly Targets GPC3 3′-UTR and Accelerates Its mRNA Degradation. Based on the above results, we focused on miR-1271. We first assessed the specificity of the miR-1271-mediated effect on *GPC3* expression. As shown in Fig. 4A, miR-1271 down-regulated GPC3-protein expression in HuH7 cells by roughly 30%. Its specific antimiR (AM1271) abrogated miR-1271-induced effects and led to a slight increase in *GPC3* expression. MiR-1271-mediated effects on *GPC3* expression were also observed at the mRNA level (Fig. 4B). Using FunREG (Fig. 4C-E), we further showed that miR-1271 acts as a *GPC3*-mRNA destabilizing factor. Similar results were obtained with SNU398 cells (data not shown). Finally, introduction of mutations in the sequence complementary to miR-1271 seed ("mut *GPC3*" transgene) or the base complementary to its 8th base ("G>U *GPC3*" transgene) completely abolished miR-

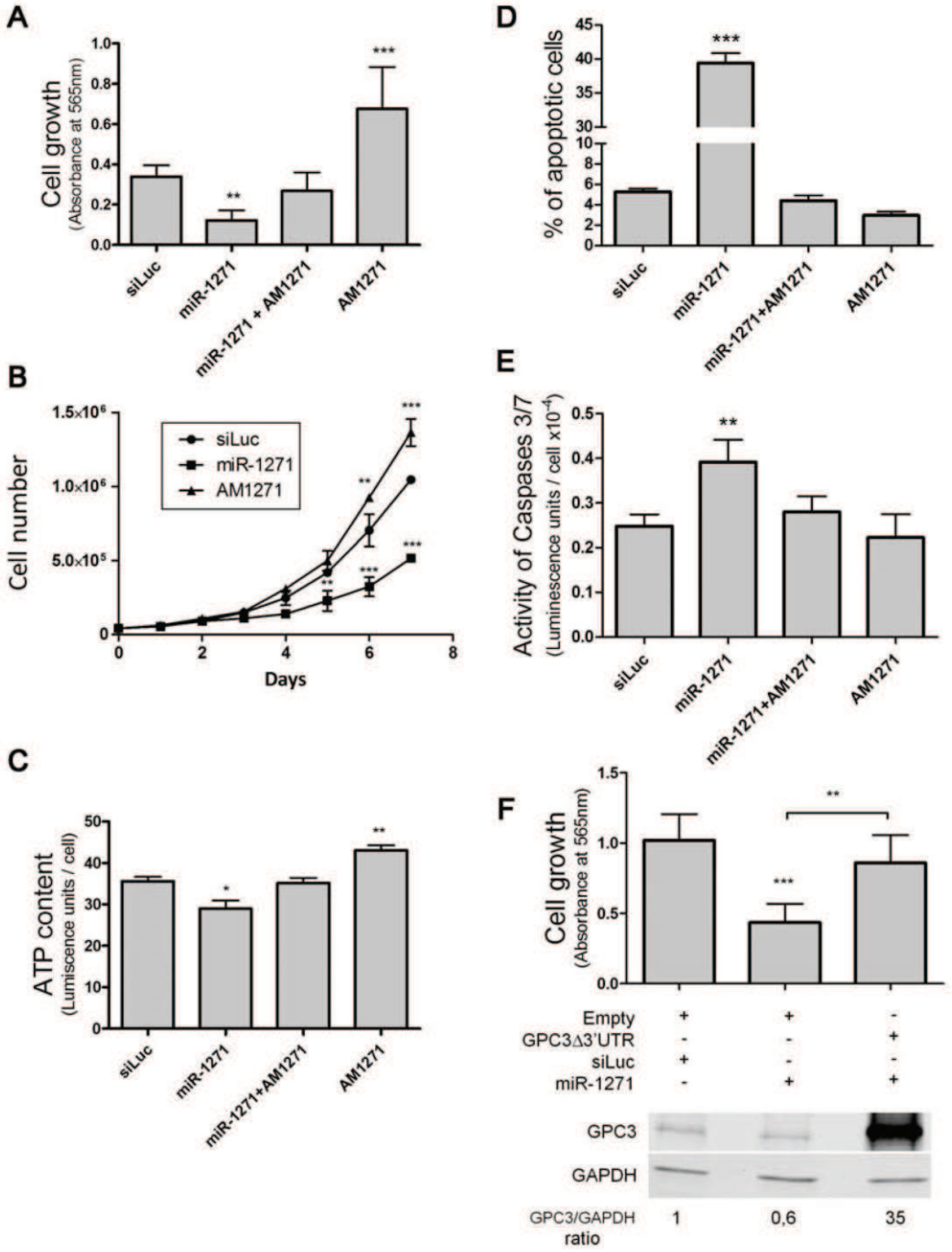


Fig. 5. MiR-1271 inhibits HCC-cell growth and induces cell death. In this figure, HuH7 cells were transfected by small RNAs and/or plasmids (expressing or not GPC3) as indicated in each panel. (A,F) Cell growth (total cellular proteins; ANOVA: $P < 0.0003$; n = 6). (B) Cell proliferation (ANOVA: $P = 0.0001$; n = 3). (C) ATP content measurement (ANOVA: $P = 0.0001$; n = 5). (D) Cell apoptosis (annexin V-positive cells; ANOVA: $P = 0.0001$; n = 3). (E) Caspases 3/7 activity (ANOVA: $P = 0.0055$; n = 3). A representative western blot experiment is shown on the bottom in (F). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

1271 activity (Fig. 4F). These data demonstrated that miR-1271 directly targets *GPC3* 3′-UTR and induces *GPC3* mRNA degradation through a seed+base 8 recognition, as does its paralog miR-96.[18]

MiR-1271 Inhibits HCC Cell Growth in a GPC3-Dependent Manner and Induces Apoptosis. Because *GPC3* promotes HCC cell growth,[6,7] we wondered whether miR-1271, which represses *GPC3* expression,

could have antitumor effects on these cells. MiR-1271 overexpression strongly inhibited the growth of HuH7 cells by decreasing their proliferation and adenosine triphosphate (ATP) content, which is an indicator of cell metabolic activity (Fig. 5A-C). Using a variety of assays, we further found that miR-1271 slightly increased the percentage of cells in G_0/G_1 phase and decreased that in S phase (Supporting Fig. S2). Concomitantly, miR-1271 markedly induced apoptosis (Fig. 5D,E) and necrosis (Supporting Fig. S3). Importantly, all these effects were abrogated by the concomitant transfection of its anti-miR AM1271. Counteracting the activity of the endogenous miR-1271 with AM1271 stimulated the growth and proliferation of HCC cells (Fig. 5A,B). Because AM1271 had no significant effects on cell cycle phases, nor cell death (Fig. 5D,E; Supporting Figs. S2, S3), we hypothesized that AM1271 increases the proliferation of HCC cells by shortening their division time. This model is supported by the higher ATP content of HCC cells transfected with AM1271 (Fig. 5C). Some of these results were obtained with the SNU398 cells (Supporting Fig. S4). Interestingly, ectopic expression of a *GPC3* transgene lacking its 3′-UTR in HuH7 cells overexpressing miR-1271 partially rescued the miR-1271-mediated inhibition of HuH7 cell growth (Fig. 5F). Altogether these results demonstrated that miR-1271 inhibits the growth of HCC cells and promotes their death, at least for a part, by lowering *GPC3* expression. We therefore concluded that the specific down-regulation of miR-1271 in HCC tumors contributes to *GPC3* overexpression and expansion of cancerous hepatic cells.

Discussion

In this work we report that *GPC3* is posttranscriptionally regulated through its 3′-UTR. More important, we show that the regulation mediated by *GPC3* 3′-UTR favors GPC3 expression in HCC cells compared to primary hepatocytes (Fig. 1B). These results further support the notion that posttranscriptional regulations are part of the events participating in HCC-associated gene deregulations and indirectly in liver carcinogenesis.[5,13]

Given the role of miRNAs in tumorigenesis[5,11,22] and the relevance of *GPC3* in HCC,[6,7] we investigated their implication in the deregulation of *GPC3* expression mediated by its 3′-UTR in HCC cells. Because of the poor accuracy of bioinformatic predictions,[18,20] we opted for a blinded screening strategy in order to systematically identify miRNAs regulating *GPC3* expression. We developed a dual reporter system deriving from FunREG,[13,16] which we termed DF-FunREG. The main difficulty encountered with miRNA screening is the pleiotropic effects of miRNAs on multiple gene targets and their subsequent cellular impacts on cellular processes (cell survival, growth, death). This led us to pay particular attention to eliminate as much as possible false-positive events. We first used a library of mature miRNAs in order to avoid problems linked to the differential processing of precursor miRNAs from a cell type to another one.[24,25] Second, by performing analyses 3 days after transfection, we minimized the effects due to miRNA overexpression on cell integrity (growth arrest, cell death) and therefore decreased the risk of losing miRNA candidates. Third, by establishing cell lines expressing two fluorescent reporter transgenes with a high degree of similarities (Fig. 2A, top panel), we get rid of false-positive events due to miRNAs either affecting transgene promoter activity by modulating expression of general transcription factors, or targeting the transgenes outside the sequence of interest. With these stringent conditions, we could not avoid eight false-positive events that were revealed by performing a secondary screen with HuH7 cells expressing the TOM and a GFP transgene lacking the *GPC3* 3′-UTR (Fig. 2A,C).

Using DF-FunREG as a three-step strategy including two successive screens and a set of validation experiments (see Fig. 2A), we tested the biological effect of 876 mature miRNAs and identified five miRNAs, which all regulate *GPC3* expression in a 3′-UTR-dependent manner (Fig. 6). We therefore concluded that DF-FunREG is a robust and accurate screening approach for identifying functional miRNAs. To our knowledge, this is one of the most exhaustive miRNA screens described so far using a gene-reporter

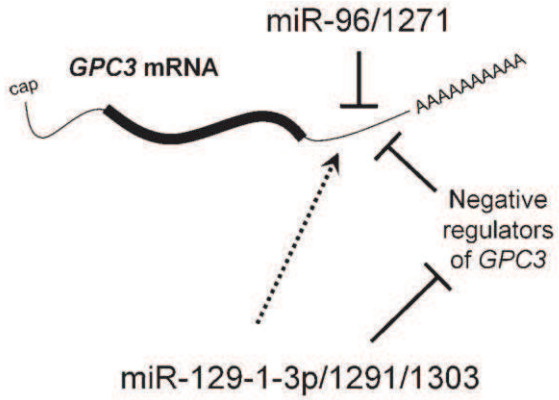


Fig. 6. Schematic representation of the five miRNAs differentially regulating GPC3 expression through its 3′-UTR.

system.[20,26,27] Among the five miRNAs, two down-regulated *GPC3* expression: miR-96 that we previously described as a negative *GPC3* regulator[18] and its paralog miR-1271.[23] As expected, both miRNAs interacted with the *GPC3* mRNA through its 3′-UTR at the expected seeding 5′-GUGCCAA$_{256}$-3′ site. We further showed that miR-96/1271 repress *GPC3* expression by triggering the decay of *GPC3* mRNA (Fig. 4).[18]

The presence of miR-96 among the selected miRNAs clearly demonstrated the reliability of our screening strategy and its specificity. Unexpectedly, none of the numerous other miRNAs predicted to target the *GPC3* 3′-UTR were found in our screen.[18,21] As highlighted elsewhere,[18,20,28,29] such discrepancies could partly be explained by the high error rate of prediction programs. Alternatively, the capacity of some miRNAs from the library to target *GPC3* 3′-UTR could depend on the cellular context (presence of other miRNAs and/or RNA-binding proteins). Interestingly, we also identified miR-129-1-3p, miR-1291, and miR-1303 as positive and 3′-UTR-dependent regulators of *GPC3* expression (Fig. 6). Based on miRWalk,[21] none of these three miRNAs were predicted to interact with *GPC3* 3′-UTR. It is thus likely that these miRNAs operate indirectly, by down-regulating the expression of negative posttranscriptional regulators of *GPC3* mRNA (Fig. 6). Of interest, several RNA-binding proteins and ribonucleases involved in mRNA degradation are potential targets of these three miRNAs. We are currently working at understanding how these three miRNAs positively control *GPC3* expression. Finally, our work contributes to highlight the fascinating notion of miRNA-associated systems biology.[19] In our opinion, systematic and blinded screening approaches such as DF-FunREG described here should, in the future, greatly contribute to the understanding of "miRNA:target" networks, as well as the individual or collective role of miRNAs in gene alteration and human pathologies.

In the context of HCC, we found that miR-1271 expression is reduced in 92% of the HCC samples analyzed compared to NTL. It would therefore be of interest to understand the molecular origin of this underexpression in HCC. Interestingly, miR-1271 expression inversely correlated with that of *GPC3* mRNA in the HBV-HCC subgroup, a result consistent with the regulatory effect of miR-1271 on the *GPC3* transcript (Fig. 4). Up to now, it is not clear why this correlation is only observed in this particular subgroup, but context-dependent correlations have been reported for miR-21 and Serpini1 in gastric cancer.[30] This could be due to the low number of samples in other subgroups, the differentiation state of these particular tumors, the influence of HBV, or a dominant role of miR-1271 over other *trans*-acting regulatory factors in this subgroup.[2,3] Our work also revealed that in the G_1-G_3 HCC subgroups (which highly express *GPC3*; Fig. 3A), miR-1271 down-regulation was associated with an increase in plasma alpha-fetoprotein (AFP) (Supporting Fig. S5A,B), a major biomarker of HCC in clinics. Interestingly both *AFP* and *GPC3* are oncofetal proteins associated with the undifferentiated state and aggressiveness of hepatic tumoral cells.[3,8,9] This suggests that miR-1271 expression could be linked to the differentiation status of hepatic cells. In terms of expression, we showed that miR-1271 is rather abundant in liver (Fig. 3D). These results are somewhat surprising compared to others,[23] but these discrepancies could be explained by the different approaches used to quantify miRNA amounts in liver tissues. Finally, we showed that miR-1271 acts as an antiproliferative and proapoptotic factor in HCC cells (Fig. 5). These results are in accordance with the tumor suppressive role of its paralog miR-96 reported in pancreatic cancer.[31] More important, we demonstrated that the antiproliferative effect of miR-1271 on HCC cells depends, at least partly, on *GPC3* expression, reinforcing the notion that *GPC3* is a key tumor driver gene in liver.[6,7]

In conclusion, we identified five miRNAs controlling *GPC3* expression in HCC cells using a functional screening approach. Among them, miR-1271 was found reproducibly underexpressed in HCC and displayed antitumoral properties *in cellulo*. MiR-1271 overexpression has been reported in head and neck cancers.[32] However, this is the first demonstration of its direct implication in human disease. Collectively, our data suggest that the underexpression of miR-1271 participates in the overexpression of *GPC3* in HCC and favors HCC cell expansion.

Acknowledgment: We thank Véronique Guyonnet-Duperat, Yuh-Shan Jou, and Roger Y. Tsien for providing experimental materials.

References

1. Jain S, Singhal S, Lee P, Xu R. Molecular genetics of hepatocellular neoplasia. Am J Transl Res 2010;2:105-118.
2. Nault JC, Zucman-Rossi J. Genetics of hepatobiliary carcinogenesis. Semin Liver Dis 2011;31:173-187.
3. Hoshida Y, Toffanin S, Lachenmayer A, Villanueva A, Minguez B, Llovet JM. Molecular classification and novel targets in hepatocellular carcinoma: recent advancements. Semin Liver Dis 2010;30: 35-51.

4. Boyault S, Rickman DS, de Reynies A, Balabaud C, Rebouissou S, Jeannot E, et al. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. Hepatology 2007;45: 42-52.
5. Hatziapostolou M, Polytarchou C, Aggelidou E, Drakaki A, Poultsides GA, Jaeger SA, et al. An HNF4alpha-miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis. Cell 2011;147:1233-1247.
6. Ho M, Kim H. Glypican-3: a new target for cancer immunotherapy. Eur J Cancer 2011;47:333-338.
7. Allegretta M, Filmus J. Therapeutic potential of targeting glypican-3 in hepatocellular carcinoma. Anticancer Agents Med Chem 2011;11: 543-548.
8. Hoshida Y, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. Cancer Res 2009;69: 7385-7392.
9. Shirakawa H, Suzuki H, Shimomura M, Kojima M, Gotohda N, Takahashi S, et al. Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. Cancer Sci 2009;100:1403-1407.
10. Sotiropoulou G, Pampalakis G, Lianidou E, Mourelatos Z. Emerging roles of microRNAs as molecular switches in the integrated circuit of the cancer cell. RNA 2009;15:1443-1461.
11. Huang S, He X. The role of microRNAs in liver cancer progression. Br J Cancer 2011;104:235-240.
12. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 2010;11:597-610.
13. Laloo B, Simon D, Veillat V, Lauzel D, Guyonnet-Duperat V, Moreau-Gaudry F, et al. Analysis of post-transcriptional regulations by a functional, integrated, and quantitative method. Mol Cell Proteomics 2009; 8:1777-1788.
14. Breving K, Esquela-Kerscher A. The complexities of microRNA regulation: mirandering around the rules. Int J Biochem Cell Biol 2010;42: 1316-1329.
15. Wahid F, Shehzad A, Khan T, Kim YY. MicroRNAs: synthesis, mechanism, function, and recent clinical trials. Biochim Biophys Acta 2010; 1803:1231-1243.
16. Laloo B, Maurel M, Jalvy-Delvaille S, Sagliocco F, Grosset CF. Analysis of post-transcriptional regulation using the FunREG method. Biochem Soc Trans 2010;38:1608-1614.
17. Simon D, Laloo B, Barillot M, Barnetche T, Blanchard C, Rooryck C, et al. A mutation in the 3′-UTR of the HDAC6 gene abolishing the post-transcriptional regulation mediated by hsa-miR-433 is linked to a new form of dominant X-linked chondrodysplasia. Hum Mol Genet 2010;19:2015-2027.
18. Jalvy-Delvaille S, Maurel M, Majo V, Pierre N, Chabas S, Combe C, et al. Molecular basis of differential target regulation by miR-96 and miR-182: the Glypican-3 as a model. Nucleic Acids Res 2012;40: 1356-1365.
19. Peter ME. Targeting of mRNAs by multiple miRNAs: the next step. Oncogene 2010;29:2161-2164.
20. Thomson DW, Bracken CP, Goodall GJ. Experimental strategies for microRNA target identification. Nucleic Acids Res 2011;39:6845-6853.
21. Dweep H, Sticht C, Pandey P, Gretz N. miRWalk—database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform 2011;44:839-847.
22. Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, et al. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology 2008;47:1955-1963.
23. Jensen KP, Covault J. Human miR-1271 is a miR-96 paralog with distinct non-conserved brain expression pattern. Nucleic Acids Res 2011; 39:701-711.
24. Thomson JM, Newman M, Parker JS, Morin-Kensicki EM, Wright T, Hammond SM. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes Dev 2006;20:2202-2207.
25. Slezak-Prochazka I, Durmus S, Kroesen BJ, van den Berg A. MicroRNAs, macrocontrol: regulation of miRNA processing. RNA 2010;16: 1087-1095.
26. Jiang Q, Feng MG, Mo YY. Systematic validation of predicted microRNAs for cyclin D1. BMC Cancer 2009;9:194.
27. Wu S, Huang S, Ding J, Zhao Y, Liang L, Liu T, et al. Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3′ untranslated region. Oncogene 2010;29:2302-2308.
28. Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. Nature 2008;455:58-63.
29. Bentwich I. Prediction and validation of microRNAs and their targets. FEBS Lett 2005;579:5904-5910.
30. Yamanaka S, Olaru AV, An F, Luvsanjav D, Jin Z, Agarwal R, et al. MicroRNA-21 inhibits Serpini1, a gene with novel tumor suppressive effects in gastric cancer. Dig Liver Dis 2012;44:589-596.
31. Yu S, Lu Z, Liu C, Meng Y, Ma Y, Zhao W, et al. miRNA-96 suppresses KRAS and functions as a tumor suppressor gene in pancreatic cancer. Cancer Res 2010;70:6015-6025.
32. Nurul-Syakima AM, Yoke-Kqueen C, Sabariah AR, Shiran MS, Singh A, Learn-Han L. Differential microRNA expression and identification of putative miRNA targets and pathways in head and neck cancers. Int J Mol Med 2011;28:327-336.

Supplementary Figure Legends

Supplementary Figure S1: Expression of miR-1303, miR-1291 and miR-96 in HCC and HCC subgroups compared to non-tumoral livers (NTL). (A-C): Relative expression of miR-1303 (Panel A; ANOVA: P=0.0047), miR-1291 (Panel B; ANOVA: P=0.9919) and miR-96 (Panel C; ANOVA: P=0.0215) in 112 HCC and 21 NTL. Expression in HCC subgroups are as indicated5. In this figure, bars represent means and error bars represent standard errors of the mean. *P<0.05; **P<0.01.

Supplementary Figure S2: miR-1271 overexpression affects the cell cycle. HuH7 cells were transfected with the indicated small RNAs. Six-days later, cells were lysed and stained with propidium iodide. Cell fluorescence and DNA repartition were analyzed by FACS (n=3; Panels A, ANOVA: P=0.0339; Panel B, ANOVA: P=0.0093; Panel C: ANOVA: P=NS). In this figure and following ones, bars represent means and error bars represent standard deviations. *P<0.05; **P<0.01.

Supplementary Figure S3: miR-1271 overexpression induces the necrosis of HuH7 cells. The percentage of necrotic cells (propidium iodide test) was measured 3-days after transfection by the indicated small RNAs (ANOVA: P<0.0001; n=3). ***P<0.001.

Supplementary Figure S4: miR-1271 negatively controls the growth of SNU398 cells. Growth of SNU398 cells was measured 6-days after transfection by the indicated small RNAs (ANOVA: P<0.0001; n=4). **P<0.01; ***P<0.001.

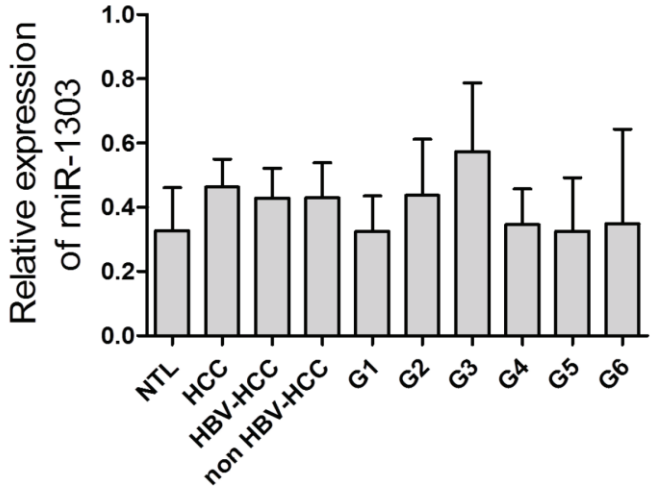
Supplementary Figure S5: Expression of miR-1271 is associated with plasmatic levels of AFP. (A) miR-1271 expressions in 36 G1-G3 HCC were divided in two groups (inferior or superior at 0.5) based on the mean of miR-1271 expression in NTL samples. Then plasmatic level of AFP was compared in the two groups (Mann-Whitney test: P=0.0013). (B) Plasmatic levels of AFP in 36 G1-G3 HCC were divided in two groups: AFP < 20ng/mL or AFP > 20ng/mL. Then miR-1271 expression was compared in the two groups (Mann-Whitney test: P=0.0028). **P<0.01.

Supplementary Table 1: Clinical and genetic characteristics of the first series of 133 liver samples.

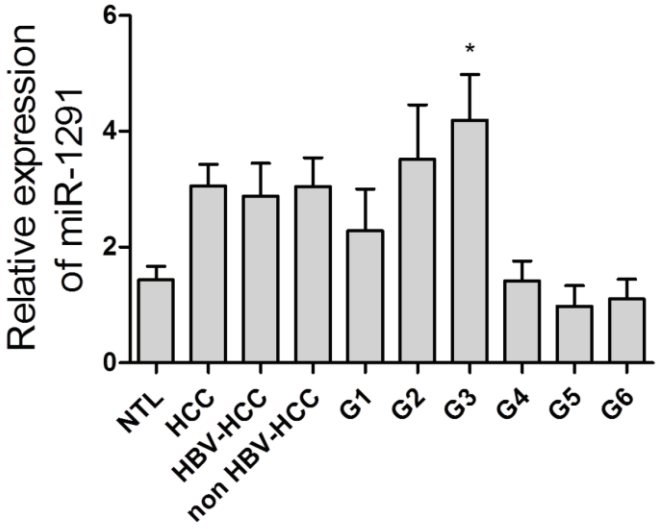
Supplementary Table 2: Clinical characteristics of the second series of 38 liver paired samples.

Supplementary Table 3: The twenty-four miRNAs having the higher probability to interact with the *GPC3* 3'UTR as predicted by miRWalk6. MiR-96 and its paralogue miR-1271 are highlighted.

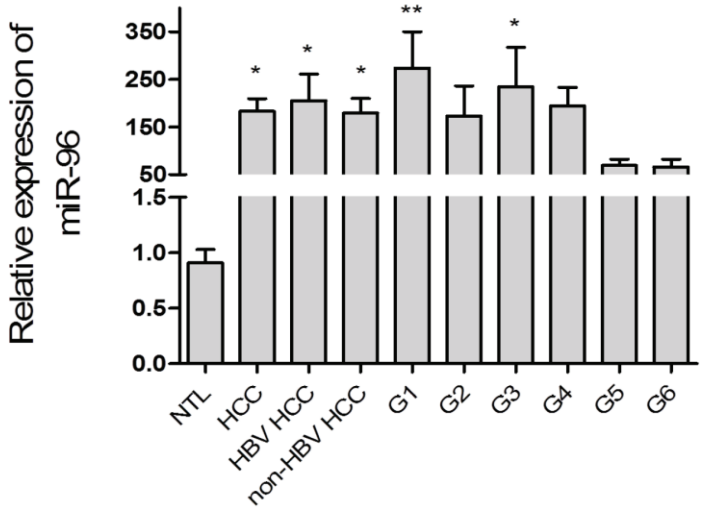
A



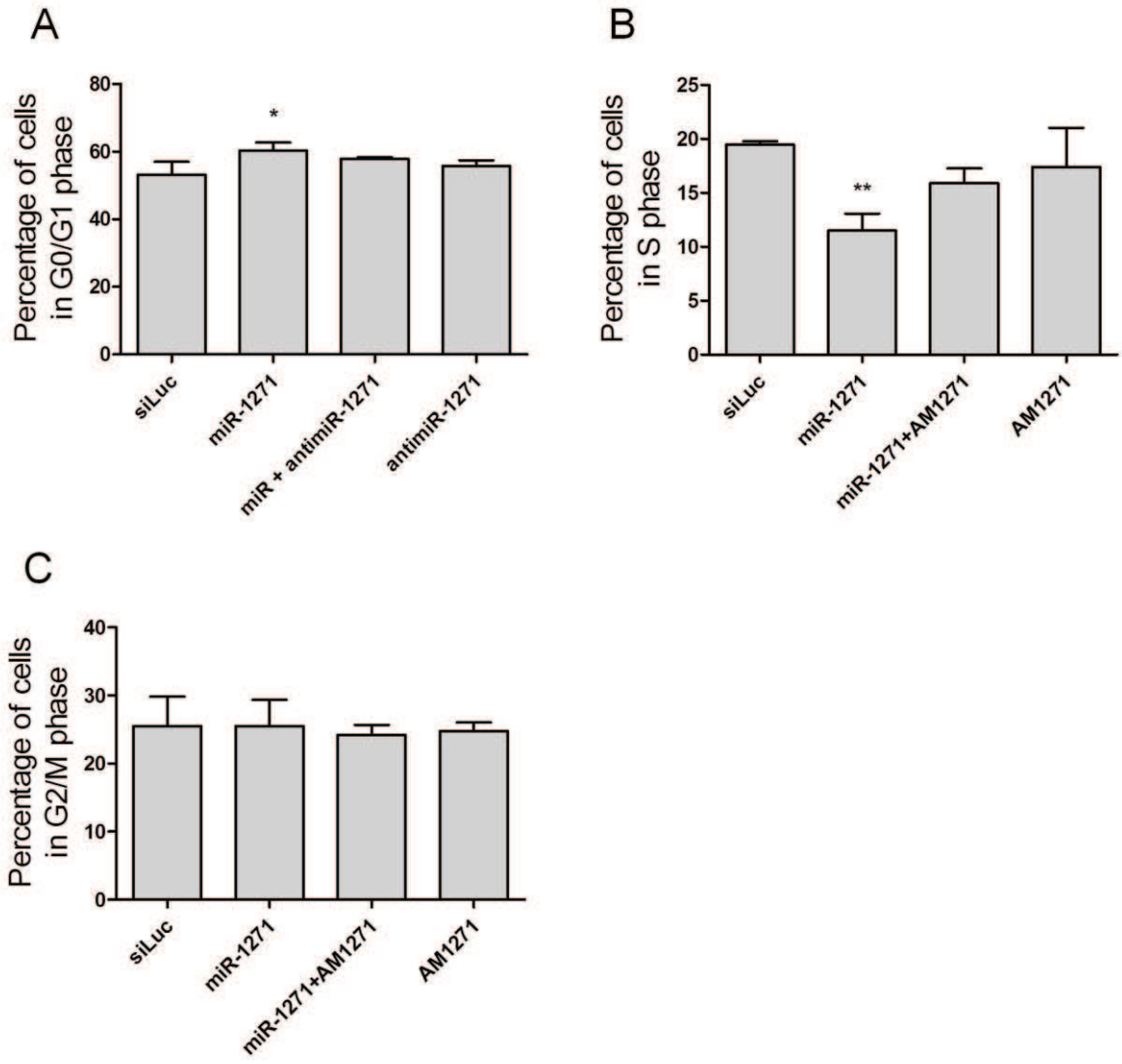
B



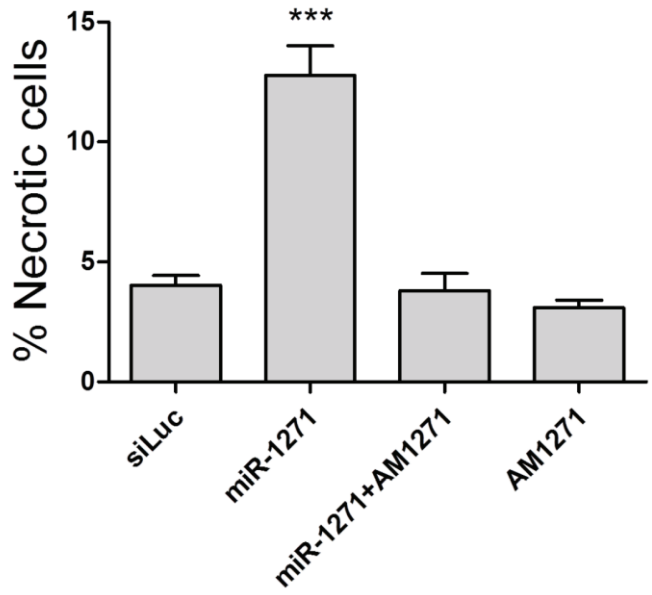
C



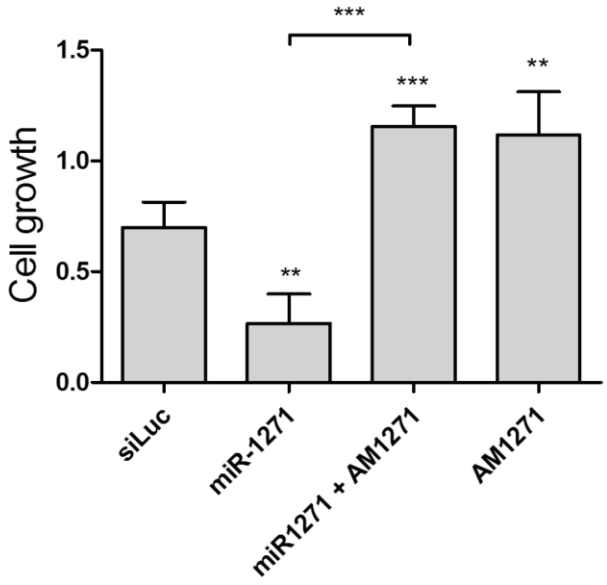
Maurel M. et al, Supplementary Figure S1



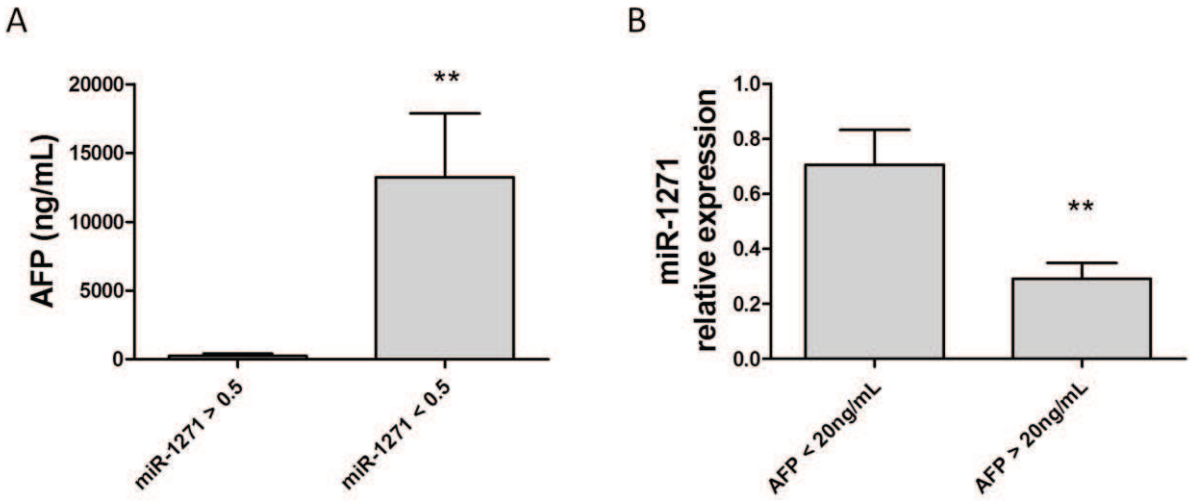
Maurel M. et al, Supplementary Figure S2



Maurel M. et al, Supplementary Figure S3



Maurel M. et al, Supplementary Figure S4



Maurel M. et al, Supplementary Figure S5

	Liver Samples (n=133)
HCC	
number of samples	112
Gender (male/female)	89/23
Mean age (years)	55,6
HBV infection (n/%)	31 (28)
HCV infection (n/%)	22 (20)
Alcohol intake(n/%)	43 (38)
Hemochromatosis (n/%)	4 (3,5)
Unknown risk factor (n/%))	21 (19)
Edmondson (I-II/III-IV/not known)	52/56/4
TP53 mutation (n/%)	21 (19)
beta-catenin mutation (n/%)	41 (37)
Transcriptomic classification (n/%) (see Suppl. Ref n°6)	
G1	16 (14)
G2	12 (11)
G3	19 (17)
G4	24 (21)
G5	20 (18)
G6	14 (13)
non classified	7 (6)
Non tumoral livers	
number of samples	21
Gender (male/female)	15/6
Mean age (years)	60,4
Cirrhotic liver (n/%)	9 (43)
non-tumoral livers asssociated with the above HCC (n/%)	14 (67)
HBV infection (n/%)	6 (29)
HCV infection (n/%)	5 (24)
Alcohol intake (n/%)	6 (29)

Maurel M. et al, Supplementary Table S1

	Liver Samples (n=38)
HCC pairs (tumoral/NTL)	
Number of paired samples	19
Gender (male/female)	15/4
Mean age (years)	66.7
HBV infection (n/%)	2 (10.5)
HCV infection (n/%)	5 (26)
Alcohol intake(n/%)	2 (10.5)
Metabolic syndrome (n/%)	4 (21)
Unknown risk factor (n/%))	8 (42)
Edmondson (I-II/II-III/III-IV/not known)	4/12/3/0

Maurel M. et al, Supplementary Table S2

MicroRNA	DIANAmT	miRanda	miRDB	miRWalk	RNAhybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	SUM
hsa-miR-96	1	1	1	0	1	0	1	0	0	1	6
hsa-miR-182	1	1	0	0	1	0	1	0	0	1	5
hsa-miR-450b-5p	1	1	1	0	0	0	1	0	0	1	5
hsa-miR-570	1	1	1	0	0	0	1	0	0	1	5
hsa-miR-518a-5p	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-7	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-765	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-140-5p	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-527	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-1271	0	1	1	0	0	0	1	0	0	1	4
hsa-miR-140-3p	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-503	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-151-5p	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-220c	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-186	1	1	0	0	1	0	1	0	0	0	4
hsa-miR-204	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-16	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-583	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-211	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-202	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-195	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-15a	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-641	1	1	0	0	0	0	1	0	0	1	4
hsa-miR-15b	1	1	0	0	0	0	1	0	0	1	4

Maurel M. et al, Supplementary Table S3

3. Discussion

Dans ce travail, j'ai démontré que l'expression du transgène « eGFP-3'NT GPC3 » est quatre fois plus importante dans des lignées de CHC par rapport aux hépatocytes normaux. Ces résultats suggèrent donc fortement que la régulation post-transcriptionnelle du GPC3 est altérée de manière différentielle dans les cellules tumorales et les cellules non transformées. Implicitement, ces résultats sous-entendent aussi que des facteurs *trans*-régulateurs modulent différemment l'expression du GPC3 entre les cellules normales et tumorales. Pour déterminer si certains miARNs sont impliqués dans le contrôle post-transcriptionnel du GPC3, j'ai par conséquent criblé une bibliothèque de 876 miARNs humains. Les résultats du crible m'ont permis de sélectionner huit candidats, puis d'en valider cinq comme étant des régulateurs de l'expression du GPC3. Parmi eux, le miR-1271 a attiré mon attention car il est sous-exprimé dans 92% des échantillons de CHC testés dans notre étude. Dans des lignées de CHC, il joue un rôle anti-prolifératif, pro-apoptotique et il diminue la stabilité de l'ARNm du GPC3 en ciblant sa région 3'NT. De plus, la sous-expression du miR-1271 corrèle avec la surexpression du GPC3 dans tous les échantillons de CHC infectés par le VHB. Ces résultats suggèrent fortement que la sous-expression du miR-1271 (i) contribuerait à la surexpression du GPC3 dans le CHC et (ii) participerait de ce fait au phénotype tumoral en augmentant la prolifération cellulaire et en diminuant l'apoptose.

En choisissant une stratégie basée sur un crible fonctionnel, nous avons éliminé un grand nombre de faux-positifs par rapport aux outils bioinformatiques. D'après miRWalk, 200 miARNs sont prédits pour cibler la région 3'NT du GPC3, parmi eux seul deux miARNs ont été validés. Cette stratégie nous a aussi permis d'identifier trois miARNs non initialement prédits qui pourtant ont bien un effet sur l'expression du GPC3 endogène :

- MiR-96 (6 prédictions / 10 algorithmes testés) et miR-1271 (4 prédictions / 10 algorithmes testés) font partie des miARNs prédits et répriment l'expression du GPC3 en ciblant directement le même site de fixation.

- MiR-129-1-3p, miR-1291 et miR-1303 ne sont pas prédits par les outils bioinformatiques comme ciblant le GPC3. Ils augmentent l'expression du GPC3 endogène probablement *via* une interaction non conventionnelle (voir section I. 2.1.2)

ou en ciblant un facteur intermédiaire régulant négativement l'expression du GPC3 (voir section I. 3.1.2.2).

Récemment le miR-219-5p a aussi été identifié comme un régulateur de l'expression du GPC3. Il est sous-exprimé dans 60% des CHC et inhibe l'expression du GPC3 en ciblant sa région 3'NT (Huang et al., 2012). Ce miARN n'a pas été identifié dans notre crible, il pourrait donc correspondre à un faux négatif. Cette différence de résultat peut s'expliquer par les différentes méthodologies utilisées dans les deux approches, nous nous sommes placés 72 heures après transfection à une concentration de 12 nM de miARNs mimics alors que Huang et collaborateurs ont transfecté 96 heures à 50 nM. Dans leur étude, l'effet du miR-219-5p endogène n'a pas été testé sur l'expression du GPC3. Le miR-219-5p n'a pas été quantifié de manière absolue dans les lignées cellulaires ou les échantillons de CHC, son taux d'expression dans le foie normal et tumoral reste donc à analyser. De plus, le travail réalisé par Huang et collaborateurs ne valide pas clairement que l'effet biologique du miR-219-5p passe bien par l'inhibition de l'expression du GPC3 (restauration phénotypique par surexpression d'une forme du GPC3 résistante au miARN). Il faudrait donc apporter d'autres preuves expérimentales pour valider miR-219-5p comme régulateur de l'expression du GPC3.

Une limite de notre processus de sélection réside dans le fait que la stratégie du crible est basée uniquement sur la région 3'NT du GPC3. En effet, les cinq miARNs validés semblent agir uniquement sur la stabilité de l'ARNm du GPC3. Aucun miARN agissant sur l'efficacité de traduction n'a été identifié. Ce résultat pourrait être dû au hasard ou à un biais en raison de l'absence de la région 5'NT du GPC3 par exemple. Les transgènes utilisés possèdent une région 5'NT minimale, mais la conformation circulaire de l'ARNm transgénique reste encore à démontrer (voir section I. 3.1.1.1). Cette conformation semble en effet indispensable pour atténuer l'efficacité de traduction de l'ARNm cible (Izaurralde and Zamore, 2009; Zipprich et al., 2009). Une amélioration possible de notre stratégie serait donc de cloner la région 5'NT du gène d'intérêt, en amont de l'eGFP, ce qui permettrait de résoudre ce problème et d'identifier simultanément les miARNs ciblant la région 5'NT.

Les perspectives de ce travail sont multiples :

- La dérégulation du miR-1271 *in vivo* pourrait permettre d'étudier son rôle anti-oncogénique/suppresseur de tumeur et de comprendre sa fonction dans la carcinogénèse hépatique

- L'implication des cinq miARNs validés dans la régulation de l'expression du GPC3 pourrait être étudiée dans d'autres pathologies associées au GPC3 (SGBS, cancers, voir section II .3.3).

II. Manuscrit 2

1. Introduction

Dans l'étude présentée dans la section 1 des résultats, j'ai démontré que le niveau d'expression du GPC3 est, par un mécanisme post-transcriptionnel, multiplié par quatre, dans les cellules tumorales, par comparaison avec les cellules non transformées (Maurel et al., 2012). La sous-expression du miR-1271 ne suffit pas à elle seule pour expliquer cette différence. Afin de mieux caractériser les mécanismes mis en jeu dans le processus de régulation post-transcriptionnelle de l'expression du GPC3, j'ai évalué le rôle de trois autres miARNs identifiés dans le crible fonctionnel mis en œuvre sur le 3'UTR du GPC3 (Maurel et al., 2012). et qui augmentent l'expression du GPC3. L'information disponible dans la littérature sur ces trois miARNs reste à ce jour très succincte.

- **MiR-129-1-3p** est surexprimé dans le cancer du côlon métastasé dans les ganglions lymphatiques (Huang et al., 2009). Son gène est localisé dans un site chromosomique fragile (FRA7H) fréquemment délété dans de nombreux cancers (Calin et al., 2004). Mais il n'est pas exprimé dans le foie normal et dans le foie tumoral, il présente donc très peu d'intérêt dans la régulation de l'expression du GPC3 dans le CHC.

- **MiR-1303** est muté dans 80% des tumeurs issues de cancer colorectal présentant un déficit du système de réparation des mésappariements (El-Murr et al., 2012). Mais il n'est pas dérégulé entre les échantillons de foie normal et de foie tumoral, par conséquent, tout comme le miR-129-1-3p, il présente peu d'intérêt dans la régulation de l'expression du GPC3 dans le CHC.

- **MiR-1291** est sous-exprimé dans le carcinome rénal (Hidaka et al., 2012) et dans le sang périphérique des patients atteints d'un infarctus aigu du myocarde (Meder et al., 2011). Il est surexprimé dans 20% des CHC **(Figure 29)**, correspondant aux groupes G2 et G3 (voir section II 2.2.1) (données non publiées de Yannick Ladeiro). Ce miARN représente donc un candidat intéressant qui pourrait contribuer à la surexpression du GPC3 dans le CHC.

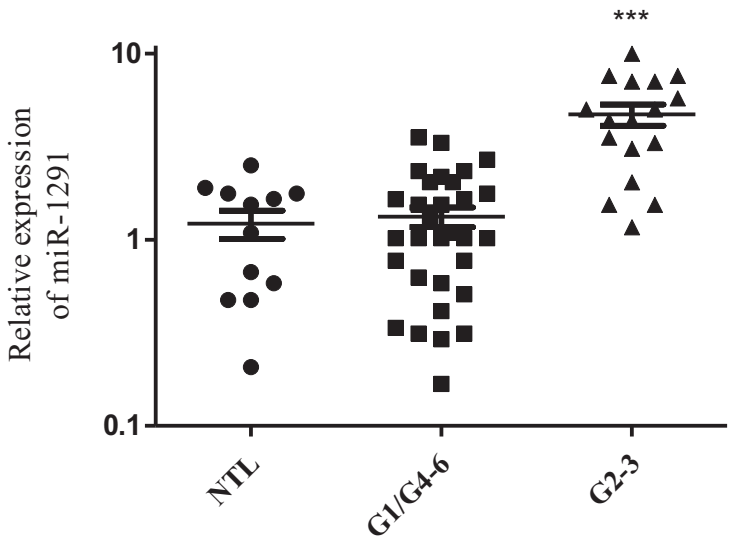


Figure 29 : Expression relative du miR-1291 dans le foie non tumoral et le foie tumoral. MiR-1291 est surexprimé d'un facteur 4 dans les groupes G2 et G3

L'objectif général de ce travail vise par conséquent à caractériser le mécanisme d'action de miR-1291 dans la régulation positive de l'expression du GPC3. L'ARNm codant pour le GPC3 ne possédant pas de site d'appariement prédit avec ce miARN, j'ai donc émis l'hypothèse que le miR-1291 pourrait induire son expression en ciblant un facteur inhibiteur intermédiaire. Afin d'identifier ce facteur intermédiaire, j'ai mis en place une approche combinant une analyse *in silico* et une validation fonctionnelle expérimentale grâce à laquelle j'ai identifié « inositol requiring enzyme-1 α » (IRE1α) comme candidat potentiel dont l'expression est réprimée par miR-1291 et qui régule négativement l'expression du GPC3.

MicroRNA-1291-mediated silencing of IRE1α enhances Glypican-3 expression

(soumis à RNA)

MicroRNA-1291-mediated silencing of IRE1α enhances Glypican-3 expression

Marion Maurel[1,2,*], Nicolas Dejeans[1,2], Eric Chevet[1,2,3] and Christophe F. Grosset[1,2,*]

[1]Groupe de Recherche sur L'étude du foie, Université Bordeaux Segalen, F-33000 Bordeaux, France. [2]INSERM, U1053, F-33000 Bordeaux, France. [3]CHU de Bordeaux, F-33000 Bordeaux, France.

*Correspondence should be addressed to Marion Maurel, m.marionmaurel@gmail.com or Christophe Grosset, christophe.grosset@u-bordeaux2.fr; INSERM U1053, Groupe de Recherche sur L'étude du Foie, Université Bordeaux-Segalen, 146 rue Léo Saignat, Bordeaux, France

Running title: miR-1291-mediated *GPC3* upregulation involves IRE1α

Keywords: microRNA, post-transcriptional up-regulation, *GPC3*, *ERN1*, RIDD, FunREG.

ABBREVATIONS

AMD, AU-rich-mediated decay; ANOVA, one-way analysis of variance; CDS, coding sequence; DF-FunREG, Dual-Fluorescence FunREG system; DTT, dithiothreitol; ER, endoplasmic reticulum; FBS, Fetal Bovine Serum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GFP, Green Fluorescent protein; GPC3, Glypican-3; GPI, glycosylphosphatidylinositol; HCC, hepatocellular carcinoma; miRISC, miRNA-induced silencing complexes; miRNAs, microRNAs; NMD, Nonsense-Mediated mRNA Decay; RIDD, Regulated IRE1α-Dependent Decay; SD, standard deviation; siRNA, small inhibitory RNA; snoRNA, small nucleolar RNA; Tun, Tunicamycin; UPR, unfolded protein response; UTR, untranslated region.

Abstract

MicroRNAs (miRNA) are generally described as negative regulators of gene expression. However rare evidences have pointed toward a positive role for these small non-coding RNAs in gene expression. Recently we reported that miR-1291 up-regulates *GPC3 mRNA* expression in hepatoma cells through a 3'-untranslated region (UTR)-dependent mechanism. In the absence of any evidence of direct interaction between miR-1291 and *GPC3* mRNA, we hypothesized that miR-1291 could act by silencing a negative regulator of *GPC3* mRNA expression. Based on *in silico* analyses and experimental validations, we demonstrate that miR-1291 down-regulates the expression of the mRNA encoding the endoplasmic reticulum-resident stress sensor *IRE1*α by interacting with a specific site located in its 5'-UTR. Moreover, we show *in vitro* and in cultured cells that IRE1α cleaves *GPC3* mRNA at a 3'-UTR consensus site, thereby prompting *GPC3* mRNA degradation. Finally, we show that the expression of a miR-1291-resistant form of IRE1α abrogates the positive effects of miR-1291 on *GPC3* mRNA expression. Collectively, our data demonstrate that miR-1291 is a biologically relevant regulator of *GPC3* expression in hepatoma cells and acts through the silencing of the endoribonuclease IRE1α.

Introduction

MicroRNAs (miRNAs) are endogenous ~22nt non-coding RNAs which regulate gene expression by controlling target mRNA translation and/or degradation (Bartel 2009; Fabian et al. 2010; Huntzinger and Izaurralde 2011). In most cases, miRNAs act as post-transcriptional repressors of gene expression through mechanisms involving sequence-specific mRNA:miRNA recognition and the regulated binding of miRNA-induced silencing complexes (miRISC) on target 3'-untranslated region (UTR) (Pillai et al. 2007). In addition, few reports also demonstrate the existence of miRNA-mediated target induction through molecular processes involving either the direct miRNA:target pairing or the indirect regulation of intermediary factors (Vasudevan 2012). Although the precise mechanisms underlying these phenomena remain elusive, several experimental evidences have been recently uncovered. For instance, some miRNAs positively regulate gene expression through a direct pairing depending on the cellular context or the site location (e.g. 5'UTR) (Vasudevan et al. 2007; Orom et al. 2008; Vasudevan 2012). This was well illustrated with miR-10a which binds to ribosomal protein RPS16, RPS6 and RPL9 encoding mRNA 5'-UTR and consequently enhances their translation (Orom et al. 2008). MiRNAs have also been implicated in gene up-regulation by targeting promoter elements. This is the case of miR-744 and miR-1186, which induce the transcription of mouse *Cyclin B1* (Huang et al. 2012). Other illustrations of miRNA-dependent gene induction were provided by recent discoveries showing that some miRNAs attenuate Nonsense-Mediated mRNA Decay (NMD) (Bruno et al. 2011) and AU-rich-mediated decay (AMD) processes (Ma et al. 2010).

In a previous study, we reported that three miRNAs promote Glypican-3 (*GPC3*) expression in hepatoma cells by a mechanism dependent on the 3'-UTR (Maurel et al. 2012). GPC3 belongs to the heparan sulfate proteoglycan family and regulates the signaling pathways mediated by WNTs, Hedgehogs, fibroblast growth factors and bone morphogenetic proteins (Fransson 2003; Filmus et al. 2008). GPC3 is a glycosylphosphatidylinositol (GPI) membrane-anchored protein that uses the secretory pathway to reach the plasma membrane. *GPC3* is a gene involved in various human diseases including type 1 Simpson-Golabi-Behmel syndrome and Wilms tumors. Moreover GPC3 is overexpressed in hepatocellular carcinoma (HCC) and hepatoblastoma (Jakubovic and Jothy 2007) in which its expression correlates with tumor aggressiveness and poor prognosis (Shirakawa et al. 2009). To characterize the miRNAs regulating *GPC3* expression in HCC-derived cells, we

screened a library of 876 human mature miRNA mimics using the *GPC3* 3'-UTR as a bait (Maurel et al. 2012). MiR-129-1-3p, miR-1291 and miR-1303 were identified as promoting the up-regulation of *GPC3* mRNA expression through uncharacterized and *GPC3* 3'-UTR-dependent mechanisms. Interestingly miR-1291 is more particularly up-regulated in HCC subgroups that express high levels of *GPC3* (Maurel et al. 2012).

In the present study, we investigated the molecular mechanisms by which miR-1291 may induce *GPC3* mRNA expression in hepatoma cells. To this end, an integrated approach combining *in silico* analyses, *in vitro* and cell-based validations was undertaken. We demonstrate that miR-1291 represses the expression of the endoplasmic reticulum (ER)-resident endoribonuclease IRE1α, which itself promotes *GPC3* mRNA decay. The latter regulation occurs through a mechanism which could be related to the Regulated IRE1α-Dependent Decay (RIDD) of mRNA (Hollien et al. 2009), therefore adding to the repertoire of miRNA-mediated decay mechanisms of repressive protein-associated machineries.

Results

MiR-1291 targets an intermediate factor that regulates *GPC3* mRNA expression

At first, to characterize miR-1291 mode of action on *GPC3* mRNA expression increase, we used GFP-*GPC3* 3'-UTR-expressing HuH7 cells and the FunREG method (Laloo et al. 2009; Laloo et al. 2010). The average number of lentiviral transgene copies per cell ('transgene copy number', TCN) was measured by quantitative PCR in HuH7 cells expressing the GFP-*GPC3* 3'-UTR transgene. Then the cells were transfected with a mature miR-1291 mimic or a control RNA. Three days later, eGFP protein (P) and mRNA (M) expression was determined using FACS and RT-qPCR, respectively. Finally P/TCN, M/TCN and P/M ratios, which respectively correspond to the global post-transcriptional regulation, the mRNA stability and the translation efficiency, were calculated (Laloo et al. 2009; Laloo et al. 2010). FunREG ratios (Fig. 1A) indicated that miR-1291 enhanced the global post-transcriptional regulation of *GPC3* expression by ~50%, as previously reported (Maurel et al. 2012). This effect exclusively resulted from an increased mRNA stability, as the translation efficiency remained unchanged (Fig. 1A). These results demonstrated that miR-1291 stabilizes *GPC3* mRNA through a mechanism involving the 3'UTR.

Then, since this miRNA up-regulates *GPC3* expression despite the absence of any predicted binding site within the mRNA, we hypothesized that miR-1291 might down-regulate the expression of an intermediate factor whose function would be to destabilize *GPC3* mRNA. To identify such candidate gene, miR-1291 targets were defined *in silico* using miRWalk predictions (Dweep et al. 2011) and yielded 2782 candidates predicted to contain at least one miR-1291 binding site in their promoter, 5'-UTR, coding sequence (CDS) and/or 3'-UTR. Among them, 83 candidates were annotated as post-transcriptional regulators in the Gene Ontology (GO) annotation database. Further analyses were carried out to determine specific functional groups among those 83 candidates. First, hierarchical clustering was performed GO annotations for Biological Processes (GOBP) based on the hypothesis that miR-1291 could target an actor of RNA catabolism. This analysis was carried out on the most represented annotations, namely GO:0006139/nucleobase-containing compound metabolic process; GO:0090304/nucleic acid metabolic process; GO:0016070/RNA metabolic process and GO:0006396/RNA processing; GO:0006401/RNA catabolic process. This revealed that the 83 candidates clustered into 5 main groups (Fig. 1B). Among the groups generated in the GOBP analysis, one contained a unique gene:

ERN1. This analysis was further completed by the manual annotation performed on the 83 candidates including the presence of a transmembrane helix (computed using the TMHMM program (Sonnhammer et al. 1998)), the ability to bind RNA and an RNA-related enzymatic activity (either direct or indirect), information retrieved from databases (see Materials and Methods) and from the literature. Clustering of this information (which was annotated on a binary mode) revealed the presence of 7 clusters substantially different from those obtained in Fig. 1B. But here again, *ERN1* clustered as the unique constituent of a group (Fig. 1C). Collectively, this reflected both the presence of a trans-membrane domain and the endoribonuclease function of *ERN1* and positions *ERN1* as a potential candidate linking miR-1291 to *GPC3* expression regulation. We further analyzed the functional organization of the 83 candidates based on their cellular component (GOCC) and integrating the information that *GPC3* mRNA encodes a extracellular membrane protein, which is exported to its final destination through the secretory pathway (GO:0043226/organelle; GO:0043227/membrane bound organelle; GO:000932/cytoplasmic RNA processing body; GO:0005634/nucleus; GO:0043233/organelle lumen). This new analysis revealed the presence of 7 main groups. This time, *ERN1* co-clustered with 6 other candidate genes, namely *TIRAP*, *BICD1*, *CHERP*, *TLR7*, *SLC11A1* and *TRMU* that belong to membranous compartments (Fig. 1D).

To test the functional relevance of this group of genes, a novel clustering analysis of those 7 candidates that integrated all the above-mentioned data was carried out and revealed, as anticipated, that *ERN1* branched out from the other candidates (Fig. 2A). Moreover, a score analysis was performed to determine which of those genes was the strongest miR-1291 potential candidate to achieve a decay function towards *GPC3* mRNA. As shown in Figure 2A (dark blue components represent the strongest scores for each information), this revealed that *ERN1* represented the strongest candidate of our selection as it belonged to the same cellular compartment as GPC3 and displayed an endogenous RNase activity. Interestingly, when the same approach was carried out on the 83 candidates, a similar result was obtained when considering *GPC3* mRNA as the target (not shown). ERN1, also known as inositol-requiring enzyme 1 (IRE1α) is an ER-resident transmembrane protein and a site-specific endoribonuclease activated upon accumulation of misfolded protein in this cellular compartment (ER stress). It is a

major sensor of the Unfolded Protein Response (UPR), an adaptive mechanism activated upon ER stress (Calfon et al. 2002; Schroder and Kaufman 2005). The cytoplasmic endoribonuclease domain of IRE1α was first described to cleave *XBP1* mRNA, yielding to its splicing and the production of an active transcription factor in response to ER stress (Yoshida et al. 2001). In addition Hollien and colleagues showed that, upon ER stress, IRE1α also induces the decay of mRNAs encoding membrane and secreted proteins in fly and mammalian cells through the RIDD pathway (Hollien and Weissman 2006; Hollien et al. 2009). GPC3 corresponds to the latter criteria as this GPI-membrane-anchored protein uses the secretory pathway to reach its final destination, i.e. the plasma membrane. Interestingly, three sites homologous to the 5'-CUGCAG-3' IRE1α consensus cleavage site previously defined by Oikawa and colleagues (Oikawa et al. 2010) are present in *GPC3* mRNA, including one in the 3'-UTR (Fig. 2B). Altogether these analyses pointed toward *ERN1/IRE1α* as a potential miR-1291 candidate target in the context of *GPC3* expression regulation. As a consequence, we hypothesized that miR-1291 could target IRE1α, thereby attenuating its endoribonuclease activity towards *GPC3* mRNA.

Based the above data, we tested the impact of miR-1291 on *IRE1α* and *GPC3* expression in HuH7 cells. Overexpression of miR-1291 down-regulated the expression of *IRE1α* mRNA (Fig. 2C) and of the corresponding protein (Fig. 2D) by ~40%, a degree of inhibition comparable to that observed for most other functional miRNAs (Avraham and Yarden 2012). As initially hypothesized, this effect was accompanied by the simultaneous increase in *GPC3* mRNA and protein expression (Fig. 2C, D). The co-transfection of HuH7 cells with miR-1291 and its specific inhibitor (AM1291) abrogated miR-1291-mediated effects, thereby demonstrating the specificity of our results and validating the first part of our hypothesis. The lack of effect of AM1291 alone could neither be attributed to the lack of AM1291 efficacy, which counteracted miR-1291 functions (not shown) nor to a very low expression of miR-1291, which we found well expressed in HuH7 cells (Fig. S1). However, a possible explanation could be that IRE1α regulates the expression of its mRNA to control its own expression (Tirasophon et al. 2000). Collectively these results indicate that miR-1291 could regulate the expression of IRE1α.

IRE1α is a direct target of miR-1291

MiRWalk predicts that *IRE1α* mRNA contains a site pairing with miR-1291 in its 5'-UTR; a miRNA:mRNA interaction that was further supported by RNAhybrid ((Rehmsmeier et al. 2004); Fig. 3A). Using GFP-expressing HuH7 cells bearing either a wild type or mutant *IRE1α* 5'-UTR (Fig. 3B), it was found that miR-1291 significantly inhibited GFP expression by ~30% with the wild-type *IRE1α* construct, whereas mutation abrogating the sequence complementary to miR-1291 seed abolished its biological activity (Fig. 3C). Using FunREG, we further demonstrated that miR-1291 post-transcriptionally destabilized *IRE1α* mRNA with no effect on translation (Fig. 3D). These data confirmed that miR-1291 directly targets *IRE1α* 5'-UTR and induces the degradation of its mRNA through a miRNA-seed recognition process.

IRE1α regulates *GPC3* mRNA expression and stability

To investigate the relationship between *IRE1α* and *GPC3* in greater detail, *IRE1α* expression was silenced in HuH7 cells and cells were then exposed or not to ER stressors. Dithiothreitol (DTT), which reduces disulfide bonds, and Tunicamycin (Tun), an N-linked glycosylation inhibitor, were used to induce ER stress and thereby IRE1α activation (Schroder and Kaufman 2005). Both DTT and Tun induced *XBP1* mRNA splicing and these effects were attenuated in *IRE1α*-silenced cells, thus confirming the activation of the IRE1α/XBP1 signaling axis upon ER stress (Fig. 4A). Under the same experimental conditions, miR-1291 expression remained unchanged (Fig. S2). Interestingly, *GPC3* expression increased at both mRNA (Fig. 4B) and protein (Fig. 4C) levels upon attenuation of *IRE1α* expression and this occurred independently of ER stress. Using actinomycin D to inhibit transcription, we further demonstrated that *IRE1α* silencing led to *GPC3* mRNA stabilization (Fig. 4D). We then transfected HuH7 cells with miR-1291 and/or its specific antimiR to assess the specificity of miR-1291 towards the IRE1α arm of the UPR. At first, the impact of miR-1291 was evaluated on the activation of *CHOP* mRNA expression, a gene whose transcription is increased downstream of the PERK arm of the UPR upon ER stress (Schroder and Kaufman 2005). In parallel we monitored *IRE1α*–mediated *XBP1* mRNA splicing under the same conditions. As shown in Figure S3A, miR-1291 had no influence on the PERK arm of the UPR as *CHOP* mRNA expression was not affected by a variation of miR-1291 expression under basal conditions or upon ER

stress. However, miR-1291 specifically affected IRE1α endoribonuclease activity as shown by the attenuation of *XBP1* mRNA splicing upon ER stress (Figure S3B). Collectively these data demonstrate that IRE1α controls *GPC3* mRNA stability and that miR-1291 selectively targets the IRE1α arm of the UPR.

IRE1α cleaves *GPC3* through a canonical site located in its 3'-UTR

IRE1α is known to act as a site-specific endoribonuclease (Oikawa et al. 2010). As mentioned above, *GPC3* mRNA sequence contains 3 sites homologous to the 5'-CUGCAG-3' IRE1α consensus cleavage site and similar to those found in *XBP1* mRNA. In addition, these 3 sites formed P-loop structures, thereby yielding potential cleavage sites for IRE1α (Oikawa et al. 2010) (Fig. 5A). To test whether IRE1α cleaves *GPC3* mRNA and to identify the IRE1α cleavage sites, total RNA from HuH7 cells was subjected to an *in vitro* IRE1α-mediated cleavage assay (Bouchecareilh et al. 2010). RT-PCR was then carried out using primers flanking the 3 consensus sites predicted above to precisely monitor *GPC3* mRNA cleavage position. Interestingly, only one site among the three predicted appeared sensitive to IRE1α endoribonuclease activity *in vitro* (Fig. 5B). Indeed, the amount of RT-PCR product corresponding to the site located at nucleotides 2039/40 in *GPC3* mRNA 3'-UTR decreased by ~60% whereas the amount of four other *GPC3* fragments, as well as the control *GAPDH* mRNA, did not change. This suggests that *GPC3* mRNA contains only one functional IRE1α cleavage site in its 3'-UTR. To further demonstrate the relevance of this cleavage site, *in vitro* transcribed *GPC3* mRNA and its mutated counterpart deleted for the predicted 2039/40 site were subjected to IRE1α-mediated cleavage. In a model where IRE1α would cleave *GPC3* mRNA at the 2039/40 site (Fig. 5C), two RNA cleavage products of 1700nt and 300nt, respectively, would be produced. As anticipated from the latter model, cleavage of wild-type *GPC3* mRNA generated two RNA fragments of the expected size (Fig. 5D, left). In contrast, deletion of the 2039/40 site in *GPC3* mRNA abrogated its cleavage by IRE1α (Fig. 5D, right). Altogether these results demonstrated that, *in vitro,* IRE1α cleaves *GPC3* in its 3'-UTR at the sole 2039/40 site.

To test the *in vivo* relevance of this observation in cultured cells, we evaluated the capacity of IRE1α to cleave *GPC3* mRNA in cultured cells. Using targeted RT-qPCR (Iqbal et al. 2008), the amount of each *GPC3* RNA fragments was measured in HuH7 cells upon siRNA-mediated downregulation of 5'-3' exonucleases *XRN1/2* or

RNA helicase *SKI2,* the latter mediating the 3'-5' degradation of mRNA through the cytoplasmic exosome (Fig. 5E). Compared to control, SKI2 siRNA, led to *SKI2* mRNA decrease as expected (data not shown) and to the accumulation of *GPC3* mRNA Fragment 1, the fragment upstream of IRE1α cleavage site, with no effect on the amount of Fragment 2 (the fragment downstream of the site) (Fig. 5F). In contrast, the silencing of *XRN1 or XRN2* led to an increase in Fragment 2 with no effect on the amount of Fragment 1 (Fig. 5F). This shows that the *GPC3* mRNA cleavage site identified *in vitro* also exists in cells. Furthermore, siRNA-mediated silencing IRE1α led to the disappearance of these fragments (Fig. 5F), consistent with an *in vivo* IRE1α-mediated cleavage of *GPC3* mRNA in its 3'-UTR at the 2039/40 site.

Our initial hypothesis raised the possibility that miR-1291 would target the expression of a negative regulator of *GPC3* mRNA stability. Our results pointed toward IRE1α as being this protein. In this context, it would be predicted that expression of a miR-1291 resistant form of *IRE1α* mRNA (i.e. devoid of its 5'UTR) would prevent miR-1291-mediated *GPC3* up-regulation. As predicted, overexpression of an *IRE1α* transgene lacking its 5'UTR in HuH7 cells (Fig. S4) inhibited miR-1291-mediated up-regulation of *GPC3* mRNA expression and led to a decrease in *GPC3* mRNA expression, thereby confirming the functional relationship between those three genes (Fig. 6A). Hence, our results demonstrated that miR-1291 targets *IRE1α* through its 5'UTR, thereby attenuating IRE1α-mediated decay of *GPC3* mRNA and consequently leading to increased *GPC3* expression in hepatoma cells.

Discussion

In this work we characterized the molecular mechanism by which miR-1291 post-transcriptionally up-regulates *GPC3* expression (Fig. 6C). Using an integrated approach combining both *in silico* analyses and *in vitro*/cell-based validations, we report that miR-1291 binds to the *IRE1α* 5'-UTR thus destabilizing its mRNA and leading to its degradation. We also demonstrate the presence of a functional IRE1α cleavage site in *GPC3* mRNA 3'-UTR, thereby indicating that *GPC3* mRNA is a genuine RIDD substrate. Collectively, our data show that miR-1291 up-regulates *GPC3* mRNA expression by down-regulating *IRE1α* mRNA expression. Therefore our results point toward a novel miRNA-dependent gene expression control mechanism through *IRE1α* silencing and RIDD attenuation.

MiRNAs generally act as negative regulators of gene expression (Bartel 2009). However, some were also shown to act positively (Vasudevan 2012). Using a functional miRNA screening, we recently identified three miRNAs enhancing *GPC3* expression through a 3'-UTR-dependent mechanism among which miR-1291 (Maurel et al. 2012). Little is known about the functional properties of miR-1291 in cells. MiRNAs loci are located in different regions of the genome. Half is intragenic and is encoded by protein-coding genes or non-coding RNA genes. This is the case of miR-1291, which is localized in the SNORA34 gene (Scott et al. 2009; Brameier et al. 2011). MiR-1291 expression is down-regulated in renal cell carcinoma (Hidaka et al. 2012) and in the peripheral blood of acute myocardial infarction patients (Meder et al. 2011). However no functional or pathophysiological roles have been associated with these observations. As there is no predicted miR-1291 binding site on *GPC3* mRNA, we hypothesized that miR-1291 could enhance *GPC3* expression by an indirect mechanism involving an intermediate regulatory factor recognizing *GPC3* mRNA 3'-UTR. Such a mechanism has for instance been illustrated with the targeting of genes involved in NMD (Bruno et al. 2011) or in the CCR4-NOT complex (Behm-Ansmant et al. 2006).

As one miRNA can target hundreds of different mRNAs, it is likely that many negative regulatory intermediates might be the target of miR-1291 to control *GPC3* expression. To restrict the number of candidates obtained *in silico* to an experimentally testable set, we focused on regulatory factors involved in RNA catabolism and likely acting on a sequence-specific site recognition basis. *In silico* data pointed towards *IRE1α* as a potential candidate as this gene fulfilled the above-

mentioned criteria (Fig. 1 and 2). The experimental validation revealed that IRE1α was a direct target of miR-1291 (Fig. 2). Remarkably, miR-1291 specifically paired with a site located in *IRE1α* 5'-UTR (Fig. 2). MiRNA:5'-UTR target interactions are currently poorly described, likely due to the fact that most of the current bioinformatics tools generate predictions on 3'-UTR sequence and not on the full mRNA sequence, which therefore may introduce a prediction bias. In contrast to most available tools miRWalk produces miRNA-targets interactions information on the complete mRNA sequence as well as on the gene promoter (Dweep et al. 2011). Finally, the identification of human miR-1291 orthologues in other mammalian species using miRBase (Griffiths-Jones et al. 2008; Kozomara and Griffiths-Jones 2011) together with the conservation of their binding sites in *IRE1α* 5'-UTRs in several species suggested the existence of a similar regulatory mechanism in mammals (data not shown).

In the past two years, several studies have identified miRNAs whose expression is regulated downstream of UPR signaling. Each one of the UPR sensors relays information on the protein folding status from the ER lumen to the nucleus, thereby controlling gene expression. The PERK branch was described to control the expression of miR-708 (Behrman et al. 2011), miR-106b-25 (Gupta et al. 2012) and miR-30c-2* (Byrd et al. 2012). In the same way, ATF6 down-regulates the expression of miR-455 (Belmont et al. 2012) and IRE1α signaling increases that of miR-346 (Bartoszewski et al. 2011). Recently, miR-122, the most abundant miRNA in the liver, which is under-expressed in hepatocellular carcinoma, was found to inhibit CDK4, which interacts and induces accumulation of PSMD10, a proteasome component and an enhancer of the UPR (Yang et al. 2011). Herein, we describe for the first time a miRNA acting as an upstream inhibitor of the UPR pathway by directly targeting *IRE1α*. Intriguingly, this regulation did not depend on ER stress, which suggests potential roles for UPR signaling components in non-stressed conditions.

Fine-tuning of the UPR plays a fundamental role in cancer cell fate decisions by determining adaptation and survival to ER stress, and eliminating irreversibly damaged cells (Moenner et al. 2007; Tabas and Ron 2011; Woehlbier and Hetz 2011). In this context, IRE1α has evolved a dual function to preserve ER homeostasis (Han et al. 2009). First, the IRE1α/XBP1 axis is thought to favor tumor cell adaptation to stress by increasing the ability of these cells to synthesize and fold large amounts of transmembrane and secreted proteins. Second, prolonged

activation of the RIDD pathway might decrease tumor growth in a cell-specific manner by degrading mRNAs encoding pro-oncogenic proteins, including PDGFR and SPARC (Hollien and Weissman 2006; Dejeans et al. 2012). In the present study, we identify *GPC3* mRNA as a novel RIDD substrate and demonstrate its cleavage by IRE1α endoribonuclease at a consensus site located in *GPC3* mRNA 3'-UTR (Fig. 5). *GPC3* is known to promote HCC cell growth by stimulating the WNT/β-catenin pathway (Capurro et al. 2005). Consequently our results suggest that miR-1291 could act as an oncomiR by attenuating IRE1α expression and RIDD, thereby leading to *GPC3* overexpression in hepatocellular tumors. This hypothesis should however be considered carefully as in the mean time miR-1291-mediated attenuation of IRE1α expression would also reduce *XBP1* mRNA splicing. The integrated signaling outcomes resulting from this initial event would therefore determine the oncogenic potential of miR-1291. Finally, although it is currently believed that IRE1α activation plays an instrumental role in tumor progression (Auf et al. 2010; Dejeans et al. 2012), somatic driver mutations in the IRE1α gene were identified in cancers (Greenman et al. 2007) and recently associated with a loss-of-signaling function of this molecule (Xue et al. 2011), thereby also associating IRE1α inactivation with cell transformation. This might be consistent with the hypothesis of a pro-oncogenic miR-1291-mediated attenuation of IRE1α signaling.

In conclusion, we report a novel mechanism of miRNA-mediated positive regulation of gene expression originating from the silencing of the endoribonuclease IRE1α (Fig. 6C). The physiological and pathophysiological consequences of such mechanism still remain to be fully investigated, especially in HCC in which *GPC3* overexpression plays a master regulatory role. However one can easily anticipate a significant contribution of *IRE1α* down-regulation to cancer development through the overexpression of cancer-associated downstream gene targets.

Materials and Methods

Plasmids and cloning

The pL-GFP-GPC3 3'-UTR lentiviral and pED-IRE1α plasmids were as described previously (Nguyen et al. 2004; Maurel et al. 2012). The pEF-hGPC3 plasmid was kindly provided by S. Mizushima and S. Nagata (Osaka Bioscience Institute, Japan) (Mizushima and Nagata 1990). The pcDNA3.1-ΔCUACAG-hGPC3 was obtained as follows: a mutant *GPC3* gene devoid of the IRE1α cleavage site was synthesized (Eurofins MWG Operon), digested by NotI and cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). The pL-wt-IRE1α 5'-UTR-GFP and pL-mut-IRE1α-5'-UTR-GFP plasmids were constructed as follows: IRE1α 5'-UTR was amplified using 5'-CACGGATCCTGCCTAGTCAGTTCTGCGTC as forward primer and either 5'-CACGGATCCGGCGAGGACTCGGCCCT or 5'-CACGGATCCGGCGAGGACTC**C**G**G**C**G**TGGCTCCGGGGG as reverse primers, respectively. Each PCR product was digested by BamHI and sub-cloned into pL-GFP plasmid (Maurel et al. 2012). Sequence was verified by DNA sequencing.

Cell culture

HuH7 cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin (1000 units/mL). Green Fluorescent protein (GFP)-expressing HuH7 cells were established using lentiviral transduction (m.o.i. =3) as previously described (Laloo et al. 2009; Jalvy-Delvaille et al. 2012).

Small RNA transfection

Small inhibitory RNAs (siRNAs, Supplementary Table S1) were designed using the Greg Hannon's webtool (http://hannonlab.cshl.edu/). The negative control RNA, mature miRNA mimics and hairpin miRNA inhibitors were from Qiagen (Hilden, Germany). Small RNAs were transfected into the target cells by reverse-transfection using Lipofectamine RNAi Max (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

RNA isolation, Reverse Transcription and qPCR analyses

Total RNA was prepared using the TRI Reagent (Sigma, StLouis, MO, USA) following the manufacturer's instructions. Mature miRNA expression was quantified

using the TaqMan microRNA Reverse Transcription Kit and TaqMan microRNA assays (Applied Biosystems, Carlsbad, CA, USA). Messenger RNA expression was quantified using the SYBR Green Supermix (Quanta Biosciences, Gaithersburg, MD, USA). Quantitative PCR reactions were performed using the Step One Plus Quantitative PCR System (Applied Biosystems, Carlsbad, CA, USA). For each data point, experiments were performed in triplicate. In all cases, each sample was normalized toward the expression of the 18S ribosomal RNA. PCR products were resolved on 1% agarose TBE 0.5x electrophoresis gels or 4% for the *XBP1* splicing experiments. Primers used are as described in Supplementary Table S2.

Antibodies and Western blot analyses

Antibody against GPC3 was from Biomosaics (Burlington, NC, USA). Anti-IRE1α and anti-GAPDH were from Santa-Cruz Biotechnology (Santa Cruz, CA, USA). Protein extraction and Western blotting were performed as previously described (Jalvy-Delvaille et al. 2012). Signals were normalized to the amount of the housekeeping protein GAPDH.

FunREG analysis

FunREG analyses were performed three days after cell transfection as previously described (Laloo et al. 2009; Maurel et al. 2012). Cells were washed with PBS, detached with trypsin/EDTA, collected and analyzed by FACS using the BD LSRFortessa cell analyzer and the BD FACSDiva software (BD Biosciences, San Jose, CA, USA). In parallel, RNA was extracted from each cell population, reverse transcribed and quantitative PCR were performed as described above.

Bioinformatic analyses

In silico analyses were performed using miRWalk (Dweep et al. 2011). This program identifies the longest consecutive complementarity between miRNA and gene sequences, produces information about miRNA:target interactions on the complete gene sequence (promoter, 5'-UTR, coding sequence and 3'-UTR) of all known genes and compares the candidate miRNA binding sites with those established by 8 miRNA-target prediction programs (i.e. DIANA-microT, miRanda, miRDB, PicTar, PITA, RNA22, RNAhybrid and TargetScan/TargetScanS). Finally miRWalk incorporates all the predicted miRNA binding sites produced by the miRWalk

algorithm and the 8 other programs into a relational database. The secondary structure of the *GPC3* mRNA:miR-1291 interaction was predicted using M-FOLD (Zuker et al. 1999). Functional classification was achieved by g:profiler (Reimand et al. 2007). Generated data were also manually sorted and integrated to generate quantitative and qualitative information on the Gene Ontology (Cellular Compartment and Biological Process components). Moreover an additional piece of information concerning candidate miR-1291 targets was retrieved from databases (GeneCard, NCBI, miRBase, miRDB, HNGC) and from the literature to constitute another annotation binary (No = 0; Yes = 1) annotation file. All these data were then clustered and represented using the CLUSTER and TREEVIEW programs (de Hoon et al. 2004; Saldanha 2004). All the clusters were built using Euclidian distances. Trees were generated using average linkage.

RNA cleavage assay

Ten µg of total RNA extracted from HuH7 were incubated at 37°C with the cytoplasmic domain of human IRE1α (5 µg) fused to GST (GST-IRE1α^{cyto}) for 4h in a buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 mM MgCl2 and 1 mM ATP, as previously described (Bouchecareilh et al. 2010; Bouchecareilh et al. 2011). Heat-denatured GST-IRE1α^{cyto} was used as control. RNA fragments were detected by RT-PCR using specific primers (Supplementary Table S2). The pcDNA3.1-ΔCUACAG-hGPC3 and pEF-hGPC3 plasmids were used as template for *in vitro* RNA transcription using the T7 polymerase (Promega, Madison, WI, USA). *In vitro* transcribed RNA was incubated at 37°C with 5 µg of GST-IRE1α^{cyto} and 1 mM ATP for 4h. Reaction products were then denatured 10 minutes at 65°C in RNA sample Buffer (56% formamide, 37% formaldehyde, 7% MOPS). Fragments resulting from the enzymatic reaction were resolved on 1% formaldehyde agarose gels and visualized by UV trans-illumination.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 software (La Jolla, CA, USA). Data are presented as the mean of the indicated number of independent experiments ± standard deviation (SD). When experiment contained three groups of values or more, regular one-way analysis of variance (ANOVA) was used for the comparison of multiple means. Means were considered significantly different when

$P<0.05$. The ANOVA test was followed by a Bonferroni's multiple-comparison post-test and selected pairs of data were compared. Significant variations are indicated by asterisks.

Acknowledgements

This work was funded in part by grants from L'Institut National du Cancer (INCa) and La Ligue Nationale contre le Cancer (LNCC) to CG and INSERM, INCa, LNCC to EC. EC was supported by an Interface contract INSERM-CHU de Bordeaux. MM was a recipient from a PhD scholarship from the French Research Ministry. ND was funded by a post-doctoral fellowship from LNCC.

References

Auf G, Jabouille A, Guerit S, Pineau R, Delugin M, Bouchecareilh M, Magnin N, Favereaux A, Maitre M, Gaiser T et al. 2010. Inositol-requiring enzyme 1alpha is a key regulator of angiogenesis and invasion in malignant glioma. *Proc Natl Acad Sci U S A* **107**(35): 15553-15558.

Avraham R, Yarden Y. 2012. Regulation of signalling by microRNAs. *Biochem Soc Trans* **40**(1): 26-30.

Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* **136**(2): 215-233.

Bartoszewski R, Brewer JW, Rab A, Crossman DK, Bartoszewska S, Kapoor N, Fuller C, Collawn JF, Bebok Z. 2011. The unfolded protein response (UPR)-activated transcription factor X-box-binding protein 1 (XBP1) induces microRNA-346 expression that targets the human antigen peptide transporter 1 (TAP1) mRNA and governs immune regulatory genes. *J Biol Chem* **286**(48): 41862-41870.

Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E. 2006. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev* **20**(14): 1885-1898.

Behrman S, Acosta-Alvear D, Walter P. 2011. A CHOP-regulated microRNA controls rhodopsin expression. *J Cell Biol* **192**(6): 919-927.

Belmont PJ, Chen WJ, Thuerauf DJ, Glembotski CC. 2012. Regulation of microRNA expression in the heart by the ATF6 branch of the ER stress response. *J Mol Cell Cardiol* **52**(5): 1176-1182.

Bouchecareilh M, Caruso ME, Roby P, Parent S, Rouleau N, Taouji S, Pluquet O, Bosse R, Moenner M, Chevet E. 2010. AlphaScreen-based characterization of the bifunctional kinase/RNase IRE1alpha: a novel and atypical drug target. *J Biomol Screen* **15**(4): 406-417.

Bouchecareilh M, Higa A, Fribourg S, Moenner M, Chevet E. 2011. Peptides derived from the bifunctional kinase/RNase enzyme IRE1alpha modulate IRE1alpha activity and protect cells from endoplasmic reticulum stress. *FASEB J* **25**(9): 3115-3129.

Brameier M, Herwig A, Reinhardt R, Walter L, Gruber J. 2011. Human box C/D snoRNAs with miRNA like functions: expanding the range of regulatory RNAs. *Nucleic Acids Res* **39**(2): 675-686.

Bruno IG, Karam R, Huang L, Bhardwaj A, Lou CH, Shum EY, Song HW, Corbett MA, Gifford WD, Gecz J et al. 2011. Identification of a microRNA that activates gene expression by repressing nonsense-mediated RNA decay. *Mol Cell* **42**(4): 500-510.

Byrd AE, Aragon IV, Brewer JW. 2012. MicroRNA-30c-2* limits expression of proadaptive factor XBP1 in the unfolded protein response. *J Cell Biol* **196**(6): 689-698.

Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D. 2002. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415**(6867): 92-96.

Capurro MI, Xiang YY, Lobe C, Filmus J. 2005. Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. *Cancer Res* **65**(14): 6245-6254.

de Hoon MJ, Imoto S, Nolan J, Miyano S. 2004. Open source clustering software. *Bioinformatics* **20**(9): 1453-1454.

Dejeans N, Pluquet O, Lhomond S, Grise F, Bouchecareilh M, Juin A, Meynard-Cadars M, Bidaud-Meynard A, Gentil C, Moreau V et al. 2012. Autocrine control of glioma cells adhesion/migration through Inositol Requiring enzyme 1alpha (IRE1alpha)-mediated cleavage of Secreted Protein Acidic Rich in Cysteine (SPARC) mRNA. *J Cell Sci*.

Dweep H, Sticht C, Pandey P, Gretz N. 2011. miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. *J Biomed Inform* **44**(5): 839-847.

Fabian MR, Sonenberg N, Filipowicz W. 2010. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* **79**: 351-379.

Filmus J, Capurro M, Rast J. 2008. Glypicans. *Genome Biol* **9**(5): 224.

Fransson LA. 2003. Glypicans. *Int J Biochem Cell Biol* **35**(2): 125-129.

Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C et al. 2007. Patterns of somatic mutation in human cancer genomes. *Nature* **446**(7132): 153-158.

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. 2008. miRBase: tools for microRNA genomics. *Nucleic Acids Res* **36**(Database issue): D154-158.

Gupta S, Read DE, Deepti A, Cawley K, Gupta A, Oommen D, Verfaillie T, Matus S, Smith MA, Mott JL et al. 2012. Perk-dependent repression of miR-106b-25 cluster is required for ER stress-induced apoptosis. *Cell Death Dis* **3**: e333.

Han D, Lerner AG, Vande Walle L, Upton JP, Xu W, Hagen A, Backes BJ, Oakes SA, Papa FR. 2009. IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. *Cell* **138**(3): 562-575.

Hidaka H, Seki N, Yoshino H, Yamasaki T, Yamada Y, Nohata N, Fuse M, Nakagawa M, Enokida H. 2012. Tumor suppressive microRNA-1285 regulates novel molecular targets: aberrant expression and functional significance in renal cell carcinoma. *Oncotarget* **3**(1): 44-57.

Hollien J, Lin JH, Li H, Stevens N, Walter P, Weissman JS. 2009. Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. *J Cell Biol* **186**(3): 323-331.

Hollien J, Weissman JS. 2006. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* **313**(5783): 104-107.

Huang V, Place RF, Portnoy V, Wang J, Qi Z, Jia Z, Yu A, Shuman M, Yu J, Li LC. 2012. Upregulation of Cyclin B1 by miRNA and its implications in cancer. *Nucleic Acids Res* **40**(4): 1695-1707.

Huntzinger E, Izaurralde E. 2011. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet* **12**(2): 99-110.

Iqbal J, Dai K, Seimon T, Jungreis R, Oyadomari M, Kuriakose G, Ron D, Tabas I, Hussain MM. 2008. IRE1beta inhibits chylomicron production by selectively degrading MTP mRNA. *Cell Metab* **7**(5): 445-455.

Jakubovic BD, Jothy S. 2007. Glypican-3: from the mutations of Simpson-Golabi-Behmel genetic syndrome to a tumor marker for hepatocellular carcinoma. *Exp Mol Pathol* **82**(2): 184-189.

Jalvy-Delvaille S, Maurel M, Majo V, Pierre N, Chabas S, Combe C, Rosenbaum J, Sagliocco F, Grosset CF. 2012. Molecular basis of differential target regulation by miR-96 and miR-182: the Glypican-3 as a model. *Nucleic Acids Res* **40**(3): 1356-1365.

Kozomara A, Griffiths-Jones S. 2011. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res* **39**(Database issue): D152-157.

Laloo B, Maurel M, Jalvy-Delvaille S, Sagliocco F, Grosset CF. 2010. Analysis of post-transcriptional regulation using the FunREG method. *Biochem Soc Trans* **38**(6): 1608-1614.

Laloo B, Simon D, Veillat V, Lauzel D, Guyonnet-Duperat V, Moreau-Gaudry F, Sagliocco F, Grosset C. 2009. Analysis of post-transcriptional regulations by a

functional, integrated, and quantitative method. *Mol Cell Proteomics* **8**(8): 1777-1788.

Ma F, Liu X, Li D, Wang P, Li N, Lu L, Cao X. 2010. MicroRNA-466l upregulates IL-10 expression in TLR-triggered macrophages by antagonizing RNA-binding protein tristetraprolin-mediated IL-10 mRNA degradation. *J Immunol* **184**(11): 6053-6059.

Maurel M, Jalvy S, Ladeiro Y, Combe C, Vachet L, Sagliocco F, Bioulac-Sage P, Pitard V, Jacquemin-Sablon H, Zucman-Rossi J et al. 2012. A functional screening identifies five miRNAs controlling Glypican-3: Role of miR-1271 down-regulation in hepatocellular carcinoma. *Hepatology*.

Meder B, Keller A, Vogel B, Haas J, Sedaghat-Hamedani F, Kayvanpour E, Just S, Borries A, Rudloff J, Leidinger P et al. 2011. MicroRNA signatures in total peripheral blood as novel biomarkers for acute myocardial infarction. *Basic Res Cardiol* **106**(1): 13-23.

Mizushima S, Nagata S. 1990. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* **18**(17): 5322.

Moenner M, Pluquet O, Bouchecareilh M, Chevet E. 2007. Integrated endoplasmic reticulum stress responses in cancer. *Cancer Res* **67**(22): 10631-10634.

Nguyen DT, Kebache S, Fazel A, Wong HN, Jenna S, Emadali A, Lee EH, Bergeron JJ, Kaufman RJ, Larose L et al. 2004. Nck-dependent activation of extracellular signal-regulated kinase-1 and regulation of cell survival during endoplasmic reticulum stress. *Mol Biol Cell* **15**(9): 4248-4260.

Oikawa D, Tokuda M, Hosoda A, Iwawaki T. 2010. Identification of a consensus element recognized and cleaved by IRE1 alpha. *Nucleic Acids Res* **38**(18): 6265-6273.

Orom UA, Nielsen FC, Lund AH. 2008. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell* **30**(4): 460-471.

Pillai RS, Bhattacharyya SN, Filipowicz W. 2007. Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol* **17**(3): 118-126.

Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R. 2004. Fast and effective prediction of microRNA/target duplexes. *RNA* **10**(10): 1507-1517.

Reimand J, Kull M, Peterson H, Hansen J, Vilo J. 2007. g:Profiler--a web-based toolset for functional profiling of gene lists from large-scale experiments. *Nucleic Acids Res* **35**(Web Server issue): W193-200.

Saldanha AJ. 2004. Java Treeview--extensible visualization of microarray data. *Bioinformatics* **20**(17): 3246-3248.

Schroder M, Kaufman RJ. 2005. The mammalian unfolded protein response. *Annu Rev Biochem* **74**: 739-789.

Scott MS, Avolio F, Ono M, Lamond AI, Barton GJ. 2009. Human miRNA precursors with box H/ACA snoRNA features. *PLoS Comput Biol* **5**(9): e1000507.

Shirakawa H, Suzuki H, Shimomura M, Kojima M, Gotohda N, Takahashi S, Nakagohri T, Konishi M, Kobayashi N, Kinoshita T et al. 2009. Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. *Cancer Sci* **100**(8): 1403-1407.

Sonnhammer EL, von Heijne G, Krogh A. 1998. A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc Int Conf Intell Syst Mol Biol* **6**: 175-182.

Tabas I, Ron D. 2011. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* **13**(3): 184-190.

Tirasophon W, Lee K, Callaghan B, Welihinda A, Kaufman RJ. 2000. The endoribonuclease activity of mammalian IRE1 autoregulates its mRNA and is required for the unfolded protein response. *Genes Dev* **14**(21): 2725-2736.

Vasudevan S. 2012. Posttranscriptional upregulation by microRNAs. *Wiley Interdiscip Rev RNA* **3**(3): 311-330.

Vasudevan S, Tong Y, Steitz JA. 2007. Switching from repression to activation: microRNAs can up-regulate translation. *Science* **318**(5858): 1931-1934.

Woehlbier U, Hetz C. 2011. Modulating stress responses by the UPRosome: a matter of life and death. *Trends Biochem Sci* **36**(6): 329-337.

Xue Z, He Y, Ye K, Gu Z, Mao Y, Qi L. 2011. A conserved structural determinant located at the interdomain region of mammalian inositol-requiring enzyme 1alpha. *J Biol Chem* **286**(35): 30859-30866.

Yang F, Zhang L, Wang F, Wang Y, Huo XS, Yin YX, Wang YQ, Sun SH. 2011. Modulation of the unfolded protein response is the core of microRNA-122-involved sensitivity to chemotherapy in hepatocellular carcinoma. *Neoplasia* **13**(7): 590-600.

Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. 2001. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107**(7): 881-891.

Zuker M, Mathews DH, Turner DH. 1999. Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide. *RNA Biochemistry and Biotechnology*.

Figure Legends

Figure 1:

MiR-1291 targets an intermediate factor that regulates *GPC3* mRNA expression. (**A**) miR-1291 enhances *GPC3* mRNA stability through its 3'-UTR. Top panel: schematic representation of the GFP-GPC3 3'-UTR transgene used in this study. Bottom panel: GFP-GPC3 3'-UTR-expressing HuH7 cells were transfected with a control RNA or miR-1291. Three days later, the transgene copy number (TCN) and the expression of eGFP protein (P) and mRNA (M) were measured. Finally the FunREG ratios were calculated as described in the Materials and Methods section. P/TCN: global post-transcriptional regulation, M/TCN: mRNA stability and P/M: translation efficiency (ANOVA: $P<0.0001$; $n=5$). **p<0.01. (**B-D**) Selection of miR-1291-predicted targets involved in mRNA destabilization. Using miRWalk, 2782 gene candidates were predicted as miR-1291 targets (p-value <0.01). Among them, 83 are described as post-transcriptional regulators. Functional clustering of those candidates was performed based on the Gene Ontology annotation for biological processes (GOBP; **B**), on molecular annotation using data from various databases and literature (MA; **C**), or on the Gene Ontology annotation for cellular compartments (GOCC; **D**). Scale bar is indicated for GOBP and GOCC clusters.

Figure 2: The mRNA destabilizing factor *IRE1α* is a direct target of miR-1291. (**A**) Information (GOBP, MA, GOCC) was integrated for seven candidate genes that were identified as relevant of a membranous compartment, namely *ERN1, TIRAP*, *BICD1*, *CHERP*, *TLR7*, *SLC11A1* and *TRMU*. Hierarchical clustering was then performed as in Figure 1. Scale bar is indicated. (**B**) Identification of three sites homologous to the 5'-CUGCAG-3' IRE1α consensus cleavage site in GPC3 mRNA (red). (**C**) MiR-1291 decreases *IRE1α* expression whereas it increases that of *GPC3*. HuH7 cells were transfected with a control miRNA, miR-1291, an anti-miR-1291 (AM1291) or a combination of miR-1291 and AM1291 (M+AM). The relative expression of *IRE1α* and GPC3 mRNA was measured using RT-qPCR (ANOVA: $P<0.0001$; $n=10$). ***p<0.001. (**D**) HuH7 cells were transfected with the above-mentionned small RNAs. Three days later, IRE1α (top panel) and GPC3 (middle panel) protein expression was detected using Western blot and normalized to that of GAPDH (bottom panel).

Figure 3: miR-1291 targets and destabilizes *IRE1α* mRNA through its 5'-UTR. (**A**) *IRE1α* 5'UTR contains a potential miR-1291 site. Schematic representation of miR-1291/*IRE1α* 5'UTR interaction using RNAhybrid. (**B**) Top panel: schematic representation of *IRE1α* 5'-UTR-GFP transgene. Bottom panel: Schematic representation of miR-1291 pairing with *IRE1α* 5'-UTR in its wild type or mutated form. (**C**) HuH7 cells expressing the indicated transgenes were transfected with the indicated small RNAs. Three days later, GFP protein expression (P/TCN ratio (Laloo et al. 2009)) was measured (ANOVA: P<0.0001; n=3). **p<0.01; ***p<0.001. (**D**) HuH7 cells expressing the wt-IRE1α 5'-UTR-GFP transgene were transfected with a control RNA or miR-1291. After 3 days, FunREG ratios were calculated as described in Figure 1A (ANOVA: P<0.0001; n=4). ***p<0.001.

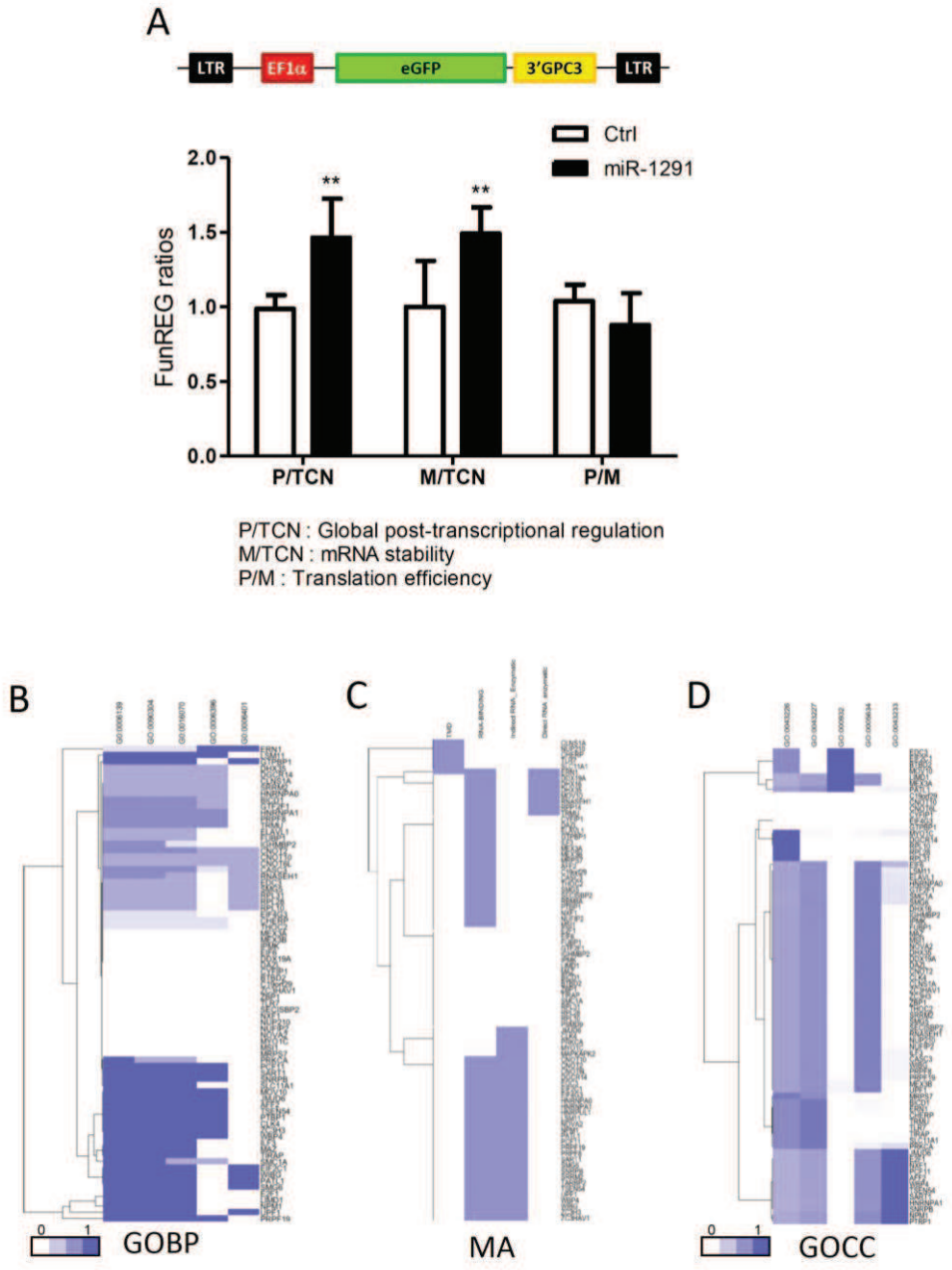
Figure 4: IRE1α destabilizes *GPC3* mRNA. (**A**) IRE1α mediates *XBP1* mRNA splicing upon ER stress. ER stress-induced splicing of *XBP1* mRNA yields a transcript with a 26-nucleotide deletion in comparison to the unspliced transcript. HuH7 cells transfected with the indicated siRNAs were exposed to dithiothreitol (DTT) for 6h or to tunicamycin (Tun) for 24h. Then *XBP1* mRNA splicing was monitored by RT-PCR. (**B-C**) *IRE1α*-silencing increases *GPC3* expression. HuH7 cells were transfected with the indicated small RNA and then treated or not with DTT or Tun. Three days later, mRNA and protein expression (ANOVA: P<0.0001; n=8) was measured by qPCR (**B**) and Western blotting (**C**). GPC3 and non-glycosylated GPC3 (ngGPC3) proteins were as shown. ***p<0.001. (**D**) IRE1α-silencing stabilizes *GPC3* mRNA. HuH7 cells were transfected with the indicated small RNA. Three days later, *GPC3* and *ALB* mRNA were measured at different times following transcription inhibition using actinomycin D (ANOVA: P<0.0001; n=3). *p<0.05; **p<0.01.

Figure 5: IRE1α cleaves *GPC3* mRNA at a canonical site located in its 3'-UTR. (**A**) Three potential IRE1α-mediated cleavage sites are present in *GPC3* mRNA. Top: Sequence alignment of IRE1α cleavage sites in *XBP1* and *GPC3* mRNAs. Bottom: Two-dimensional M-Fold RNA modeling of potential IRE1α cleavage sites in *GPC3* mRNA. (**B**) IRE1α cleaves *GPC3* mRNA within its 3'-UTR *in vitro*. Total RNA extracted from HuH7 cells was incubated with heat-inactivated or not GST-IRE1α^{cyto}. IRE1α-mediated cleavage of *GPC3* mRNA was monitored by PCR at the indicated

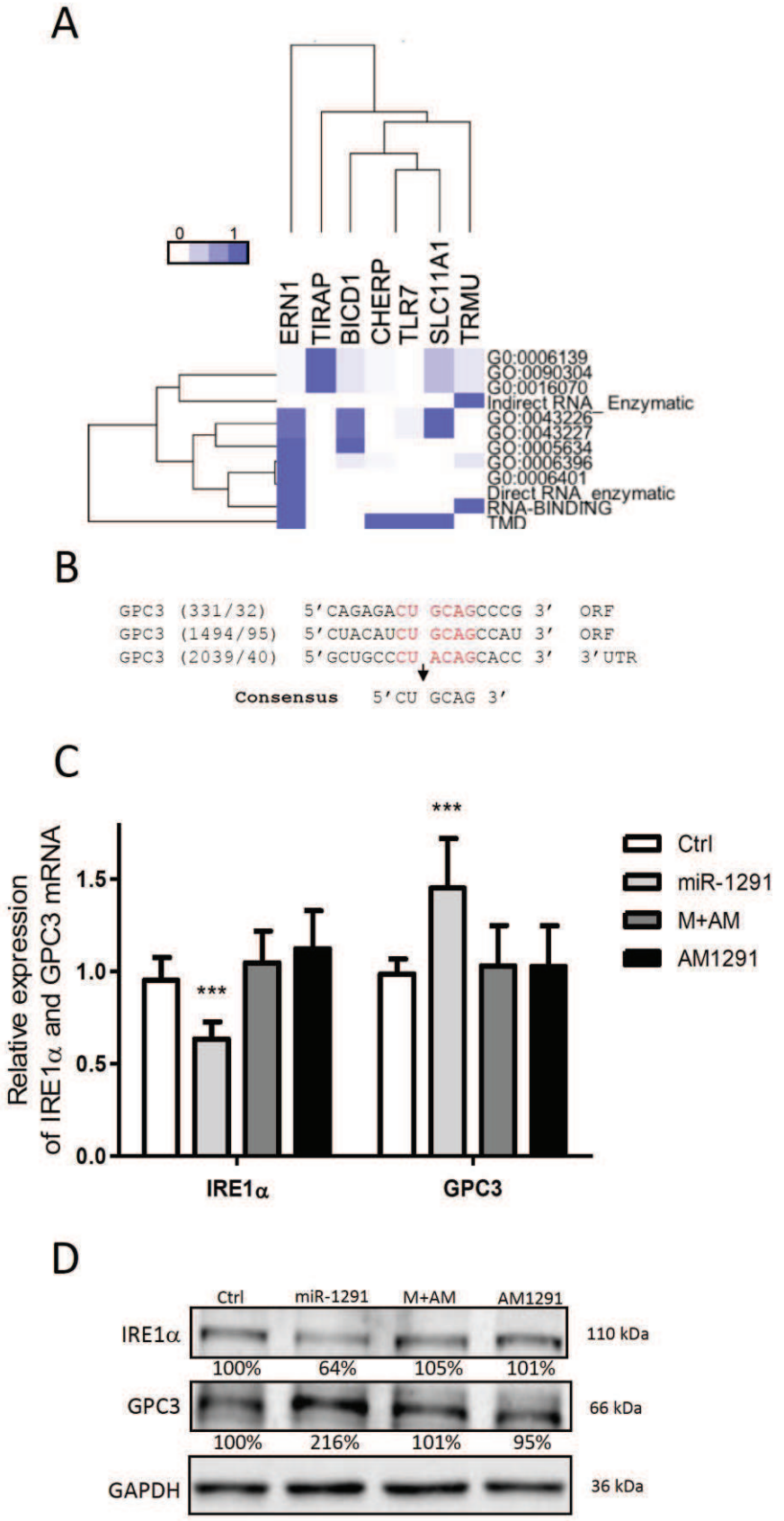
site. (**C-D**) IRE1α directly targets the 2039/40 cleavage site in *GPC3* 3'-UTR. (**C**) Expected *GPC3* mRNA products following IRE1α–mediated cleavage at the 2039/40 site. (**D**) *In vitro*-transcribed wild-type and IRE1α-site deleted *GPC3* RNAs were incubated or not with heat-inactivated or not GST-IRE1α^{cyto} in presence or absence of RNase H. Resulting reaction products were resolved on denaturing agarose gels. (**E**) Schematic representation of the expected *GPC3* mRNA products following IRE1α-mediated cleavage and their subsequent degradation by the exonucleases SKI2, XRN1 and XRN2. (**F**) HuH7 cells were transfected with the indicated small RNAs. Three days later, presence of the two mRNA fragments was measured using RT-qPCR (ANOVA: $P<0.0001$; n=6). *p<0.05; **p<0.01; ***p<0.001.

Figure 6: miR-1291 up-regulates *GPC3* through inhibition of *IRE1α* expression. (**A**) Overexpression of *IRE1α* lacking its mRNA 5'-UTR rescues miR-1291-mediated increase in *GPC3* mRNA. HuH7 cells were transfected with the indicated plasmid and small RNA. Then *GPC3* mRNA expression was measured using RT-qPCR (ANOVA: $P<0.0001$; n=5). *p<0.05; **p<0.01; ***p<0.001. (**B**) Schematic representation of miR-1291-mediated *GPC3* up-regulation through inhibition of IRE1α expression. The grey frame indicates the original observation that miR-1291 upregulates *GPC3* mRNA expression through its 3'-UTR. The green dashed arrow indicates a positive and indirect regulation of *GPC3* mRNA expression. Red signs indicate negative and direct regulations.

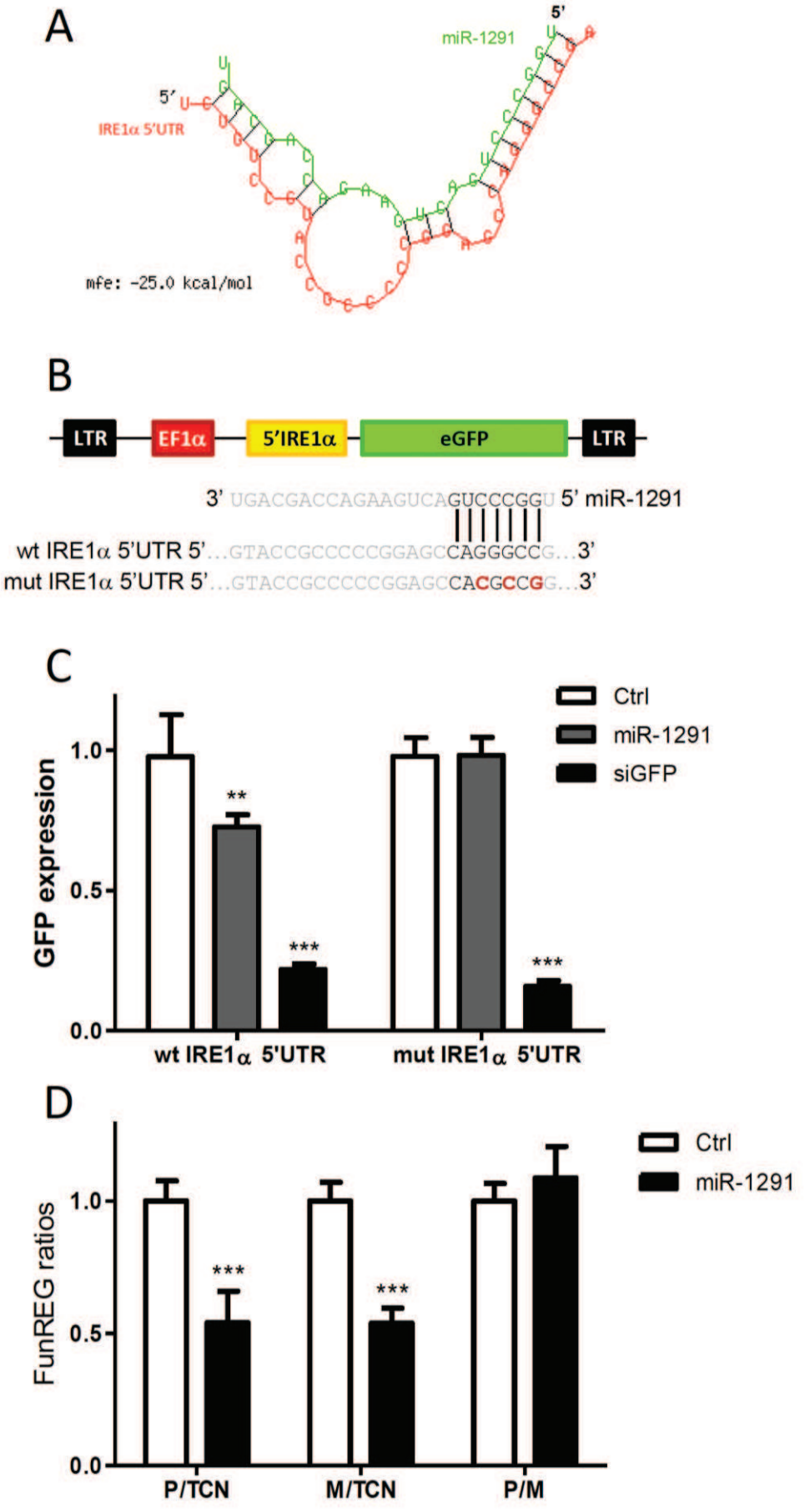
Maurel M. et al Figure 1



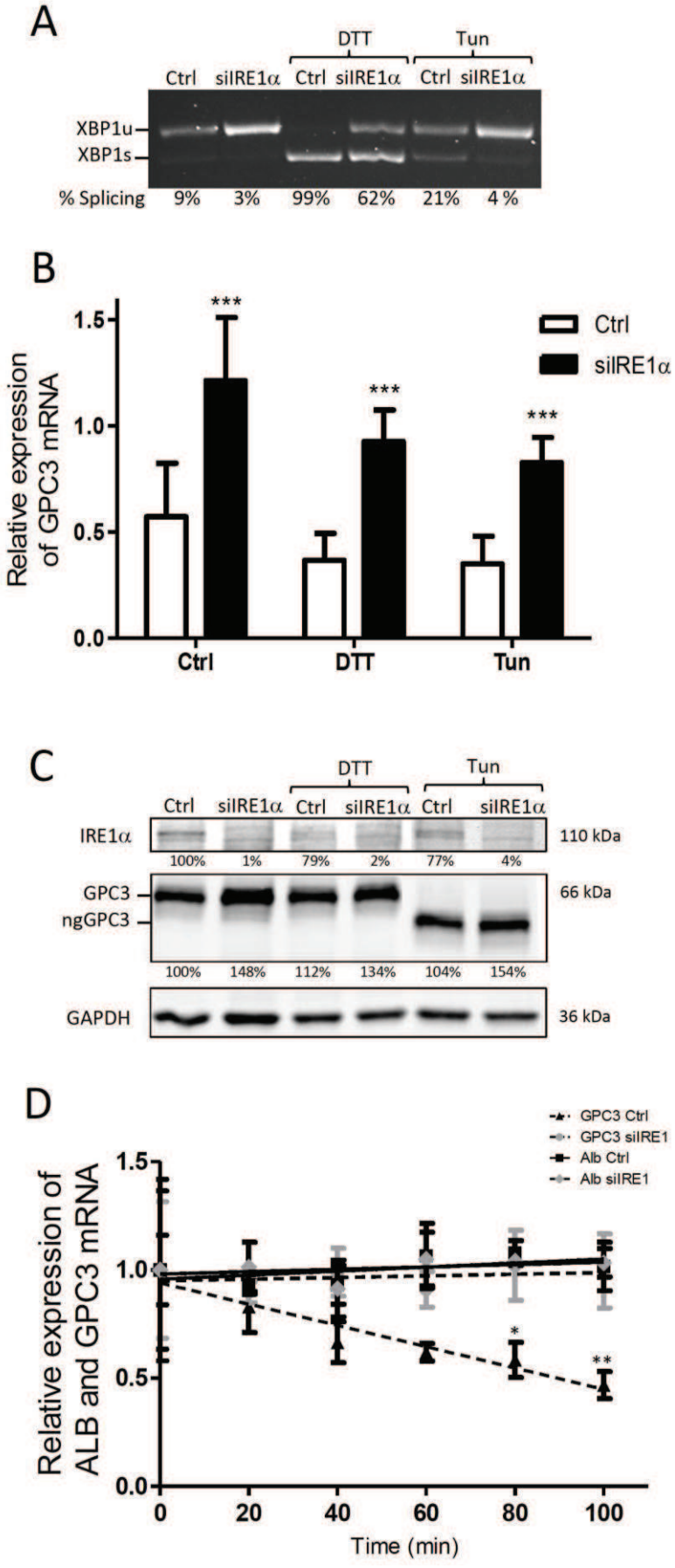
Maurel M. et al Figure 2



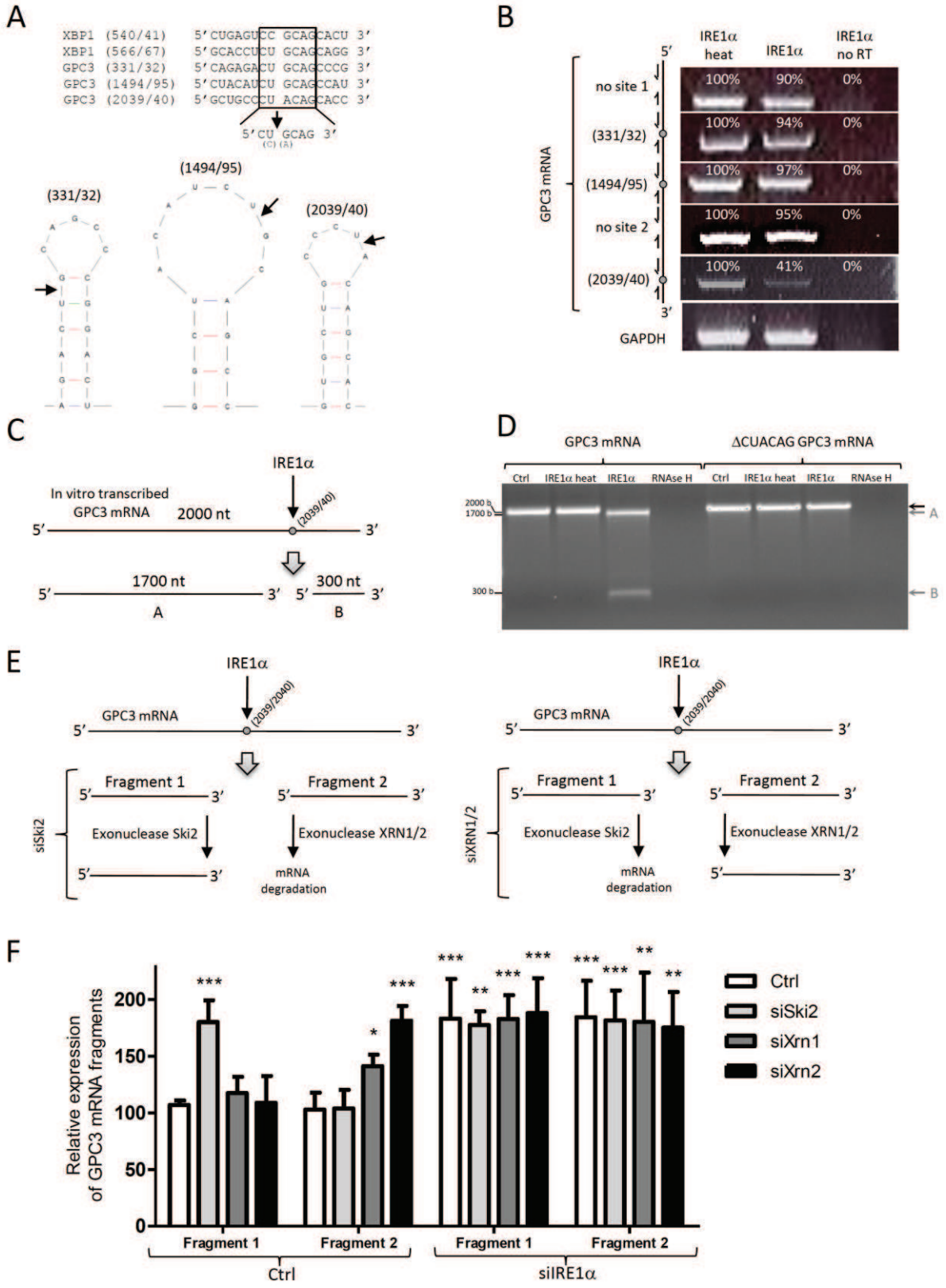
Maurel M. et al Figure 3



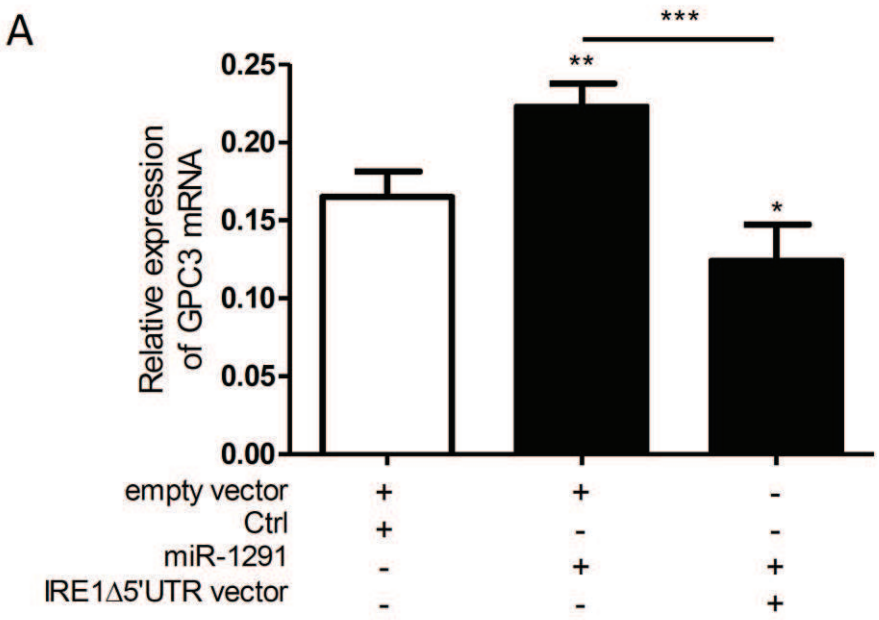
Maurel M. et al Figure 4



Maurel M. et al Figure 5



Maurel M. et al Figure 6



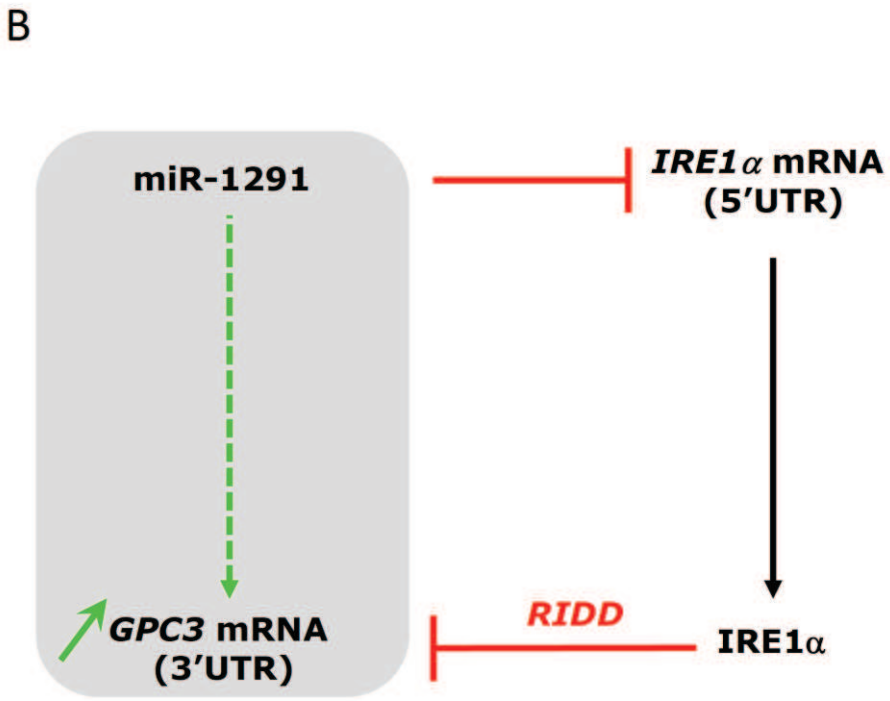


Table S1: siRNA sequences

Gene ID	Sequence
IRE1α	5′-UUACUGGCUUCUGAUAGGA-3′
XRN1	5′-GCCUUUGUCUCCUAACUGA-3′
	5′-UCCUCAAUGUGUAUAAUUA-3′
XRN2	5′-UUCAGUAUUUCUUUGGAAA-3′
	5′-CAGUGGAUCUGAAUUUAUU-3′
SKI2	5′-CAGAGAAACGGCUAUGAGA-3′
	5′-CCGUUAUCCUGCUCUGCAA-3′

Table S2: Primer pairs used for PCR and qPCR studies

Gene ID	Forward	Reverse
PCR		
XBP1	5′-GGAACAGCAAGTGGTAGA-3′	5′-CTCAGTGTAGCCCAGGATGC-3′
no site 1	5′- GATGCTGCTCAGCTTGGACT-3'	5′- GAAGAAGGAGCGGACTTGGT-3'
(331/332)	5′- ACCAAGTCCGCTCCTTCTTC -3'	5′- TGGAGTCAGGCTTGGGTAGT-3'
(1494/1495)	5′- CCAGCCGAAGAAGGGAACTA -3'	5′- TGGCTGTATCTCTCCACGAG-3'
no site 2	5′- AACTCCGAAGGACAACGAGA -3'	5′- GCACCAGGAAGAAGAAGCAC-3'
(2039/2040)	5′- TCATTCCCCGCTGAAGCTTC -3'	5′- GAGAGGTACCCAAAGAAATCCATGCAAAGA-3'
GAPDH	5′-TTGGTATCGTGGAAGGACTCATG-3'	5′-GGATGATGTTCTGGAGAGCCC-3'
qPCR		
18S	5'-GGATCCATTGGAGGGCAAGT-3'	5'-CCGCTCCCAAGATCCAACTA-3'
IRE1α	5′-GGCCTGGTCACCACAATTAG-3′	5′-ATTCCCTCCTCTCCCTTCCT-3′
GTPBP1	5'-TAGAGGAGCTGATGGGTTGG-3'	5'-GGCACCTTCACAGTCACCTT-3'
EDC3	5'-GCTACAGATTGGCTGGGAAG-3'	5'-GACCTGATCCACAGCTGACA-3'
GPC3 1	5'-AACTCCGAAGGACAACGAGA-3'	5'-GCACCAGGAAGAAGAAGCAC-3'
GPC3 2	5'-ACCCTGTGGTCTTCCTCGAT-3'	5'-CTTCATGGCTGGAGGAGGTA-3'
ALB	5'-CTTACGTGCATCTCGACGAA-3'	5'-TGCTAATTTCCCTCCGTTTG-3'
CHOP	5'-ATTGACCGAATGGTGAATCTGC-3'	5'-AGCTGAGACCTTTCCTTTTGTCTA-3'

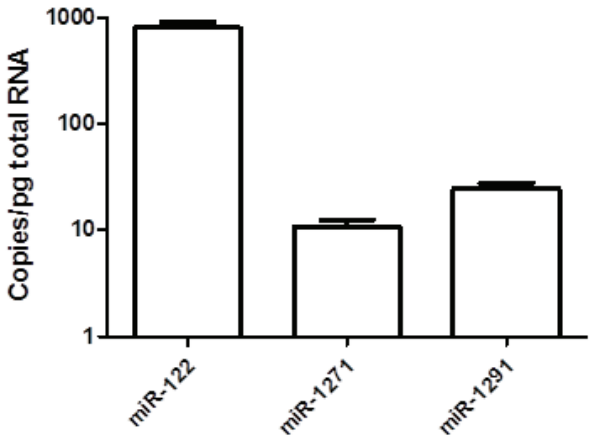
Supplemental Figure Legends

Supplementary Figure S1: Absolute quantification of miR-122, miR-1271 and miR-1291 expression in HuH7 cell line.

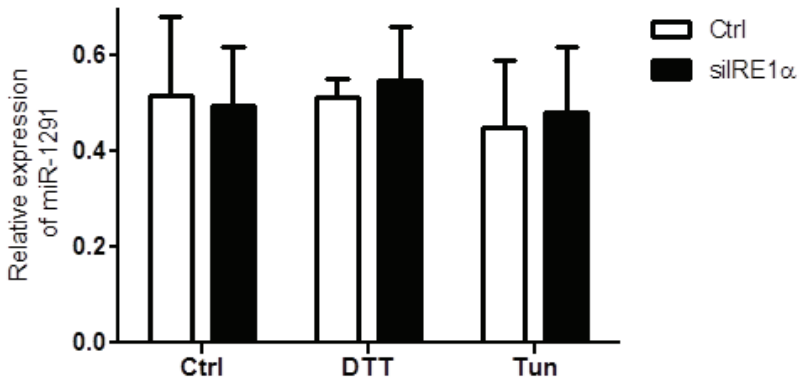
Supplementary Figure S2: miR-1291 expression is independent of *IRE1α* expression and of ER stress induction. HuH7 cells transfected with the indicated siRNAs were exposed to DTT (6h) or Tun (24h). Then miR-1291 expression was measured (ANOVA: P<0.0001; n=5).

Supplementary Figure S3: miR-1291 specifically alters IRE1α signaling independently of ER stress. (**A**) Overexpression of miR-1291 does not affect PERK signaling upon Tun treatment. HuH7 cells transfected with the indicated small RNA were exposed to Tun (24h). Then *CHOP* mRNA expression was measured (ANOVA: P<0.0001; n=5). ***p<0.001. (**B**) miR-1291 selectively affects IRE1α signaling. HuH7 cells transfected with the indicated small RNA were exposed to DTT (6h). Then *XBP1* mRNA splicing was monitored.

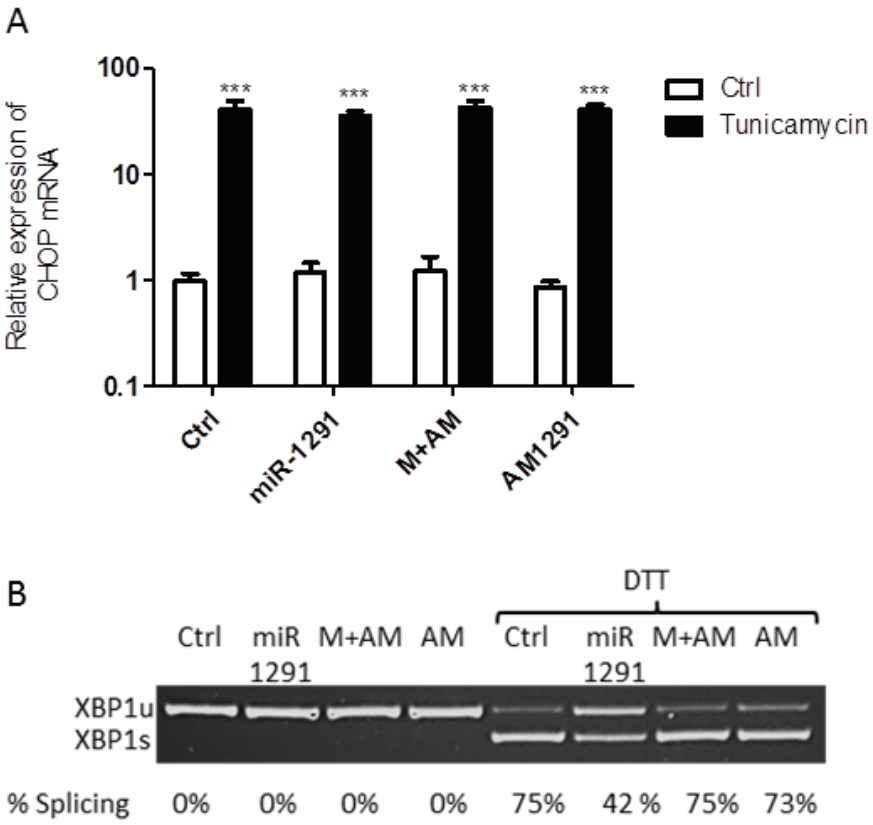
Supplementary Figure 4: Overexpression of *IRE1α* lacking the 5'UTR-located miR-1291 site. HuH7 cells were transfected with the indicated vector and small RNA. Then *IRE1α* mRNA expression was measured (ANOVA: P<0.0001; n=5). **p<0.01.



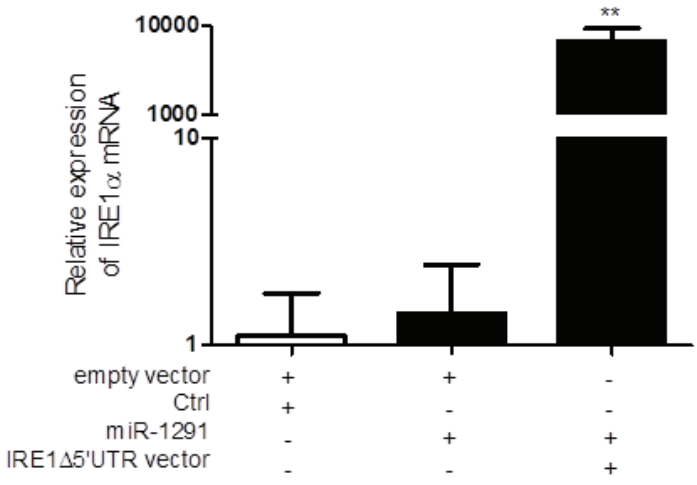
Maurel M et al. Supplementary Figure 1



Maurel M et al. Supplementary Figure 2



Maurel M et al. Supplementary Figure 3



Maurel M et al. Supplementary Figure 4

3. Discussion

Dans ce travail, j'ai démontré que miR-1291 augmente l'expression du GPC3 en inhibant un facteur inhibiteur intermédiaire. Une analyse *in silico* a permis d'identifier IRE1α comme candidat. IRE1α est une protéine transmembranaire du réticulum endoplasmique (RE) qui participe à « l'Unfolded Protein Response » (UPR), une réponse adaptative activée lors de l'accumulation de protéines mal conformées dans le RE **(Figure 30)**.

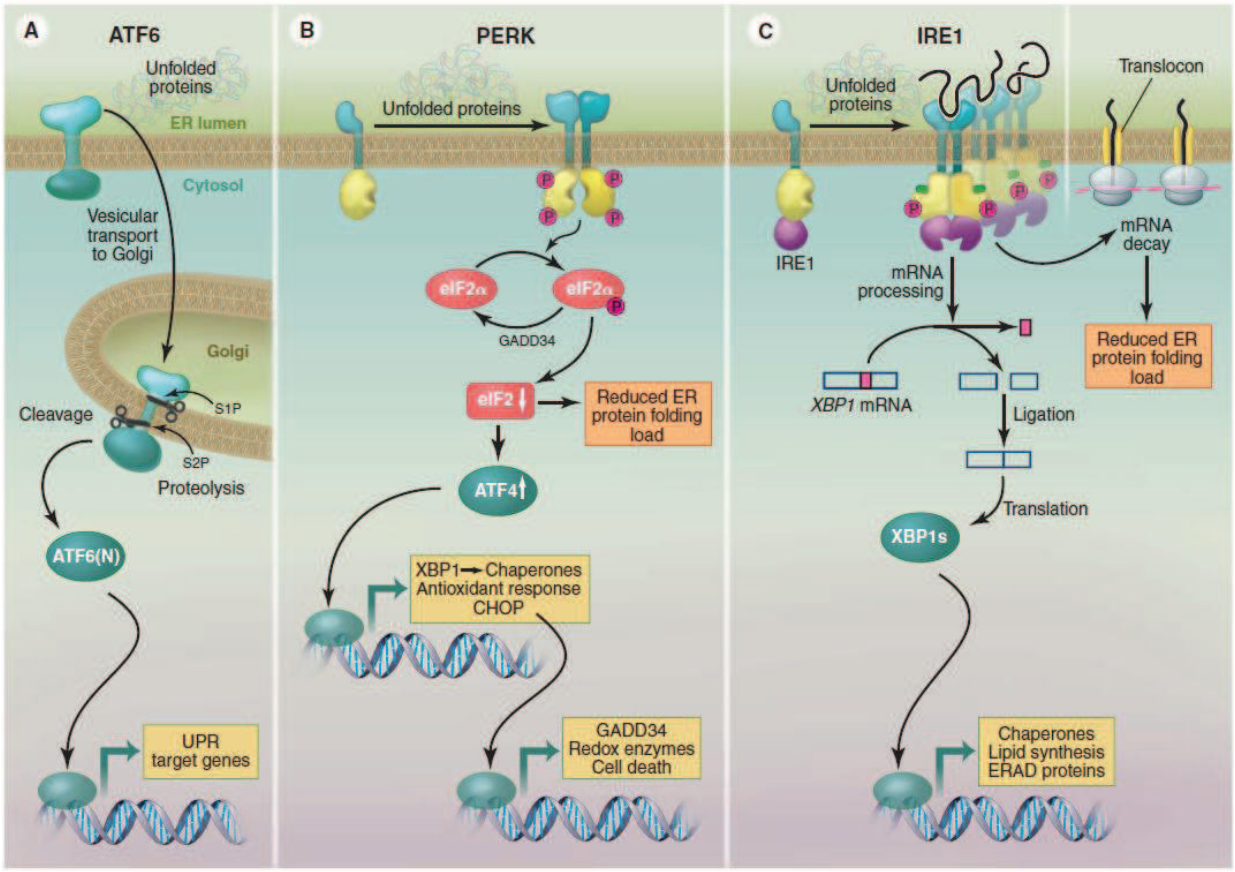


Figure 30 : La voie "Unfolded Protein Response"

Différentes modifications environnementales des cellules (carences en nutriments, en oxygène, exposition à des toxiques) peuvent perturber l'homéostasie du RE en affectant les fonctions basales de repliement des protéines membranaires et/ou sécrétées, s'en suit une accumulation anormale de protéines mal conformées dans le lumen du RE et un stress. La voie UPR est alors activée afin de rétablir l'homéostasie du RE *via* (i) l'induction de la transcription de gènes codant pour des protéines chaperonnes ou pour des protéines impliquées dans les mécanismes de dégradation associés au RE et (ii) l'inhibition globale de la synthèse protéique (Schroder and Kaufman, 2005). Ces événements tendent à limiter

l'entrée de nouvelles protéines dans le RE et à faciliter le repliement et/ou la clairance de celles qui y sont accumulées. La voie UPR est modulée par trois protéines transmembranaires du RE **(Figure 30)** : la « protein kinase RNA-like ER kinase » (PERK), l'« activation transcription factor-6 » (ATF6) et IRE1α (Ron and Walter, 2007 ; Walter and Ron, 2011). Lors d'un stress du RE, la protéine chaperonne GRP78, la plus abondante du RE, se dissocie de ces trois protéines, aboutissant à leur activation.

- **La voie ATF6 :**

ATF6 est un facteur de transcription qui, dissocié de GRP78, est ensuite exporté vers l'appareil de Golgi où il est clivé dans sa forme active. ATF6 clivé transloque dans le noyau où il induit la transcription des gènes présentant un promoteur avec le « ER Stress Response Element » (ERSE), dont le gène « X-box Binding Protein 1 » (XBP1) **(Figure 30A)**.

- **La voie PERK :**

PERK s'active suite à la dissociation de GRP78 par un mécanisme de dimérisation et de trans-autophosphorylation. Une fois activée, PERK phosphoryle eIF2α, un facteur qui active la traduction de protéines, ce qui entraîne son inhibition et une diminution globale de la synthèse protéique (Harding et al., 2000). De plus, ATF4, qui contient un cadre de lecture ouvert dans son 5'NT, est préférentiellement traduit quand eIF2α est limitant. ATF4 est un facteur de transcription contrôlant l'expression de gènes impliqués dans l'apoptose (comme CHOP/GADD153, GADD34) **(Figure 30B)**.

- **La voie IRE1α :**

IRE1α tout comme PERK, s'active suite à la dissociation de GRP78 par un mécanisme impliquant son oligomérisation et sa trans-autophosphorylation. Ceci conduit à un changement conformationnel qui, lui-même, induit une activité endoribonucléase intrinsèque qui permet l'épissage non conventionnel de l'ARNm codant pour XBP1. L'épissage de 26 nt de l'ARNm codant pour XBP1 peut se produire grâce à l'intervention d'une ligase encore inconnue chez les mammifères et entraîne un décalage de son cadre de lecture et aboutissant à la traduction d'un facteur de transcription XBP1 « spliced » (XBP1s). Les gènes cibles de XBP1s codent pour des chaperonnes du RE, dont GRP78, et des protéines impliquées dans la dégradation des protéines mal repliées (Walter and Ron, 2011). De plus, l'activité endoribonucléasique d'IRE1α a récemment été impliquée dans un mécanisme nommé « Regulated IRE1α Dependent Decay of mRNA » (RIDD) qui induit la dégradation d'ARNm

codant pour des protéines sécrétées et des protéines membranaires (Hollien et al., 2009; Hollien and Weissman, 2006) **(Figure 30C)**.

Le GPC3 étant une protéine membranaire à ancre GPI, elle utilise la voie de sécrétion pour atteindre la membrane plasmique. J'ai donc émis l'hypothèse que l'ARNm du GPC3 pourrait être un nouveau substrat du RIDD. En testant expérimentalement cette hypothèse, j'ai démontré qu'IRE1α clive l'ARNm codant pour le *GPC3* dans sa région 3'NT grâce à son activité endoribonucléase. D'autre part, le miR-1291 cible directement l'ARNm codant pour *IRE1α* dans sa région 5'NT. Cette étude a permis de démontrer que l'inhibition de l'expression d'IRE1α par le miR-1291 induit une surexpression du GPC3 dans des lignées cellulaires humaines dérivées de CHC.

MiR-1291 est surexprimé dans 20% des CHC, correspondant aux groupes G2 et G3 **(présenté en Figure 29)**. D'après des données préliminaires d'analyse transcriptomique (données non publiées de Yannick Ladeiro), (i) l'expression d'IRE1α et du miR-1291 corrèlent négativement dans les groupes G2 et G3 **(Figure 31A)**, (ii) l'expression d'IRE1α et du GPC3 corrèlent négativement dans l'ensemble des échantillons de CHC **(Figure 31B)** et l'expression du miR-1291 et du GPC3 corrèlent positivement dans les groupes G2 et G3 **(Figure 31C)**.

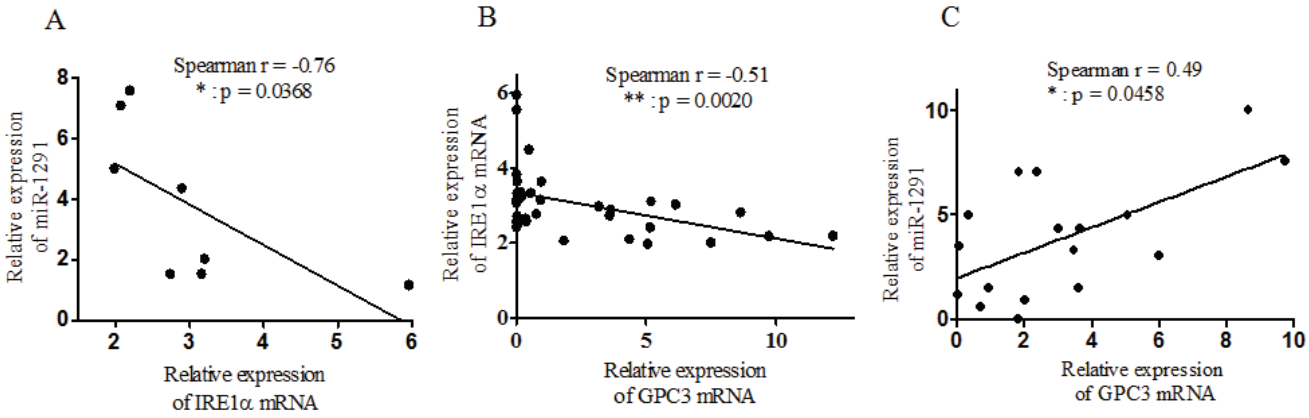


Figure 31 : Analyse corrélative de l'expression du miR-1291, du GPC3 et d'IRE1α dans les échantillons de CHC (B) ou dans les groupes G2 et G3 (A et C).

Cependant, les données présentées ci-dessus ont été obtenues à partir d'un faible nombre d'échantillons de CHC et nécessiteraient une validation sur un plus grand nombre de tumeurs. Ces premiers résultats sont concordants avec nos résultats obtenus dans des cellules

en culture, et nous permettent d'émettre l'hypothèse que le miR-1291 participe à la surexpression du GPC3 dans les groupes G2 et G3 en inhibant l'expression d'IRE1α. Une perspective intéressante de ce projet serait donc d'étudier le rôle du miR-1291 dans la modulation de la voie UPR et son implication dans le CHC.

Un des principaux obstacles à la survie des cellules cancéreuses est de devoir faire face à des conditions environnementales hostiles qui affectent le bon repliement des protéines néo-synthétisées. L'initiation, la promotion et la progression des cancers nécessitent une adaptation constante des cellules cancéreuses à ces différents stresses afin d'échapper à la mort cellulaire. Dans les cancers, l'activation de la voie UPR représente un avantage sélectif, car elle est cytoprotective : elle accroît les capacités d'adaptation des cellules cancéreuses et augmente leur survie dans des conditions hostiles (Lhomond and Chevet, 2012 ; Moenner et al., 2007).

L'activation de la voie UPR dans les hépatocytes a été décrite dans différentes pathologies associées au CHC, les hépatites virales, l'alcoolisme, NAFLD, NASH (voir section II 1.1) (Malhi and Kaufman, 2011). Dans le CHC, GRP78, ATF6/ATF6 clivé, XBP1/XBP1s sont surexprimés dans les tumeurs peu différenciées et sont impliqués dans la progression et l'invasion tumorale ((Arai et al., 2006 ; Shuda et al., 2003 ; Su et al., 2010). IRE1α a deux fonctions contradictoires dans l'oncogenèse (Han et al., 2009). L'axe IRE1/XBP1 favoriserait l'adaptation des cellules tumorales au stress en augmentant les capacités de repliement et de synthèse protéique du RE. À l'opposé, l'activation du RIDD réduirait la croissance tumorale en dégradant des protéines pro-oncogéniques (Dejeans et al., 2012 ; Hollien and Weissman, 2006). Dans les cancers, l'activation d'IRE1α est majoritairement décrite comme pro-oncogénique (Auf et al., 2010 ; Dejeans et al., 2012; Drogat et al., 2007). Pourtant, des mutations somatiques ponctuelles d'IRE1α ont été retrouvées dans des cancers du rein, des poumons, de l'ovaire, de l'estomac, du cerveau et du foie (Greenman et al., 2007 ; Guichard et al., 2012; Parsons et al., 2008)). Certaines sont associées à des pertes de fonction d'IRE1α ((Xue et al., 2011)et données non publiées de Stéphanie Lhomond). Le rôle de la surexpression du miR-1291 au cours de la progression du CHC reste donc à élucider. Selon le modèle suivant **(Figure 32)**, il pourrait représenter un avantage sélectif pour les cellules cancéreuses.

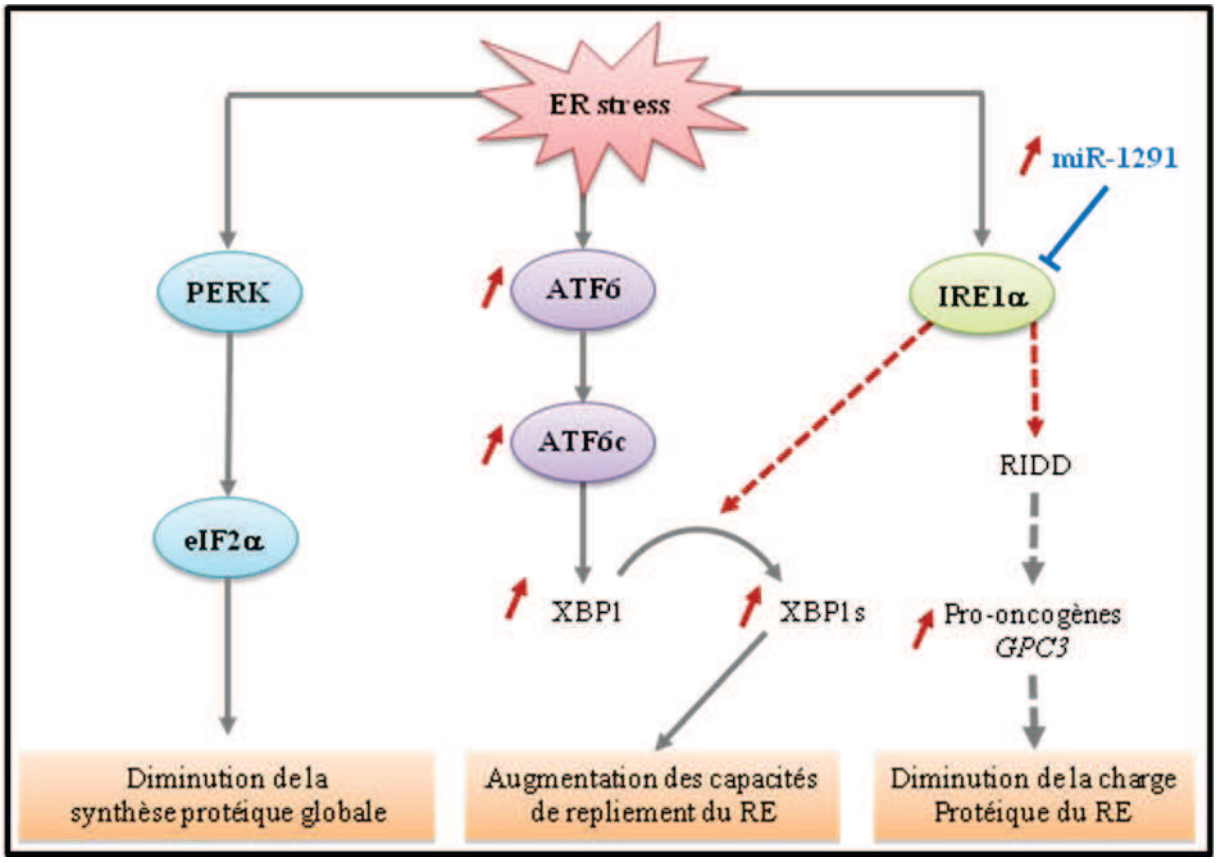


Figure 32 : ATF6 et miR-1291 modulent la voie UPR pour augmenter les capacités de repliement du RE et l'expression de pro-oncogènes dans les cellules cancéreuses.

Les miARNs sont des régulateurs fins de l'expression génique et sont décrits comme des modulateurs de nombreuses voies de signalisation. Nos résultats indiquent que miR-1291 réduit de ~40% le RIDD et l'épissage de XBP1 dans des lignées de CHC. Ces résultats nous permettent de proposer un modèle dans lequel l'atténuation du RIDD représenterait un avantage pour les cellules tumorales, car elle permettrait d'augmenter l'expression de protéines pro-oncogéniques telle que le GPC3. En revanche, la diminution de l'épissage de l'ARNm codant pour XBP1 pourrait réduire les capacités de repliement du RE. Cet obstacle pourrait être surmonté par la surexpression d'ATF6 dans le CHC qui induit une augmentation de la quantité de la forme non épissée de XBP1 (Arai et al., 2006; Shuda et al., 2003). Cette augmentation pourrait alors compenser l'effet répresseur du miR-1291 sur l'épissage de XBP1 en augmentant la quantité de substrat pour IRE1α. Ainsi la surexpression/activation d'ATF6 et du miR-1291 apporterait un avantage sélectif aux cellules cancéreuses (i) en augmentant la résistance des cellules aux stresses *via* l'augmentation de XBP1 et (ii) en augmentant l'expression de pro-oncogènes *via* l'atténuation du RIDD.

CONCLUSION GÉNÉRALE ET PERSPECTIVES

Les deux projets présentés dans la section des résultats m'ont permis d'identifier des régulateurs post-transcriptionnels du GPC3 et de caractériser leur mode d'action. Sur la base des corrélations effectuées entre l'expression des miARNs et du GPC3 dans différents groupes de CHC, il est possible d'envisager que ces deux miARNs participeraient aux mécanismes conduisant à la surexpression du GPC3 dans le CHC. Pour obtenir ces résultats, j'ai réalisé un crible fonctionnel permettant d'évaluer l'impact des miARNs sur le 3'NT du GPC3, puis combiné les résultats avec une analyse globale *in silico* et différentes étapes de validation expérimentale. Cette approche intégrée m'a permis d'identifier cinq miARNs régulateurs positifs ou négatifs de l'expression du GPC3. De plus, dans la perspective de caractériser les mécanismes de régulation positive de l'expression du GPC3 qui dépendent de ces miARNs, j'ai pu identifier la protéine IRE1α comme un nouvel acteur de ces mécanismes. La **Figure 33** résume l'ensemble des régulateurs connus de l'expression du GPC3 décrits dans la section II 3.4 ou identifiés au cours de ma thèse.

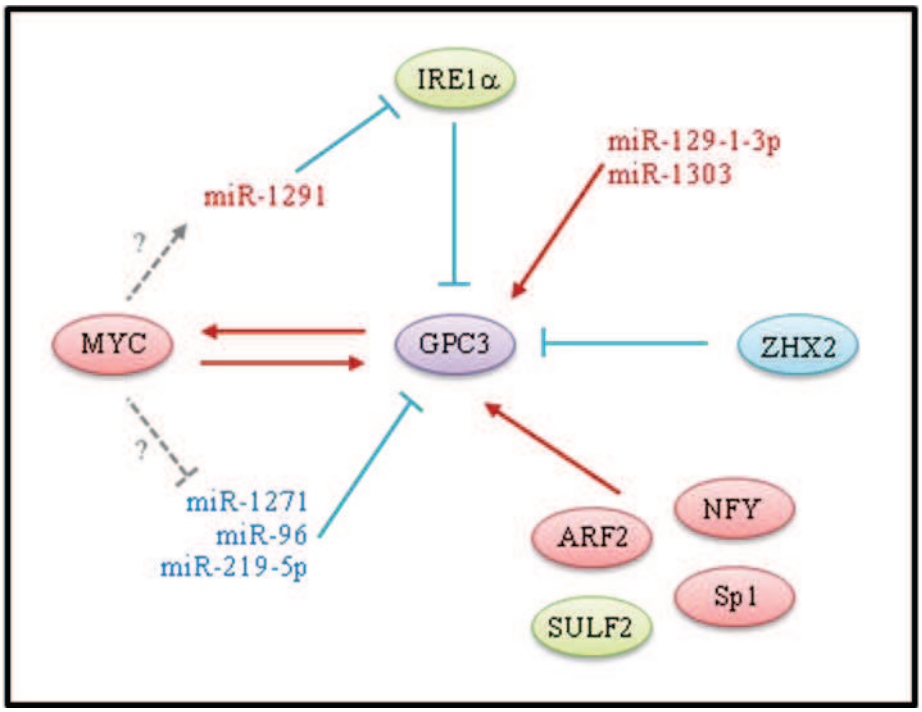


Figure 33 : Représentation schématique de l'ensemble des régulateurs connus de l'expression du GPC3.

Les facteurs de transcription et les miARNs sont représentés en rouge s'ils régulent positivement l'expression du GPC3 ou en bleu s'ils régulent négativement son expression. Les enzymes sont en vert. Les flèches grises indiquent des régulations potentielles non démontrées expérimentalement.

Parmi les différents régulateurs identifiés, deux miARNs ont plus particulièrement attiré mon attention, car leur expression corrèle avec la surexpression du GPC3 dans certains groupes spécifiques de CHC :

- **miR-1271** cible directement la région 3'NT du GPC3. Il est globalement sous-exprimé dans 92% des CHC et sa sous-expression corrèle avec la surexpression du GPC3 dans les CHC de patients infectés par le VHB.

- **miR-1291** régule positivement l'expression du GPC3 en réprimant l'expression d'**IRE1α.** Il est surexprimé dans 20% des CHC, c'est-à-dire ceux correspondant aux groupes G2 et G3 et d'après des résultats préliminaires **(présentés en Figure 29)** sa surexpression corrèle positivement avec celle du GPC3.

Les perspectives de travail engendrées par ces résultats sont multiples :

La première perspective possible viserait à comprendre comment **l'intégration des signaux médiés par l'ensemble de ces régulateurs peut conduire à moduler l'expression du GPC3**.

MiR-1271 et l'axe de régulation miR-1291/IRE1α sont co-exprimés dans le CHC. Il serait par exemple intéressant d'étudier l'effet global/intégré de ces deux mécanismes de régulation sur l'expression du GPC3. Ils sont simultanément dérégulés dans les groupes G2 et G3 qui correspondent à des groupes de CHC dans lesquels le GPC3 est le plus surexprimé. Ces deux voies de régulation pourraient donc agir de manière synergique pour augmenter l'expression du GPC3 dans le CHC et de ce fait donner aux cellules cancéreuses/à la tumeur un avantage sélectif. Les différentes combinaisons d'expression des régulateurs du GPC3 **(Figure 33)** pourraient par conséquent justifier la complexité des profils d'expression du GPC3 observés dans le foie fœtal, le foie adulte et le foie tumoral. Par ailleurs, l'implication de la protéine IRE1α et des cinq miARNs validés dans la régulation de l'expression du GPC3 pourrait être étudiée dans d'autres pathologies associées au GPC3 (SGBS, cancers, voir section II .3.3).

Une deuxième perspective intéressante découlant de mon travail viserait à **identifier les mécanismes impliqués dans la dérégulation de miR-1271 et miR-1291.**

Jusqu'à présent, différents mécanismes, décrits dans la section I.4.3., ont été impliqués dans la dérégulation de l'expression des miARNs dans les cancers. D'après les données de la littérature, certains d'entre eux pourraient être responsables de la dérégulation du miR-1271 et miR-1291 dans le CHC.

- **La modification des mécanismes épigénétiques ?**

Les gènes des miR-1271 et miR-1291 sont respectivement localisés aux régions génomiques 5q35 et 12q13 qui pourraient être soumises à des réarrangements chromosomiques (Feitelson and Lee, 2007). En effet, les gènes codant pour ces deux miARNs sont localisés dans des sites chromosomiques fragiles (respectivement FRA5G et FRA12A) (Calin et al., 2004). Qui plus est, les groupes G2 et G3, dans lesquels miR-1291 est surexprimé, sont associés à une forte instabilité chromosomique (Boyault et al., 2007) et le chromosome 12q est amplifié dans le CHC (Zondervan et al., 2000). De plus, les gènes des miR-1271 et miR-1291 sont proches de sites récurrents d'intégration du VHB (Murakami et al., 2005; Zondervan et al., 2000) qui peut aussi provoquer une instabilité génomique (voir section II 1.1), ce qui pourrait aussi expliquer la forte corrélation de la surexpression du miR-1271 et de celle du GPC3 dans des CHC résultant de l'infection par le virus de l'hépatite B.

- **La variation de l'activité de facteurs de transcription ?**

Les facteurs de transcription c-MYC et p53 contrôlent l'expression de nombreux miARNs et pourraient participer à leur dérégulation dans le CHC (voir section I 4.3.2). Une étude récente a révélé que c-MYC participe à la surexpression du GPC3 dans le CHC (Li et al., 2012). Il serait donc intéressant de voir si cet effet est uniquement transcriptionnel. C-Myc pourrait par exemple contrôler l'expression de miR-1271 et miR-1291 et ainsi augmenter l'expression du GPC3 par un mécanisme post-transcriptionnel **(Figure 33)**. Par ailleurs, p53 pourrait réguler l'expression du miR-1291, les groupes G2 et G3 étant caractérisés par des mutations inactivatrices de p53 (Boyault et al., 2007), il pourrait participer à sa surexpression.

- **L'altération de la biogénèse des miARNs ?**

L'inhibition de la maturation des miARNs est un mécanisme impliqué dans la diminution globale des miARNs dans les cancers (voir section I 4.3.3). Dicer, une protéine impliquée dans la biogénèse des miARNs, est sous-exprimée dans le CHC et diminue la

production de miARNs matures (Wu et al., 2011). Cette altération pourrait contribuer à la sous-expression globale du miR-1271 dans les CHC. De plus, p53 est aussi impliqué dans la biogénèse des miARNs, il favorise la maturation de certains pri-miARNs (Bates et al., 2005; Suzuki et al., 2009) et il pourrait diminuer l'expression du miR-1271 de manière post-transcriptionnelle. Bien sûr, ces résultats n'expliqueraient pas la surexpression observée du miR-1291 dont l'expression pourrait, quant à elle, être dépendante de mécanismes alternatifs de régulation.

Nos travaux soulignent l'importance de l'utilisation d'approches systémiques pour étudier les réseaux miARNs : ARNm cible ainsi que la complexité des systèmes biologiques étudiés qui reflète probablement l'hétérogénéité tumorale observée dans les CHC. En effet, les miARNs sont des régulateurs fins de l'expression génique. L'altération de leur expression dans les cancers module de nombreuses voies de signalisation et affecte différents processus biologiques liés au phénotype tumoral. Au cours de ma thèse, j'ai démontré que miR-1271 réprime l'expression du GPC3 alors qu'à l'opposé miR-1291 l'augmente en atténuant l'expression d'IRE1α. Ces deux mécanismes pourraient être impliqués dans la surexpression du GPC3 dans le CHC et pourraient ainsi participer à la carcinogénèse hépatique. A terme, il est envisageable de proposer que la compréhension des mécanismes de régulation de l'expression du GPC3, impliquant les miARNs et l'identification des acteurs moléculaires impliqués, pourraient constituer une source de cibles thérapeutiques applicables pour le traitement du CHC.

REFERENCES

Arai, M., Kondoh, N., Imazeki, N., Hada, A., Hatsuse, K., Kimura, F., Matsubara, O., Mori, K., Wakatsuki, T., and Yamamoto, M. (2006). Transformation-associated gene regulation by ATF6alpha during hepatocarcinogenesis. FEBS Lett *580*, 184-190.

Asare, G.A., Mossanda, K.S., Kew, M.C., Paterson, A.C., Kahler-Venter, C.P., and Siziba, K. (2006). Hepatocellular carcinoma caused by iron overload: a possible mechanism of direct hepatocarcinogenicity. Toxicology *219*, 41-52.

Auf, G., Jabouille, A., Guerit, S., Pineau, R., Delugin, M., Bouchecareilh, M., Magnin, N., Favereaux, A., Maitre, M., Gaiser, T.*, et al.* (2010). Inositol-requiring enzyme 1alpha is a key regulator of angiogenesis and invasion in malignant glioma. Proc Natl Acad Sci U S A *107*, 15553-15558.

Baeg, G.H., Lin, X., Khare, N., Baumgartner, S., and Perrimon, N. (2001). Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. Development *128*, 87-94.

Baek, D., Villen, J., Shin, C., Camargo, F.D., Gygi, S.P., and Bartel, D.P. (2008). The impact of microRNAs on protein output. Nature *455*, 64-71.

Bandiera, S., Ruberg, S., Girard, M., Cagnard, N., Hanein, S., Chretien, D., Munnich, A., Lyonnet, S., and Henrion-Caude, A. (2011). Nuclear outsourcing of RNA interference components to human mitochondria. PLoS One *6*, e20746.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215-233.

Bates, G.J., Nicol, S.M., Wilson, B.J., Jacobs, A.M., Bourdon, J.C., Wardrop, J., Gregory, D.J., Lane, D.P., Perkins, N.D., and Fuller-Pace, F.V. (2005). The DEAD box protein p68: a novel transcriptional coactivator of the p53 tumour suppressor. EMBO J *24*, 543-553.

Bazzini, A.A., Lee, M.T., and Giraldez, A.J. (2012). Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. Science *336*, 233-237.

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. (2006). mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev *20*, 1885-1898.

Bhattacharyya, S.N., Habermacher, R., Martine, U., Closs, E.I., and Filipowicz, W. (2006). Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell *125*, 1111-1124.

Boily, G., Ouellet, S., Langlois, S., Lariviere, M., Drouin, R., and Sinnett, D. (2007). In vivo footprinting analysis of the Glypican 3 (GPC3) promoter region in neuroblastoma cells. Biochim Biophys Acta *1769*, 182-193.

Boily, G., Saikali, Z., and Sinnett, D. (2004). Methylation analysis of the glypican 3 gene in embryonal tumours. Br J Cancer *90*, 1606-1611.

Bolondi, L., Sofia, S., Siringo, S., Gaiani, S., Casali, A., Zironi, G., Piscaglia, F., Gramantieri, L., Zanetti, M., and Sherman, M. (2001). Surveillance programme of cirrhotic patients for early diagnosis and treatment of hepatocellular carcinoma: a cost effectiveness analysis. Gut *48*, 251-259.

Borchert, G.M., Lanier, W., and Davidson, B.L. (2006). RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol *13*, 1097-1101.

Bosch, F.X., Ribes, J., and Borras, J. (1999). Epidemiology of primary liver cancer. Semin Liver Dis *19*, 271-285.

Boyault, S., Rickman, D.S., de Reynies, A., Balabaud, C., Rebouissou, S., Jeannot, E., Herault, A., Saric, J., Belghiti, J., Franco, D.*, et al.* (2007). Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. Hepatology *45*, 42-52.

Brechot, C., Thiers, V., Kremsdorf, D., Nalpas, B., Pol, S., and Paterlini-Brechot, P. (2001). Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen: clinically significant or purely "occult"? Hepatology *34*, 194-203.

Brennecke, J., Stark, A., Russell, R.B., and Cohen, S.M. (2005). Principles of microRNA-target recognition. PLoS Biol *3*, e85.

Bressac, B., Galvin, K.M., Liang, T.J., Isselbacher, K.J., Wands, J.R., and Ozturk, M. (1990). Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. Proc Natl Acad Sci U S A *87*, 1973-1977.

Bruno, S., Crosignani, A., Maisonneuve, P., Rossi, S., Silini, E., and Mondelli, M.U. (2007). Hepatitis C virus genotype 1b as a major risk factor associated with hepatocellular carcinoma in patients with cirrhosis: a seventeen-year prospective cohort study. Hepatology *46*, 1350-1356.

Bueno, M.J., Gomez de Cedron, M., Gomez-Lopez, G., Perez de Castro, I., Di Lisio, L., Montes-Moreno, S., Martinez, N., Guerrero, M., Sanchez-Martinez, R., Santos, J.*, et al.* (2011). Combinatorial effects of microRNAs to suppress the Myc oncogenic pathway. Blood *117*, 6255-6266.

Bui, T.V., and Mendell, J.T. (2010). Myc: Maestro of MicroRNAs. Genes Cancer *1*, 568-575.

Cai, X., Hagedorn, C.H., and Cullen, B.R. (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA *10*, 1957-1966.

Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., *et al.* (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A *99*, 15524-15529.

Calin, G.A., Sevignani, C., Dumitru, C.D., Hyslop, T., Noch, E., Yendamuri, S., Shimizu, M., Rattan, S., Bullrich, F., Negrini, M., *et al.* (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A *101*, 2999-3004.

Cantley, L.C., and Neel, B.G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A *96*, 4240-4245.

Capurro, M., Wanless, I.R., Sherman, M., Deboer, G., Shi, W., Miyoshi, E., and Filmus, J. (2003). Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology *125*, 89-97.

Capurro, M.I., Xiang, Y.Y., Lobe, C., and Filmus, J. (2005). Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res *65*, 6245-6254.

Capurro, M.I., Xu, P., Shi, W., Li, F., Jia, A., and Filmus, J. (2008). Glypican-3 inhibits Hedgehog signaling during development by competing with patched for Hedgehog binding. Dev Cell *14*, 700-711.

Caturelli, E., Biasini, E., Bartolucci, F., Facciorusso, D., Decembrino, F., Attino, V., and Bisceglia, M. (2002). Diagnosis of hepatocellular carcinoma complicating liver cirrhosis: utility of repeat ultrasound-guided biopsy after unsuccessful first sampling. Cardiovasc Intervent Radiol *25*, 295-299.

Cernilogar, F.M., Onorati, M.C., Kothe, G.O., Burroughs, A.M., Parsi, K.M., Breiling, A., Lo Sardo, F., Saxena, A., Miyoshi, K., Siomi, H., *et al.* (2011). Chromatin-associated RNA interference components contribute to transcriptional regulation in Drosophila. Nature *480*, 391-395.

Chang, T.C., Yu, D., Lee, Y.S., Wentzel, E.A., Arking, D.E., West, K.M., Dang, C.V., Thomas-Tikhonenko, A., and Mendell, J.T. (2008). Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet *40*, 43-50.

Chen, C.Y., Zheng, D., Xia, Z., and Shyu, A.B. (2009). Ago-TNRC6 triggers microRNA-mediated decay by promoting two deadenylation steps. Nat Struct Mol Biol *16*, 1160-1166.

Chen, J.D., Yang, H.I., Iloeje, U.H., You, S.L., Lu, S.N., Wang, L.Y., Su, J., Sun, C.A., Liaw, Y.F., and Chen, C.J. (2010). Carriers of inactive hepatitis B virus are still at risk for hepatocellular carcinoma and liver-related death. Gastroenterology *138*, 1747-1754.

Chendrimada, T.P., Finn, K.J., Ji, X., Baillat, D., Gregory, R.I., Liebhaber, S.A., Pasquinelli, A.E., and Shiekhattar, R. (2007). MicroRNA silencing through RISC recruitment of eIF6. Nature *447*, 823-828.

Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., and Shiekhattar, R. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature *436*, 740-744.

Corcoran, D.L., Pandit, K.V., Gordon, B., Bhattacharjee, A., Kaminski, N., and Benos, P.V. (2009). Features of mammalian microRNA promoters emerge from polymerase II chromatin immunoprecipitation data. PLoS One *4*, e5279.

Cordes, K.R., Sheehy, N.T., White, M.P., Berry, E.C., Morton, S.U., Muth, A.N., Lee, T.H., Miano, J.M., Ivey, K.N., and Srivastava, D. (2009). miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature *460*, 705-710.

David, G. (1993). Integral membrane heparan sulfate proteoglycans. FASEB J *7*, 1023-1030.

Dejeans, N., Pluquet, O., Lhomond, S., Grise, F., Bouchecareilh, M., Juin, A., Meynard-Cadars, M., Bidaud-Meynard, A., Gentil, C., Moreau, V.*, et al.* (2012). Autocrine control of glioma cells adhesion/migration through Inositol Requiring enzyme 1alpha (IRE1alpha)-mediated cleavage of Secreted Protein Acidic Rich in Cysteine (SPARC) mRNA. J Cell Sci.

Didiano, D., and Hobert, O. (2006). Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions. Nat Struct Mol Biol *13*, 849-851.

Djuranovic, S., Nahvi, A., and Green, R. (2012). miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. Science *336*, 237-240.

Doench, J.G., and Sharp, P.A. (2004). Specificity of microRNA target selection in translational repression. Genes Dev *18*, 504-511.

Drogat, B., Auguste, P., Nguyen, D.T., Bouchecareilh, M., Pineau, R., Nalbantoglu, J., Kaufman, R.J., Chevet, E., Bikfalvi, A., and Moenner, M. (2007). IRE1 signaling is essential for ischemia-induced vascular endothelial growth factor-A expression and contributes to angiogenesis and tumor growth in vivo. Cancer Res *67*, 6700-6707.

Dweep, H., Sticht, C., Pandey, P., and Gretz, N. (2011). miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform *44*, 839-847.

El-Murr, N., Abidi, Z., Wanherdrick, K., Svrcek, M., Gaub, M.P., Flejou, J.F., Hamelin, R., Duval, A., and Lesuffleur, T. (2012). MiRNA genes constitute new targets for microsatellite instability in colorectal cancer. PLoS One *7*, e31862.

El-Serag, H.B., and Rudolph, K.L. (2007). Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology *132*, 2557-2576.

Elmen, J., Lindow, M., Silahtaroglu, A., Bak, M., Christensen, M., Lind-Thomsen, A., Hedtjarn, M., Hansen, J.B., Hansen, H.F., Straarup, E.M.*, et al.* (2008). Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. Nucleic Acids Res *36*, 1153-1162.

Eulalio, A., Huntzinger, E., and Izaurralde, E. (2008). GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nat Struct Mol Biol *15*, 346-353.

Eulalio, A., Huntzinger, E., Nishihara, T., Rehwinkel, J., Fauser, M., and Izaurralde, E. (2009). Deadenylation is a widespread effect of miRNA regulation. RNA *15*, 21-32.

Eulalio, A., Rehwinkel, J., Stricker, M., Huntzinger, E., Yang, S.F., Doerks, T., Dorner, S., Bork, P., Boutros, M., and Izaurralde, E. (2007). Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. Genes Dev *21*, 2558-2570.

Fabian, M.R., Mathonnet, G., Sundermeier, T., Mathys, H., Zipprich, J.T., Svitkin, Y.V., Rivas, F., Jinek, M., Wohlschlegel, J., Doudna, J.A.*, et al.* (2009). Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol Cell *35*, 868-880.

Fabian, M.R., Sonenberg, N., and Filipowicz, W. (2010). Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem *79*, 351-379.

Farazi, P.A., and DePinho, R.A. (2006). Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer *6*, 674-687.

Farazi, T.A., Juranek, S.A., and Tuschl, T. (2008). The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. Development *135*, 1201-1214.

Farh, K.K., Grimson, A., Jan, C., Lewis, B.P., Johnston, W.K., Lim, L.P., Burge, C.B., and Bartel, D.P. (2005). The widespread impact of mammalian MicroRNAs on mRNA repression and evolution. Science *310*, 1817-1821.

Fattovich, G., Stroffolini, T., Zagni, I., and Donato, F. (2004). Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology *127*, S35-50.

Feitelson, M.A., and Lee, J. (2007). Hepatitis B virus integration, fragile sites, and hepatocarcinogenesis. Cancer Lett *252*, 157-170.

Filmus, J., Capurro, M., and Rast, J. (2008). Glypicans. Genome Biol *9*, 224.

Filmus, J., and Selleck, S.B. (2001). Glypicans: proteoglycans with a surprise. J Clin Invest *108*, 497-501.

Fontana, L., Fiori, M.E., Albini, S., Cifaldi, L., Giovinazzi, S., Forloni, M., Boldrini, R., Donfrancesco, A., Federici, V., Giacomini, P.*, et al.* (2008). Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. PLoS One *3*, e2236.

Forner, A., Llovet, J.M., and Bruix, J. (2012). Hepatocellular carcinoma. Lancet *379*, 1245-1255.

Friedman, R.C., Farh, K.K., Burge, C.B., and Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. Genome Res *19*, 92-105.

Gao, W., and Ho, M. (2011). The role of glypican-3 in regulating Wnt in hepatocellular carcinomas. Cancer Rep *1*, 14-19.

Gaur, A., Jewell, D.A., Liang, Y., Ridzon, D., Moore, J.H., Chen, C., Ambros, V.R., and Israel, M.A. (2007). Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. Cancer Res *67*, 2456-2468.

Gehrke, S., Imai, Y., Sokol, N., and Lu, B. (2010). Pathogenic LRRK2 negatively regulates microRNA-mediated translational repression. Nature *466*, 637-641.

Ghosh, T., Soni, K., Scaria, V., Halimani, M., Bhattacharjee, C., and Pillai, B. (2008). MicroRNA-mediated up-regulation of an alternatively polyadenylated variant of the mouse cytoplasmic {beta}-actin gene. Nucleic Acids Res *36*, 6318-6332.

Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Van Dongen, S., Inoue, K., Enright, A.J., and Schier, A.F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science *312*, 75-79.

Gonzalez, A.D., Kaya, M., Shi, W., Song, H., Testa, J.R., Penn, L.Z., and Filmus, J. (1998). OCI-5/GPC3, a glypican encoded by a gene that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, induces apoptosis in a cell line-specific manner. J Cell Biol *141*, 1407-1414.

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G.L., Hunter, C., Bignell, G., Davies, H., Teague, J., Butler, A., Stevens, C.*, et al.* (2007). Patterns of somatic mutation in human cancer genomes. Nature *446*, 153-158.

Griffiths-Jones, S., Saini, H.K., van Dongen, S., and Enright, A.J. (2008). miRBase: tools for microRNA genomics. Nucleic Acids Res *36*, D154-158.

Grimson, A., Farh, K.K., Johnston, W.K., Garrett-Engele, P., Lim, L.P., and Bartel, D.P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell *27*, 91-105.

Gu, J., He, T., Pei, Y., Li, F., Wang, X., Zhang, J., Zhang, X., and Li, Y. (2006). Primary transcripts and expressions of mammal intergenic microRNAs detected by mapping ESTs to their flanking sequences. Mamm Genome *17*, 1033-1041.

Guichard, C., Amaddeo, G., Imbeaud, S., Ladeiro, Y., Pelletier, L., Maad, I.B., Calderaro, J., Bioulac-Sage, P., Letexier, M., Degos, F.*, et al.* (2012). Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet *44*, 694-698.

Guo, H., Ingolia, N.T., Weissman, J.S., and Bartel, D.P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature *466*, 835-840.

Han, D., Lerner, A.G., Vande Walle, L., Upton, J.P., Xu, W., Hagen, A., Backes, B.J., Oakes, S.A., and Papa, F.R. (2009). IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. Cell *138*, 562-575.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell *6*, 1099-1108.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805-816.

He, L., Thomson, J.M., Hemann, M.T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S.W., Hannon, G.J.*, et al.* (2005). A microRNA polycistron as a potential human oncogene. Nature *435*, 828-833.

Hendrickson, D.G., Hogan, D.J., McCullough, H.L., Myers, J.W., Herschlag, D., Ferrell, J.E., and Brown, P.O. (2009). Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA. PLoS Biol *7*, e1000238.

Henke, J.I., Goergen, D., Zheng, J., Song, Y., Schuttler, C.G., Fehr, C., Junemann, C., and Niepmann, M. (2008). microRNA-122 stimulates translation of hepatitis C virus RNA. EMBO J *27*, 3300-3310.

Hidaka, H., Seki, N., Yoshino, H., Yamasaki, T., Yamada, Y., Nohata, N., Fuse, M., Nakagawa, M., and Enokida, H. (2012). Tumor suppressive microRNA-1285 regulates novel molecular targets: aberrant expression and functional significance in renal cell carcinoma. Oncotarget *3*, 44-57.

Ho, M., and Kim, H. (2011). Glypican-3: a new target for cancer immunotherapy. Eur J Cancer *47*, 333-338.

Hollien, J., Lin, J.H., Li, H., Stevens, N., Walter, P., and Weissman, J.S. (2009). Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. J Cell Biol *186*, 323-331.

Hollien, J., and Weissman, J.S. (2006). Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. Science *313*, 104-107.

Hsu, H.C., Cheng, W., and Lai, P.L. (1997). Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular carcinoma: biological significance and temporospatial distribution. Cancer Res *57*, 5179-5184.

Hsu, I.C., Metcalf, R.A., Sun, T., Welsh, J.A., Wang, N.J., and Harris, C.C. (1991). Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature *350*, 427-428.

Huang, N., Lin, J., Ruan, J., Su, N., Qing, R., Liu, F., He, B., Lv, C., Zheng, D., and Luo, R. (2012). MiR-219-5p inhibits hepatocellular carcinoma cell proliferation by targeting glypican-3. FEBS Lett *586*, 884-891.

Huang, S., and He, X. (2011). The role of microRNAs in liver cancer progression. Br J Cancer *104*, 235-240.

Huang, V., Place, R.F., Portnoy, V., Wang, J., Qi, Z., Jia, Z., Yu, A., Shuman, M., Yu, J., and Li, L.C. (2011). Upregulation of Cyclin B1 by miRNA and its implications in cancer. Nucleic Acids Res *40*, 1695-1707.

Huang, Z.M., Yang, J., Shen, X.Y., Zhang, X.Y., Meng, F.S., Xu, J.T., Zhang, B.F., and Gao, H.J. (2009). MicroRNA expression profile in non-cancerous colonic tissue associated with lymph node metastasis of colon cancer. J Dig Dis *10*, 188-194.

Huber, R., Schlessinger, D., and Pilia, G. (1998). Multiple Sp1 sites efficiently drive transcription of the TATA-less promoter of the human glypican 3 (GPC3) gene. Gene *214*, 35-44.

Huntzinger, E., Braun, J.E., Heimstadt, S., Zekri, L., and Izaurralde, E. (2010). Two PABPC1-binding sites in GW182 proteins promote miRNA-mediated gene silencing. EMBO J *29*, 4146-4160.

Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., and Zamore, P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science *293*, 834-838.

Hutvagner, G., and Zamore, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. Science *297*, 2056-2060.

Imbeaud, S., Ladeiro, Y., and Zucman-Rossi, J. (2010). Identification of novel oncogenes and tumor suppressors in hepatocellular carcinoma. Semin Liver Dis *30*, 75-86.

Ishiguro, T., Sugimoto, M., Kinoshita, Y., Miyazaki, Y., Nakano, K., Tsunoda, H., Sugo, I., Ohizumi, I., Aburatani, H., Hamakubo, T., *et al.* (2008). Anti-glypican 3 antibody as a potential antitumor agent for human liver cancer. Cancer Res *68*, 9832-9838.

Izaurralde, E., and Zamore, P.D. (2009). Nucleus and gene expression. Curr Opin Cell Biol *21*, 331-334.

Jakubovic, B.D., and Jothy, S. (2007). Glypican-3: from the mutations of Simpson-Golabi-Behmel genetic syndrome to a tumor marker for hepatocellular carcinoma. Exp Mol Pathol *82*, 184-189.

Jakymiw, A., Lian, S., Eystathioy, T., Li, S., Satoh, M., Hamel, J.C., Fritzler, M.J., and Chan, E.K. (2005). Disruption of GW bodies impairs mammalian RNA interference. Nat Cell Biol *7*, 1267-1274.

Jalvy-Delvaille, S., Maurel, M., Majo, V., Pierre, N., Chabas, S., Combe, C., Rosenbaum, J., Sagliocco, F., and Grosset, C.F. (2012). Molecular basis of differential target regulation by miR-96 and miR-182: the Glypican-3 as a model. Nucleic Acids Res *40*, 1356-1365.

John, B., Enright, A.J., Aravin, A., Tuschl, T., Sander, C., and Marks, D.S. (2004). Human MicroRNA targets. PLoS Biol *2*, e363.

Jones, M.R., Quinton, L.J., Blahna, M.T., Neilson, J.R., Fu, S., Ivanov, A.R., Wolf, D.A., and Mizgerd, J.P. (2009). Zcchc11-dependent uridylation of microRNA directs cytokine expression. Nat Cell Biol *11*, 1157-1163.

Jopling, C.L., Yi, M., Lancaster, A.M., Lemon, S.M., and Sarnow, P. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science *309*, 1577-1581.

Kahvejian, A., Svitkin, Y.V., Sukarieh, R., M'Boutchou, M.N., and Sonenberg, N. (2005). Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. Genes Dev *19*, 104-113.

Kedde, M., Strasser, M.J., Boldajipour, B., Oude Vrielink, J.A., Slanchev, K., le Sage, C., Nagel, R., Voorhoeve, P.M., van Duijse, J., Orom, U.A., *et al.* (2007). RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. Cell *131*, 1273-1286.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev *15*, 2654-2659.

Khvorova, A., Reynolds, A., and Jayasena, S.D. (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell *115*, 209-216.

Kim, D.H., Saetrom, P., Snove, O., Jr., and Rossi, J.J. (2008). MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc Natl Acad Sci U S A *105*, 16230-16235.

Kim, V.N., Han, J., and Siomi, M.C. (2009). Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol *10*, 126-139.

Kiriakidou, M., Tan, G.S., Lamprinaki, S., De Planell-Saguer, M., Nelson, P.T., and Mourelatos, Z. (2007). An mRNA m7G cap binding-like motif within human Ago2 represses translation. Cell *129*, 1141-1151.

Klein, U., Lia, M., Crespo, M., Siegel, R., Shen, Q., Mo, T., Ambesi-Impiombato, A., Califano, A., Migliazza, A., Bhagat, G.*, et al.* (2010). The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. Cancer Cell *17*, 28-40.

Kloosterman, W.P., Wienholds, E., Ketting, R.F., and Plasterk, R.H. (2004). Substrate requirements for let-7 function in the developing zebrafish embryo. Nucleic Acids Res *32*, 6284-6291.

Kong, Y.W., Cannell, I.G., de Moor, C.H., Hill, K., Garside, P.G., Hamilton, T.L., Meijer, H.A., Dobbyn, H.C., Stoneley, M., Spriggs, K.A.*, et al.* (2008). The mechanism of micro-RNA-mediated translation repression is determined by the promoter of the target gene. Proc Natl Acad Sci U S A *105*, 8866-8871.

Krek, A., Grun, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., MacMenamin, P., da Piedade, I., Gunsalus, K.C., Stoffel, M.*, et al.* (2005). Combinatorial microRNA target predictions. Nat Genet *37*, 495-500.

Kumar, M.S., Lu, J., Mercer, K.L., Golub, T.R., and Jacks, T. (2007). Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet *39*, 673-677.

Kumar, M.S., Pester, R.E., Chen, C.Y., Lane, K., Chin, C., Lu, J., Kirsch, D.G., Golub, T.R., and Jacks, T. (2009). Dicer1 functions as a haploinsufficient tumor suppressor. Genes Dev *23*, 2700-2704.

Kunej, T., Godnic, I., Ferdin, J., Horvat, S., Dovc, P., and Calin, G.A. (2011). Epigenetic regulation of microRNAs in cancer: an integrated review of literature. Mutat Res *717*, 77-84.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. Science *294*, 853-858.

Lai, J.P., Oseini, A.M., Moser, C.D., Yu, C., Elsawa, S.F., Hu, C., Nakamura, I., Han, T., Aderca, I., Isomoto, H.*, et al.* (2010). The oncogenic effect of sulfatase 2 in human hepatocellular carcinoma is mediated in part by glypican 3-dependent Wnt activation. Hepatology *52*, 1680-1689.

Lai, J.P., Sandhu, D.S., Yu, C., Han, T., Moser, C.D., Jackson, K.K., Guerrero, R.B., Aderca, I., Isomoto, H., Garrity-Park, M.M.*, et al.* (2008). Sulfatase 2 up-regulates glypican 3,

promotes fibroblast growth factor signaling, and decreases survival in hepatocellular carcinoma. Hepatology *47*, 1211-1222.

Laloo, B., Simon, D., Veillat, V., Lauzel, D., Guyonnet-Duperat, V., Moreau-Gaudry, F., Sagliocco, F., and Grosset, C. (2009). Analysis of post-transcriptional regulations by a functional, integrated, and quantitative method. Mol Cell Proteomics *8*, 1777-1788.

Lanford, R.E., Hildebrandt-Eriksen, E.S., Petri, A., Persson, R., Lindow, M., Munk, M.E., Kauppinen, S., and Orum, H. (2010). Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science *327*, 198-201.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science *294*, 858-862.

Lee, R.C., and Ambros, V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. Science *294*, 862-864.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843-854.

Lee, Y., Hur, I., Park, S.Y., Kim, Y.K., Suh, M.R., and Kim, V.N. (2006). The role of PACT in the RNA silencing pathway. EMBO J *25*, 522-532.

Lee, Y., Jeon, K., Lee, J.T., Kim, S., and Kim, V.N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. EMBO J *21*, 4663-4670.

Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. EMBO J *23*, 4051-4060.

Lehmann, U., Hasemeier, B., Christgen, M., Muller, M., Romermann, D., Langer, F., and Kreipe, H. (2008). Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol *214*, 17-24.

Levine, A.J., Hu, W., and Feng, Z. (2006). The P53 pathway: what questions remain to be explored? Cell Death Differ *13*, 1027-1036.

Lewis, B.P., Burge, C.B., and Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell *120*, 15-20.

Lhomond, S., and Chevet, E. (2012). Signaling the Unfolded Protein Response in cancer (Dordrecht Springer).

Li, L., Jin, R., Zhang, X., Lv, F., Liu, L., Liu, D., Liu, K., Li, N., and Chen, D. (2012). Oncogenic activation of GPC3 by c-Myc in human hepatocellular carcinoma. Hepatology.

Li, L.C., Okino, S.T., Zhao, H., Pookot, D., Place, R.F., Urakami, S., Enokida, H., and Dahiya, R. (2006). Small dsRNAs induce transcriptional activation in human cells. Proc Natl Acad Sci U S A *103*, 17337-17342.

Li, M., Choo, B., Wong, Z.M., Filmus, J., and Buick, R.N. (1997). Expression of OCI-5/glypican 3 during intestinal morphogenesis: regulation by cell shape in intestinal epithelial cells. Exp Cell Res *235*, 3-12.

Liao, J.Y., Ma, L.M., Guo, Y.H., Zhang, Y.C., Zhou, H., Shao, P., Chen, Y.Q., and Qu, L.H. (2010). Deep sequencing of human nuclear and cytoplasmic small RNAs reveals an unexpectedly complex subcellular distribution of miRNAs and tRNA 3' trailers. PLoS One *5*, e10563.

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S., and Johnson, J.M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature *433*, 769-773.

Lim, L.P., Lau, N.C., Weinstein, E.G., Abdelhakim, A., Yekta, S., Rhoades, M.W., Burge, C.B., and Bartel, D.P. (2003). The microRNAs of Caenorhabditis elegans. Genes Dev *17*, 991-1008.

Lin, C.C., Liu, L.Z., Addison, J.B., Wonderlin, W.F., Ivanov, A.V., and Ruppert, J.M. (2011). A KLF4-miRNA-206 autoregulatory feedback loop can promote or inhibit protein translation depending upon cell context. Mol Cell Biol *31*, 2513-2527.

Lin, H., Huber, R., Schlessinger, D., and Morin, P.J. (1999). Frequent silencing of the GPC3 gene in ovarian cancer cell lines. Cancer Res *59*, 807-810.

Lind, T., Tufaro, F., McCormick, C., Lindahl, U., and Lidholt, K. (1998). The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. J Biol Chem *273*, 26265-26268.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., and Hannon, G.J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science *305*, 1437-1441.

Liu, J., Rivas, F.V., Wohlschlegel, J., Yates, J.R., 3rd, Parker, R., and Hannon, G.J. (2005). A role for the P-body component GW182 in microRNA function. Nat Cell Biol *7*, 1261-1266.

Llovet, J.M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.F., de Oliveira, A.C., Santoro, A., Raoul, J.L., Forner, A.*, et al.* (2008). Sorafenib in advanced hepatocellular carcinoma. N Engl J Med *359*, 378-390.

Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A.*, et al.* (2005). MicroRNA expression profiles classify human cancers. Nature *435*, 834-838.

Lujambio, A., and Lowe, S.W. (2012). The microcosmos of cancer. Nature *482*, 347-355.

Lytle, J.R., Yario, T.A., and Steitz, J.A. (2007). Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci U S A *104*, 9667-9672.

Malhi, H., and Kaufman, R.J. (2011). Endoplasmic reticulum stress in liver disease. J Hepatol *54*, 795-809.

Mathonnet, G., Fabian, M.R., Svitkin, Y.V., Parsyan, A., Huck, L., Murata, T., Biffo, S., Merrick, W.C., Darzynkiewicz, E., Pillai, R.S.*, et al.* (2007). MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science *317*, 1764-1767.

Matsui, M., Sakurai, F., Elbashir, S., Foster, D.J., Manoharan, M., and Corey, D.R. (2010). Activation of LDL receptor expression by small RNAs complementary to a noncoding transcript that overlaps the LDLR promoter. Chem Biol *17*, 1344-1355.

Mattick, J.S. (2004). RNA regulation: a new genetics? Nat Rev Genet *5*, 316-323.

Maurel, M., Jalvy, S., Ladeiro, Y., Combe, C., Vachet, L., Sagliocco, F., Bioulac-Sage, P., Pitard, V., Jacquemin-Sablon, H., Zucman-Rossi, J.*, et al.* (2012). A functional screening identifies five miRNAs controlling glypican-3: Role of miR-1271 down-regulation in hepatocellular carcinoma. Hepatology.

Meder, B., Keller, A., Vogel, B., Haas, J., Sedaghat-Hamedani, F., Kayvanpour, E., Just, S., Borries, A., Rudloff, J., Leidinger, P.*, et al.* (2011). MicroRNA signatures in total peripheral blood as novel biomarkers for acute myocardial infarction. Basic Res Cardiol *106*, 13-23.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell *15*, 185-197.

Melo, S.A., Moutinho, C., Ropero, S., Calin, G.A., Rossi, S., Spizzo, R., Fernandez, A.F., Davalos, V., Villanueva, A., Montoya, G.*, et al.* (2010). A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. Cancer Cell *18*, 303-315.

Melo, S.A., Ropero, S., Moutinho, C., Aaltonen, L.A., Yamamoto, H., Calin, G.A., Rossi, S., Fernandez, A.F., Carneiro, F., Oliveira, C.*, et al.* (2009). A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. Nat Genet *41*, 365-370.

Mendell, J.T., and Olson, E.N. (2012). MicroRNAs in stress signaling and human disease. Cell *148*, 1172-1187.

Meng, F., Henson, R., Lang, M., Wehbe, H., Maheshwari, S., Mendell, J.T., Jiang, J., Schmittgen, T.D., and Patel, T. (2006). Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology *130*, 2113-2129.

Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S.T., and Patel, T. (2007). MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology *133*, 647-658.

Metzler, M., Wilda, M., Busch, K., Viehmann, S., and Borkhardt, A. (2004). High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma. Genes Chromosomes Cancer *39*, 167-169.

Midorikawa, Y., Ishikawa, S., Iwanari, H., Imamura, T., Sakamoto, H., Miyazono, K., Kodama, T., Makuuchi, M., and Aburatani, H. (2003). Glypican-3, overexpressed in hepatocellular carcinoma, modulates FGF2 and BMP-7 signaling. Int J Cancer *103*, 455-465.

Min, H., and Yoon, S. (2010). Got target? Computational methods for microRNA target prediction and their extension. Exp Mol Med *42*, 233-244.

Moenner, M., Pluquet, O., Bouchecareilh, M., and Chevet, E. (2007). Integrated endoplasmic reticulum stress responses in cancer. Cancer Res *67*, 10631-10634.

Moll, U.M., and Petrenko, O. (2003). The MDM2-p53 interaction. Mol Cancer Res *1*, 1001-1008.

Monteys, A.M., Spengler, R.M., Wan, J., Tecedor, L., Lennox, K.A., Xing, Y., and Davidson, B.L. (2010). Structure and activity of putative intronic miRNA promoters. RNA *16*, 495-505.

Morford, L.A., Davis, C., Jin, L., Dobierzewska, A., Peterson, M.L., and Spear, B.T. (2007). The oncofetal gene glypican 3 is regulated in the postnatal liver by zinc fingers and homeoboxes 2 and in the regenerating liver by alpha-fetoprotein regulator 2. Hepatology *46*, 1541-1547.

Mortensen, R.D., Serra, M., Steitz, J.A., and Vasudevan, S. (2011). Posttranscriptional activation of gene expression in Xenopus laevis oocytes by microRNA-protein complexes (microRNPs). Proc Natl Acad Sci U S A *108*, 8281-8286.

Murakami, Y., Saigo, K., Takashima, H., Minami, M., Okanoue, T., Brechot, C., and Paterlini-Brechot, P. (2005). Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas. Gut *54*, 1162-1168.

Nakano, K., Orita, T., Nezu, J., Yoshino, T., Ohizumi, I., Sugimoto, M., Furugaki, K., Kinoshita, Y., Ishiguro, T., Hamakubo, T.*, et al.* (2009). Anti-glypican 3 antibodies cause ADCC against human hepatocellular carcinoma cells. Biochem Biophys Res Commun *378*, 279-284.

Nakato, H., Futch, T.A., and Selleck, S.B. (1995). The division abnormally delayed (dally) gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in Drosophila. Development *121*, 3687-3702.

Newman, M.A., and Hammond, S.M. (2010a). Emerging paradigms of regulated microRNA processing. Genes Dev *24*, 1086-1092.

Newman, M.A., and Hammond, S.M. (2010b). Lin-28: an early embryonic sentinel that blocks Let-7 biogenesis. Int J Biochem Cell Biol *42*, 1330-1333.

Nielsen, C.B., Shomron, N., Sandberg, R., Hornstein, E., Kitzman, J., and Burge, C.B. (2007). Determinants of targeting by endogenous and exogenous microRNAs and siRNAs. RNA *13*, 1894-1910.

Nottrott, S., Simard, M.J., and Richter, J.D. (2006). Human let-7a miRNA blocks protein production on actively translating polyribosomes. Nat Struct Mol Biol *13*, 1108-1114.

O'Connor, L., Strasser, A., O'Reilly, L.A., Hausmann, G., Adams, J.M., Cory, S., and Huang, D.C. (1998). Bim: a novel member of the Bcl-2 family that promotes apoptosis. EMBO J *17*, 384-395.

O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V., and Mendell, J.T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. Nature *435*, 839-843.

Okamura, K., Phillips, M.D., Tyler, D.M., Duan, H., Chou, Y.T., and Lai, E.C. (2008). The regulatory activity of microRNA* species has substantial influence on microRNA and 3' UTR evolution. Nat Struct Mol Biol *15*, 354-363.

Olsen, P.H., and Ambros, V. (1999). The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol *216*, 671-680.

Ota, A., Tagawa, H., Karnan, S., Tsuzuki, S., Karpas, A., Kira, S., Yoshida, Y., and Seto, M. (2004). Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res *64*, 3087-3095.

Ozen, M., Creighton, C.J., Ozdemir, M., and Ittmann, M. (2008). Widespread deregulation of microRNA expression in human prostate cancer. Oncogene *27*, 1788-1793.

Ozsolak, F., Poling, L.L., Wang, Z., Liu, H., Liu, X.S., Roeder, R.G., Zhang, X., Song, J.S., and Fisher, D.E. (2008). Chromatin structure analyses identify miRNA promoters. Genes Dev *22*, 3172-3183.

Parsons, D.W., Jones, S., Zhang, X., Lin, J.C., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Siu, I.M., Gallia, G.L.*, et al.* (2008). An integrated genomic analysis of human glioblastoma multiforme. Science *321*, 1807-1812.

Petersen, C.P., Bordeleau, M.E., Pelletier, J., and Sharp, P.A. (2006). Short RNAs repress translation after initiation in mammalian cells. Mol Cell *21*, 533-542.

Pichiorri, F., Suh, S.S., Rocci, A., De Luca, L., Taccioli, C., Santhanam, R., Zhou, W., Benson, D.M., Jr., Hofmainster, C., Alder, H.*, et al.* (2010). Downregulation of p53-inducible

microRNAs 192, 194, and 215 impairs the p53/MDM2 autoregulatory loop in multiple myeloma development. Cancer Cell *18*, 367-381.

Pilia, G., Hughes-Benzie, R.M., MacKenzie, A., Baybayan, P., Chen, E.Y., Huber, R., Neri, G., Cao, A., Forabosco, A., and Schlessinger, D. (1996). Mutations in GPC3, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome. Nat Genet *12*, 241-247.

Piskounova, E., Viswanathan, S.R., Janas, M., LaPierre, R.J., Daley, G.Q., Sliz, P., and Gregory, R.I. (2008). Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28. J Biol Chem *283*, 21310-21314.

Place, R.F., Li, L.C., Pookot, D., Noonan, E.J., and Dahiya, R. (2008). MicroRNA-373 induces expression of genes with complementary promoter sequences. Proc Natl Acad Sci U S A *105*, 1608-1613.

Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W.J., and Pandolfi, P.P. (2010). A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature *465*, 1033-1038.

Portnoy, V., Huang, V., Place, R.F., and Li, L.C. (2011). Small RNA and transcriptional upregulation. Wiley Interdiscip Rev RNA *2*, 748-760.

Qian, G.S., Ross, R.K., Yu, M.C., Yuan, J.M., Gao, Y.T., Henderson, B.E., Wogan, G.N., and Groopman, J.D. (1994). A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. Cancer Epidemiol Biomarkers Prev *3*, 3-10.

Rehwinkel, J., Behm-Ansmant, I., Gatfield, D., and Izaurralde, E. (2005). A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. RNA *11*, 1640-1647.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., and Nusse, R. (1987). The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. Cell *50*, 649-657.

Ro, S., Park, C., Young, D., Sanders, K.M., and Yan, W. (2007). Tissue-dependent paired expression of miRNAs. Nucleic Acids Res *35*, 5944-5953.

Robaglia, C., and Caranta, C. (2006). Translation initiation factors: a weak link in plant RNA virus infection. Trends Plant Sci *11*, 40-45.

Robb, G.B., Brown, K.M., Khurana, J., and Rana, T.M. (2005). Specific and potent RNAi in the nucleus of human cells. Nat Struct Mol Biol *12*, 133-137.

Rodriguez, A., Griffiths-Jones, S., Ashurst, J.L., and Bradley, A. (2004). Identification of mammalian microRNA host genes and transcription units. Genome Res *14*, 1902-1910.

Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol *8*, 519-529.

Saini, H.K., Griffiths-Jones, S., and Enright, A.J. (2007). Genomic analysis of human microRNA transcripts. Proc Natl Acad Sci U S A *104*, 17719-17724.

Sasisekharan, R., Shriver, Z., Venkataraman, G., and Narayanasami, U. (2002). Roles of heparan-sulphate glycosaminoglycans in cancer. Nat Rev Cancer *2*, 521-528.

Schroder, M., and Kaufman, R.J. (2005). The mammalian unfolded protein response. Annu Rev Biochem *74*, 739-789.

Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P.D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell *115*, 199-208.

Seggerson, K., Tang, L., and Moss, E.G. (2002). Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Dev Biol *243*, 215-225.

Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. Nature *455*, 58-63.

Sevignani, C., Calin, G.A., Siracusa, L.D., and Croce, C.M. (2006). Mammalian microRNAs: a small world for fine-tuning gene expression. Mamm Genome *17*, 189-202.

Sherman, M. (2001). Alphafetoprotein: an obituary. J Hepatol *34*, 603-605.

Sherman, M. (2010). Hepatocellular carcinoma: epidemiology, surveillance, and diagnosis. Semin Liver Dis *30*, 3-16.

Shuda, M., Kondoh, N., Imazeki, N., Tanaka, K., Okada, T., Mori, K., Hada, A., Arai, M., Wakatsuki, T., Matsubara, O.*, et al.* (2003). Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis. J Hepatol *38*, 605-614.

Song, H.H., Shi, W., and Filmus, J. (1997). OCI-5/rat glypican-3 binds to fibroblast growth factor-2 but not to insulin-like growth factor-2. J Biol Chem *272*, 7574-7577.

Stark, A., Brennecke, J., Bushati, N., Russell, R.B., and Cohen, S.M. (2005). Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution. Cell *123*, 1133-1146.

Starley, B.Q., Calcagno, C.J., and Harrison, S.A. (2010). Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. Hepatology *51*, 1820-1832.

Su, H., Trombly, M.I., Chen, J., and Wang, X. (2009). Essential and overlapping functions for mammalian Argonautes in microRNA silencing. Genes Dev *23*, 304-317.

Su, R., Li, Z., Li, H., Song, H., Bao, C., Wei, J., and Cheng, L. (2010). Grp78 promotes the invasion of hepatocellular carcinoma. BMC Cancer *10*, 20.

Sun, G., Li, H., and Rossi, J.J. (2010). Sequence context outside the target region influences the effectiveness of miR-223 target sites in the RhoB 3'UTR. Nucleic Acids Res *38*, 239-252.

Sutcliffe, R., Maguire, D., Murphy, P., Portmann, B., Rela, M., O'Sullivan, G., Mufti, G., and Heaton, N. (2005). Detection and clinical significance of bone marrow micrometastases in patients undergoing liver transplantation for hepatocellular carcinoma. Transplantation *80*, 88-94.

Suzuki, H.I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., and Miyazono, K. (2009). Modulation of microRNA processing by p53. Nature *460*, 529-533.

Tan, G.S., Garchow, B.G., Liu, X., Yeung, J., Morris, J.P.t., Cuellar, T.L., McManus, M.T., and Kiriakidou, M. (2009). Expanded RNA-binding activities of mammalian Argonaute 2. Nucleic Acids Res *37*, 7533-7545.

Tarun, S.Z., Jr., Wells, S.E., Deardorff, J.A., and Sachs, A.B. (1997). Translation initiation factor eIF4G mediates in vitro poly(A) tail-dependent translation. Proc Natl Acad Sci U S A *94*, 9046-9051.

Tay, Y., Zhang, J., Thomson, A.M., Lim, B., and Rigoutsos, I. (2008). MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature *455*, 1124-1128.

Thermann, R., and Hentze, M.W. (2007). Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature *447*, 875-878.

Traister, A., Shi, W., and Filmus, J. (2008). Mammalian Notum induces the release of glypicans and other GPI-anchored proteins from the cell surface. Biochem J *410*, 503-511.

Tsai, N.P., Lin, Y.L., and Wei, L.N. (2009a). MicroRNA mir-346 targets the 5'-untranslated region of receptor-interacting protein 140 (RIP140) mRNA and up-regulates its protein expression. Biochem J *424*, 411-418.

Tsai, W.C., Hsu, P.W., Lai, T.C., Chau, G.Y., Lin, C.W., Chen, C.M., Lin, C.D., Liao, Y.L., Wang, J.L., Chau, Y.P.*, et al.* (2009b). MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma. Hepatology *49*, 1571-1582.

Tsai, W.L., and Chung, R.T. (2010). Viral hepatocarcinogenesis. Oncogene *29*, 2309-2324.

Ueda, H., Ullrich, S.J., Gangemi, J.D., Kappel, C.A., Ngo, L., Feitelson, M.A., and Jay, G. (1995). Functional inactivation but not structural mutation of p53 causes liver cancer. Nat Genet *9*, 41-47.

Vasudevan, S. (2012). Posttranscriptional upregulation by microRNAs. Wiley Interdiscip Rev RNA *3*, 311-330.

Vasudevan, S., Tong, Y., and Steitz, J.A. (2007). Switching from repression to activation: microRNAs can up-regulate translation. Science *318*, 1931-1934.

Vella, M.C., Choi, E.Y., Lin, S.Y., Reinert, K., and Slack, F.J. (2004). The C. elegans microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3'UTR. Genes Dev *18*, 132-137.

Verdel, A., Vavasseur, A., Le Gorrec, M., and Touat-Todeschini, L. (2009). Common themes in siRNA-mediated epigenetic silencing pathways. Int J Dev Biol *53*, 245-257.

Villanueva, A., Newell, P., Chiang, D.Y., Friedman, S.L., and Llovet, J.M. (2007). Genomics and signaling pathways in hepatocellular carcinoma. Semin Liver Dis *27*, 55-76.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307-310.

Vousden, K.H. (2002). Switching from life to death: the Miz-ing link between Myc and p53. Cancer Cell *2*, 351-352.

Wakiyama, M., Takimoto, K., Ohara, O., and Yokoyama, S. (2007). Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. Genes Dev *21*, 1857-1862.

Walter, P., and Ron, D. (2011). The unfolded protein response: from stress pathway to homeostatic regulation. Science *334*, 1081-1086.

Wang, X.W., Heegaard, N.H., and Orum, H. (2012). MicroRNAs in liver disease. Gastroenterology *142*, 1431-1443.

Weinmann, L., Hock, J., Ivacevic, T., Ohrt, T., Mutze, J., Schwille, P., Kremmer, E., Benes, V., Urlaub, H., and Meister, G. (2009). Importin 8 is a gene silencing factor that targets argonaute proteins to distinct mRNAs. Cell *136*, 496-507.

White, B.D., Chien, A.J., and Dawson, D.W. (2012). Dysregulation of Wnt/beta-catenin signaling in gastrointestinal cancers. Gastroenterology *142*, 219-232.

Whitelock, J.M., and Iozzo, R.V. (2005). Heparan sulfate: a complex polymer charged with biological activity. Chem Rev *105*, 2745-2764.

Wicklund, C.L., Pauli, R.M., Johnston, D., and Hecht, J.T. (1995). Natural history study of hereditary multiple exostoses. Am J Med Genet *55*, 43-46.

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell *75*, 855-862.

Wilhelm, S.M., Carter, C., Tang, L., Wilkie, D., McNabola, A., Rong, H., Chen, C., Zhang, X., Vincent, P., McHugh, M.*, et al.* (2004). BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res *64*, 7099-7109.

Witkos, T.M., Koscianska, E., and Krzyzosiak, W.J. (2011). Practical Aspects of microRNA Target Prediction. Curr Mol Med *11*, 93-109.

Wu, J.F., Shen, W., Liu, N.Z., Zeng, G.L., Yang, M., Zuo, G.Q., Gan, X.N., Ren, H., and Tang, K.F. (2011). Down-regulation of Dicer in hepatocellular carcinoma. Med Oncol *28*, 804-809.

Wu, L., Fan, J., and Belasco, J.G. (2006). MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A *103*, 4034-4039.

Xiang, Y.Y., Ladeda, V., and Filmus, J. (2001). Glypican-3 expression is silenced in human breast cancer. Oncogene *20*, 7408-7412.

Xiao, C., Srinivasan, L., Calado, D.P., Patterson, H.C., Zhang, B., Wang, J., Henderson, J.M., Kutok, J.L., and Rajewsky, K. (2008). Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. Nat Immunol *9*, 405-414.

Xue, Z., He, Y., Ye, K., Gu, Z., Mao, Y., and Qi, L. (2011). A conserved structural determinant located at the interdomain region of mammalian inositol-requiring enzyme 1alpha. J Biol Chem *286*, 30859-30866.

Yan, L.X., Huang, X.F., Shao, Q., Huang, M.Y., Deng, L., Wu, Q.L., Zeng, Y.X., and Shao, J.Y. (2008). MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA *14*, 2348-2360.

Yang, J.S., Phillips, M.D., Betel, D., Mu, P., Ventura, A., Siepel, A.C., Chen, K.C., and Lai, E.C. (2011). Widespread regulatory activity of vertebrate microRNA* species. RNA *17*, 312-326.

Younger, S.T., and Corey, D.R. (2011). Transcriptional gene silencing in mammalian cells by miRNA mimics that target gene promoters. Nucleic Acids Res *39*, 5682-5691.

Zakhari, S., and Li, T.K. (2007). Determinants of alcohol use and abuse: Impact of quantity and frequency patterns on liver disease. Hepatology *46*, 2032-2039.

Zekri, L., Huntzinger, E., Heimstadt, S., and Izaurralde, E. (2009). The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. Mol Cell Biol *29*, 6220-6231.

Zhu, H., Hu, F., Wang, R., Zhou, X., Sze, S.H., Liou, L.W., Barefoot, A., Dickman, M., and Zhang, X. (2011). Arabidopsis Argonaute10 specifically sequesters miR166/165 to regulate shoot apical meristem development. Cell *145*, 242-256.

Zhu, Z.W., Friess, H., Wang, L., Abou-Shady, M., Zimmermann, A., Lander, A.D., Korc, M., Kleeff, J., and Buchler, M.W. (2001). Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. Gut *48*, 558-564.

Zipprich, J.T., Bhattacharyya, S., Mathys, H., and Filipowicz, W. (2009). Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression. RNA *15*, 781-793.

Zondervan, P.E., Wink, J., Alers, J.C., JN, I.J., Schalm, S.W., de Man, R.A., and van Dekken, H. (2000). Molecular cytogenetic evaluation of virus-associated and non-viral hepatocellular carcinoma: analysis of 26 carcinomas and 12 concurrent dysplasias. J Pathol *192*, 207-215.

Zucman-Rossi, J. (2010). Molecular classification of hepatocellular carcinoma. Dig Liver Dis *42 Suppl 3*, S235-241.

ANNEXE 1: Closing the gap on Drug Induced Liver Injury

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Closing the Gap on Drug-Induced Liver Injury

Patel SJ, Milwid JM, King KR, Bohr S, Iracheta-Velle A, Li M, et al. Gap junction inhibition prevents drug-induced liver toxicity and fulminant hepatic failure. Nat Biotechnol 2012;30:179-183. Available at: www.nature.com (Reprinted with permission.)

Abstract

Drug-induced liver injury (DILI) limits the development and application of many therapeutic compounds and presents major challenges to the pharmaceutical industry and clinical medicine. Acetaminophen-containing compounds are among the most frequently prescribed drugs and are also the most common cause of DILI. Here we describe a pharmacological strategy that targets gap junction communication to prevent amplification of fulminant hepatic failure and acetaminophen-induced hepatotoxicity. We demonstrate that connexin 32 (Cx32), a key hepatic gap junction protein, is an essential mediator of DILI by showing that mice deficient in Cx32 are protected against liver damage, acute inflammation and death caused by liver-toxic drugs. We identify a small-molecule inhibitor of Cx32 that protects against liver failure and death in wild-type mice when co-administered with known hepatotoxic drugs. These findings indicate that gap junction inhibition could provide a pharmaceutical strategy to limit DILI and improve drug safety.

Comment

Drug-induced liver injury (DILI) is the most frequent reason for drug withdrawal either during early development or later from the market. It can also lead to a recommendation of limited dosage.[1,2] DILI is initiated by direct hepatotoxic effects of a drug or a reactive metabolite of a drug. Overdosing on N-acetyl-para-amino-phenol (APAP; acetaminophen), a widely used antipyretic and analgesic agent, is the most frequent cause of acute liver failure in young adults in the United States. *CYP1A2*, *CYP2E1*, and *CYP3A4* drive conversion of APAP into N-acetyl-p-benzoquinone imine (NAPQI), which is then conjugated to glutathione and detoxified to mercapturic acid.[3] When excessive doses of APAP are ingested, the glucuronidation pathway is overwhelmed, and the rapid generation of NAPQI can lead to the depletion of intrahepatic glutathione stores and reduced detoxification of the drug. NAPQI can also covalently bind to intracellular proteins, interfere with mitochondrial and nuclear function, generate reactive oxidative species, and ultimately lead to apoptosis and necrosis in the liver.[3] Currently, the only available therapies are N-acetylcysteine (NAC), supportive care, and liver transplantation. Because of the zonation of the liver that confines most P450 enzymes to perivenular hepatocytes, APAP toxicity initially affects those, and then spreads into the parenchyma and the entire hepatic lobule. Published evidence suggests that liver gap junctions are involved in such spreading. Gap junctions are plasma membrane spatial microdomains constituted by assemblies of channel proteins called connexins (Cx). The channels provide direct intercellular communication pathways, allowing cell-to-cell rapid exchange of ions and metabolites up to approximately 1 Kd in size. Cx32 is the major gap junction protein in the liver, but it is also found in pancreas, kidney, and nervous tissue. Several studies have shown that interfering with liver gap junctions function greatly reduces liver damage due to several toxic agents including carbon tetrachloride, D-galactosamine,[4] and APAP itself.[5] In the latter study, the authors used a line of transgenic rats carrying a dominant negative mutant of Cx32 and found a reduced hepatotoxic effect of APAP compared to wild-type rats.[5] In this new study, Patel et al.[6] have confirmed and expanded these results, and most importantly, have been able to devise a pharmacological strategy to limit DILI spreading through the blockade of gap junctions.

Patel et al. first showed that mice deficient in Cx32 ($Cx32^{-/-}$) are protected against liver damage, acute inflammation, and death caused by thioacetamide (TAA), a hepatotoxin model. They carefully checked that phase I and phase II drug metabolism efficiency was similar in $Cx32^{+/+}$ and $Cx32^{-/-}$ mice, ruling out the possibility that $Cx32^{-/-}$ mice were protected through defective drug metabolism.

Because hepatotoxin exposure leads to oxidative stress and because free radicals propagate through gap junctions,[7,8] Patel et al. hypothesized that gap junctions might be responsible for the dissemination and amplification of oxidative stress in the liver after TAA treatment. They first verified that drug-induced hepatotoxicity was indeed dependent on oxidative stress by showing that antioxidants (dimethyl sulfoxide, N-acetylcysteine) reduced liver injury. They also validated that Cx32 gap junctions propagated oxidative stress from hepatotoxin-injured cells to neighboring hepatocytes by comparing the accumulation of intracellular free radicals within the livers of $Cx32^{-/-}$ and $Cx32^{+/+}$ mice.

Patel et al. then sought to identify small molecule inhibitors of Cx32 and selected 2-aminoethoxydipenylborate (2APB). 2APB is a membrane-permeable reagent widely used to block inositol 1,4,5-trisphosphate–induced calcium release,[9] and that also activates or inhibits various transient receptor potential channels.[10] Tao et al. previously showed that 2APB directly and

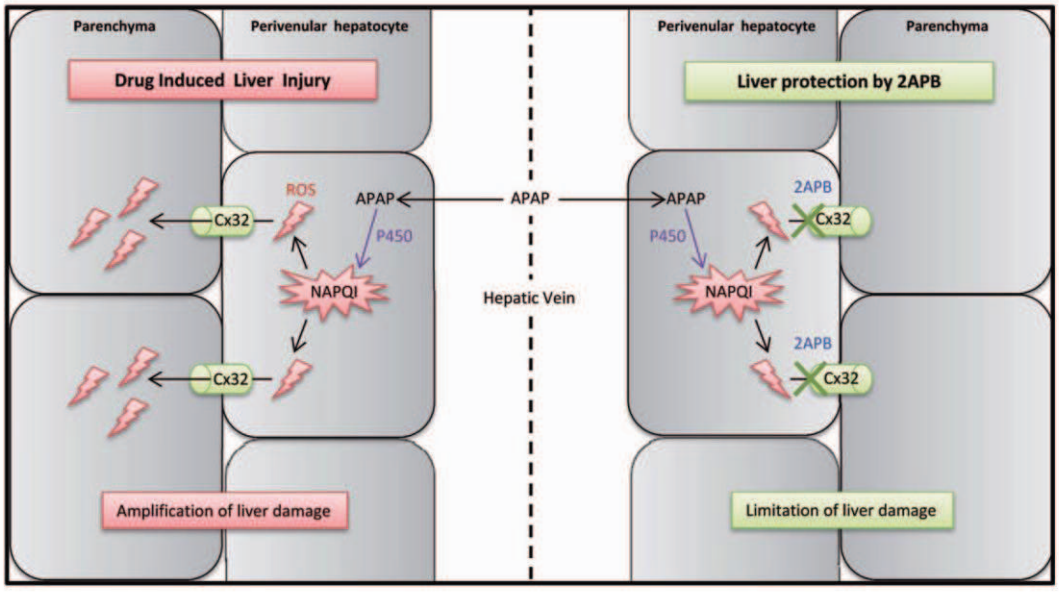


Fig. 1. (Left) Acetaminophen (APAP) is converted to the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) in perivenular hepatocytes, leading to the generation of reactive oxygen species (ROS). ROS spread to parenchymal hepatocytes through connexin 32 (Cx32) gap junctions, thus propagating liver damage. (Right) in the presence of the Cx32 blocker 2APB, diffusion of ROS is blocked and the liver damage is limited.

reversibly inhibited connexin channels composed of Cx26 and Cx32 in vitro.[11] Here, Patel et al. first confirmed using liver slices that 2APB very efficiently blocked gap junction function. They then pretreated mice with 2APB before administration of TAA or APAP. This experiment showed a dramatic protection against the hepatotoxic effects of both drugs in the same range as that seen in $Cx32^{-/-}$ mice. Because pretreatment is not an option in clinical practice, they then tested the coformulation of 2APB with APAP or TAA. In that case, mice were still largely preserved from severe liver damage as judged by a variety of parameters. Most importantly, 2APB reduced APAP-induced mortality from 80% to 30% and completely abolished TAA-induced mortality.

Even though coformulation might in theory be applicable to drugs with a high incidence of DILI, the most common situation is that of patients seeking treatment after ingestion of compounds at hepatotoxic doses. Patel et al. thus treated mice with 2APB at various time points following APAP or TAA administration. At 1.5 hours after intoxication, protection by 2APB was still almost complete. Remarkably, even when administered 6 hours after hepatotoxin exposure, at a time where hepatic necrosis is already evident, the treatment could reduce serum alanine aminotransferase (ALT) levels and hepatocellular damage and necrosis, although data on mortality were not reported.

Altogether, these results strongly argue for a role of liver gap junctions in the propagation of DILI and suggest for the first time that this mechanism can be targeted with drugs (Fig. 1). A number of issues will likely have to be solved before human trials. For instance, 2APB has other targets besides Cx32, and its toxicity needs to be investigated further and/or new blockers need to be identified. Tao et al. have shown that 2APB also block Cx32 hemichannels that connect the cytosol to the extracellular space[11]; because there is now accumulated evidence that connexin hemichannels are involved in cell death signaling,[12] 2APB might protect from cell death at least partly independently of blocking gap junctions. Very interestingly, promising results were obtained by Patel et al. in a postabsorption setting, suggesting that their strategy could be used in patients seen after intoxication with the hope of limiting the requirement for liver transplantation. However, and intriguingly, previous studies have shown that administration of hepatotoxic chemicals such as dimethylnitrosamine or carbon tetrachloride decreased the protein levels of Cx32 in cultured hepatocytes[13] and in rat liver.[14] Remarkably, Cx32 levels became undetectable as soon as 24 hours following dimethylnitrosamine treatment, before the peak of ALT,[14] which may be a cellular response to injury designed to protect healthy cells from the diffusion of toxic molecules. However, if such a decreased Cx32

expression was also to be found in the TAA or APAP setting, it could indicate that the protective effect of 2APB may be partly related to an effect on other targets, such as those mentioned above. In any case, it suggests that the therapeutic window for using Cx32 blockers in the course of an acute intoxication may be narrow. On the other hand, Cx32 blockers used in coformulation may help rescue a number of hepatotoxic drugs in the course of their clinical development.

MARION MAUREL, PH.D.
JEAN ROSENBAUM, M.D.
INSERM U1053, Université Bordeaux Segalen, Bordeaux, France

References

1. Lee WM. Drug-induced hepatotoxicity. N Engl J Med 2003;349:474-485.
2. Wysowski DK, Swartz L. Adverse drug event surveillance and drug withdrawals in the United States, 1969-2002: the importance of reporting suspected reactions. Arch Intern Med 2005;165:1363-1369.
3. Tujios S, Fontana RJ. Mechanisms of drug-induced liver injury: from bedside to bench. Nat Rev Gastroenterol Hepatol 2011;8:202-211.
4. Asamoto M, Hokaiwado N, Murasaki T, Shirai T. Connexin 32 dominant-negative mutant transgenic rats are resistant to hepatic damage by chemicals. HEPATOLOGY 2004;40:205-210.
5. Naiki-Ito A, Asamoto M, Naiki T, Ogawa K, Takahashi S, Sato S, et al. Gap junction dysfunction reduces acetaminophen hepatotoxicity with impact on apoptotic signaling and connexin 43 protein induction in rat. Toxicol Pathol 2010;38:280-286.
6. Patel SJ, Milwid JM, King KR, Bohr S, Iracheta-Velle A, Li M, et al. Gap junction inhibition prevents drug-induced liver toxicity and fulminant hepatic failure. Nat Biotechnol 2012;30:179-183.
7. Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. Toxicol Sci 2002;65:166-176.
8. Ferret PJ, Hammoud R, Tulliez M, Tran A, Trebeden H, Jaffray P, et al. Detoxification of reactive oxygen species by a nonpeptidyl mimic of superoxide dismutase cures acetaminophen-induced acute liver failure in the mouse. HEPATOLOGY 2001;33:1173-1180.
9. Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K. 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P3-induced Ca2+ release. J Biochem 1997;122:498-505.
10. Hu HZ, Gu Q, Wang C, Colton CK, Tang J, Kinoshita-Kawada M, et al. 2-aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2, and TRPV3. J Biol Chem 2004;279:35741-35748.
11. Tao L, Harris AL. 2-aminoethoxydiphenyl borate directly inhibits channels composed of connexin26 and/or connexin32. Mol Pharmacol 2007;71:570-579.
12. Decrock E, Vinken M, De Vuyst E, Krysko DV, D'Herde K, Vanhaecke T, et al. Connexin-related signaling in cell death: to live or let die? Cell Death Differ 2009;16:524-536.
13. Saez JC, Bennett MV, Spray DC. Carbon tetrachloride at hepatotoxic levels blocks reversibly gap junctions between rat hepatocytes. Science 1987;236:967-969.
14. Miyashita T, Takeda A, Iwai M, Shimazu T. Single administration of hepatotoxic chemicals transiently decreases the gap-junction-protein levels of connexin 32 in rat liver. Eur J Biochem 1991;196:37-42.

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.25779
Potential conflict of interest: Nothing to report.

ANNEXE 2: Analysis of post-transcriptional regulation using the FunREG method

Biochemical Society Transactions (2010) Volume 38, part 6

Analysis of post-transcriptional regulation using the FunREG method

Benoît Laloo, Marion Maurel, Sandra Jalvy-Delvaille, Francis Sagliocco and Christophe F. Grosset[1]
INSERM, U889, Groupe de Recherche pour l'Etude du Foie (GREF), Université Victor Segalen Bordeaux 2, Bordeaux cedex, F-33076 France

Abstract

An increasing number of arguments, including altered microRNA expression, support the idea that post-transcriptional deregulation participates in gene disturbances found in diseased tissues. To evaluate this hypothesis, we developed a method which facilitates post-transcriptional investigations in a wide range of human cells and experimental conditions. This method, called FunREG (functional, integrated and quantitative method to measure post-transcriptional regulation), connects lentiviral transduction with a fluorescent reporter system and quantitative PCR. Using FunREG, we efficiently measured post-transcriptional regulation mediated either by selected RNA sequences or regulatory factors (microRNAs), and then evaluated the contribution of mRNA decay and translation efficiency in the observed regulation. We demonstrated the existence of gene-specific post-transcriptional deregulation in liver tumour cells, and also reported a molecular link between a transcript variant abrogating HDAC6 (histone deacetylase 6) regulation by *miR-433* and a rare familial genetic disease. Because FunREG is sensitive, quantitative and easy to use, many applications can be envisioned in fundamental and pathophysiological research.

Introduction

Post-transcriptional regulation is a set of biological processes controlling cell transcripts from their birth to their death (see [1,2] for more detailed reviews). This regulation plays a critical role in gene expression by providing the adequate amount of proteins for the functioning, growth, survival or contextual adaptability of cells. As a consequence, post-transcriptionally controlled genes are usually involved in transient and adaptable cellular processes such as signal transduction, cell proliferation, gene transcription, cell communication, metabolism or stimulus response [1,3]. Among the post-transcriptional processes, mRNA turnover and translation play a central role as these mechanisms govern directly, in a spatiotemporal manner, the quantity of proteins being produced by and distributed throughout the whole cell. The two key elements involved in these regulatory mechanisms are: (i) intrinsic *cis*ARSs (*cis*-acting RNA sequences); and (ii) their specific and specialized partners, called *trans*RFs (*trans*-regulatory factors). Functional *cis*ARSs can be found throughout the mRNA sequence [4–6]. However the best-described ones, namely the ARE (AU-rich element) and the miRNA (microRNA) site, are mainly, but not exclusively, located in the 3′-UTR (untranslated region) (see the miRWalk website at http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/) [7,8]. *Trans*RFs are mostly represented by RBPs (RNA-binding proteins), small non-coding miRNAs and their cofactors [1,9,10]. RBPs belong to a very large family of proteins (an estimated 500 RBPs in yeast) bearing various types of RNA-binding domains, and involved in all steps of RNA metabolism and post-transcriptional control [2,11]. Post-translational modifications of RBPs (phosphorylation, methylation, etc.) provide another level of complexity in RNA-dependent mechanisms either by modulating the affinity of RBPs for their target RNAs or controlling their subcellular localization [12,13]. miRNAs are ~22-nt-long non-coding RNAs, generated nuclearly from either introns or specific primary transcripts (pri-miRNA) by a multistep process using the pre-mRNA splicing machinery or a dedicated microprocessor complex respectively [10]. Following their maturation by Dicer in the cytoplasm, miRNAs are incorporated into the RISC (RNA-induced silencing complex) and associated with the functional core protein Argonaute. Both RBPs and miRNAs control gene expression at a post-transcriptional level by modulating mRNA decay and/or translation efficiency. These controls are mainly linked to the polyadenylated and capped status of the target transcript [14,15].

Key words: functional, integrated and quantitative method to measure post-transcriptional regulation (FunREG), hepatocellular carcinoma, microRNA (miRNA), mRNA, post-transcriptional regulation, RNA-binding protein.

Abbreviations used: ARE, AU-rich element; *cis*ARS, *cis*-acting RNA sequence; eGFP, enhanced green fluorescent protein; FunREG, functional, integrated and quantitative method to measure post-transcriptional regulation; HB, hepatoblastoma; HCC, hepatocellular carcinoma; HDAC6, histone deacetylase 6; miRNA, microRNA; qPCR, real-time quantitative PCR; RBP, RNA-binding protein; siRNA, small interfering RNA; TCN, transgene copy number per cell; *trans*RF, *trans*-regulatory factor; UTR, untranslated region.

[1]To whom correspondence should be addressed (email christophe.grosset@u-bordeaux2.fr).

Post-transcriptional regulation and pathology

Over the last decade, arguments underlying the importance of post-transcriptional deregulation in human diseases have accumulated [1,16,17]. Two major post-transcriptional

defects have been reported in pathological cells: (i) loss of *cis*ARSs by sequence mutation or deletion; and (ii) aberrant expression of *trans*RFs, exemplified by the HuR protein or miRNAs in cancerous tissue [1,10,18]. As RBPs can be phosphorylated or methylated, and miRNAs edited [10,12,13], it should be emphasized that such mechanisms may also influence gene expression and participate in 'normal to pathological' cell transition.

Sequence mutations or deletions of *cis*ARSs are the result of genetic alterations or aberrations due to viral infections, chromosomal rearrangements, gains/losses of genetic information or, more rarely, to deficiencies in DNA surveillance and repair [1]. Genetic changes influencing mRNA decay or translation could also originate from normal events such as polymorphism [1]. In a collaborative work performed with our team, Benoît Arveiler and colleagues recently reported an example of such a genetic defect, which abrogates the regulation of HDAC6 (histone deacetylase 6) expression by *miR-433* (see below and [19]). On the other hand, the expression of numerous *trans*RFs (either RBP or miRNA) is altered in diseased tissues [1,10,17]. Concerning RBPs, most studies have focused on ARE-BPs (ARE-binding proteins) in cancer, particularly on HuR [1,12,20]. But other RBPs, such as PTBP1 (polypyrimidine-tract-binding protein 1) in viral infection, can display altered expression in pathological tissues [21]. For miRNAs, numerous large-scale expression analyses have been performed in most diseased tissues, revealing specific marks or signatures which validate their use as biological markers in diagnosis [1,10,17,22].

Post-transcriptional deregulation in liver cancer

HCC (hepatocellular carcinoma) is the primary malignancy of the liver, and one of the most common and aggressive cancers worldwide (0.5–1 million deaths annually) [23,24]. Its childhood counterpart is HB (hepatoblastoma), a fetal-like liver cancer arising from undifferentiated liver stem cells. HCC is a heterogeneous tumour that develops on a diseased liver harbouring severe fibrosis or cirrhosis, themselves originating from hepatitis B or C virus infection, aflatoxin or alcohol consumption [25,26]. Its diagnosis is generally made at advanced stages of the disease when curative solutions, mostly based on surgery, can no longer be proposed, and patients are often affected with a recurrence of the disease. Several recent observations have argued in favour of an active participation of post-transcriptional mechanisms in HCC-associated gene alterations. First, Acevedo et al. [27] speculated that 40–50% of the changes in gene expression observed in HCC could originate from aberrant post-transcriptional regulation. Concomitantly, a comparative proteomic and transcriptomic profiling showed that the abundance of numerous proteins, whose expression varies between HCC and the adjacent non-tumour tissue, was poorly correlated with mRNA expression changes [28]. Finally, the altered expression of many *trans*RFs (either protein or miRNA) has been reported in HCC tissues [12,20,22,26]. However, important questions remain unanswered. How can it be demonstrated experimentally that post-transcriptional deregulation definitively participates in HCC-associated gene variations? How can this deregulation be studied in a laboratory context and the molecular factors involved be identified?

Methods for measuring post-transcriptional regulation

Many of the strategies summarized in Table 1 have been developed to study the molecular processes mediated either by *cis*ARSs (located in mRNA coding or non-coding regions) or by *trans*RFs in mammalian cells. The methods first developed were devoted to the measurement of mRNA stability and the identification of *cis*-regulatory elements and *trans*-factors involved in mRNA decay, as exemplified by the transcriptional pulse assay [29]. Although these methods are very accurate, they have very limited application and have been used in only a small number of cell types (i.e. HeLa). Moreover, whereas some have profound indirect effects on mRNA decay (i.e. transcription inhibitors [30]), others require very specialized methodologies (i.e. cell-free extracts [31]) or time-consuming kinetics [6,29]. More recently, the dual-reporter system, either using fluorescent protein or luciferase, has allowed investigators to routinely and rapidly introduce post-transcriptional studies into a broader range of cells and conditions. The advantages and disadvantages of all of these methods in terms of the investigatory questioning and experimental context are summarized in Table 1. More detailed information can be found in the corresponding references. In the present paper, we focus on FunREG (functional, integrated and quantitative method to measure post-transcriptional regulation), a recently described method [32], and its potential applications in laboratories.

FunREG method: principle and experimental pipeline

FunREG was developed in order to determine whether or not the expression of particular genes (i.e. oncogenes) is post-transcriptionally altered in cancerous hepatic cells when compared with normal hepatocytes. However, we had to address several problems. First, the transgene reporter (post-transcriptionally regulated or not) had to be transferred into various human cell types, including primary human hepatocytes. Secondly, the interferences or biases due to transgene expression in host cells should be minimized as far as possible. Thirdly, the post-transcriptional mechanisms, mediated either by *cis*ARSs or *trans*RFs, had to be measured in a quick, accurate and quantitative way, considering the limited amount of biological materials available from hepatocytes. Fourthly, to limit the use of laborious methodologies (i.e. kinetics in Table 1 and [6,29]), the measurements had to be achieved in 'one time point'. Finally, the contribution of mRNA decay or translation

Table 1 | Methods for analysing post-transcriptional regulation through mRNA degradation, translational control or both

Method	Kinetics (K)/ end-point (E)	Global post-transcriptional regulation	mRNA stability	Translation efficiency	Advantages	Disadvantages	Reference(s)
Transcription inhibitors	K	–	+	–	Easy and quick to perform; study of endogenous transcripts	Multiple non-specific effects; some inhibitors are cell-specific	[5,30]
Pulse-labelling and chasing assay	K	–	+	–	Study of endogenous transcripts; no transcriptional interference	Some assays use radioisotopes; possible low sensitivity	[5]
Transcriptional-pulse assay	K	–	+	–	Optimized for studying mRNA decay and poly(A) shortening	Transgene copy number unknown; possible saturation of the system and transcriptional interference	[5,29]
Mammalian cell-free extracts	K	–	+	+	Optimized for deciphering the molecular basis of mRNA decay and translation	*In vitro* study; lower decay rate than *in vivo*; specific experimental requirements; possible saturation of the system	[5,31]
Fluorescent- or light-dual-reporter system	E	+	–	+	Easy and quick to perform; commercial plasmid libraries	Transgene copy number unknown; possible saturation of the system and transcriptional interference; relative quantification	[34,36]
PCR-derived reporter system	E	+	+	+	mRNA stability and translation efficiency are both evaluated; easy and quick to perform	Transgene copy number unknown; possible saturation of the system and transcriptional interference; depends on DNA polymerase fidelity; relative quantification	[37]
FunREG method	E	+	+	+	mRNA stability and translation efficiency are both evaluated; stable transgene expression; no cell toxicity; large cellular spectrum including non-dividing cells; comparative cell analyses; transcription-independent	Not adaptable to the study of endogenous mRNAs; lentiviral facilities; relative quantification	[32]

Figure 1 | Schematic representation of the two transgenes used with FunREG

Upper panel: reference transgene. Lower panel: *cis*ARS-containing test transgene. EF1α, elongation factor 1α; LTR, long terminal repeats; pA, polyadenylation site; ΔU3, U3 deletion.

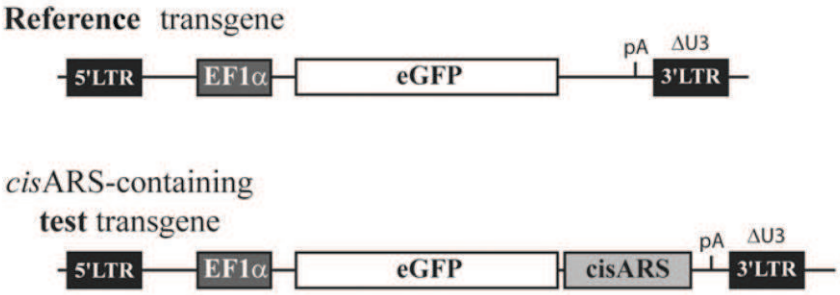
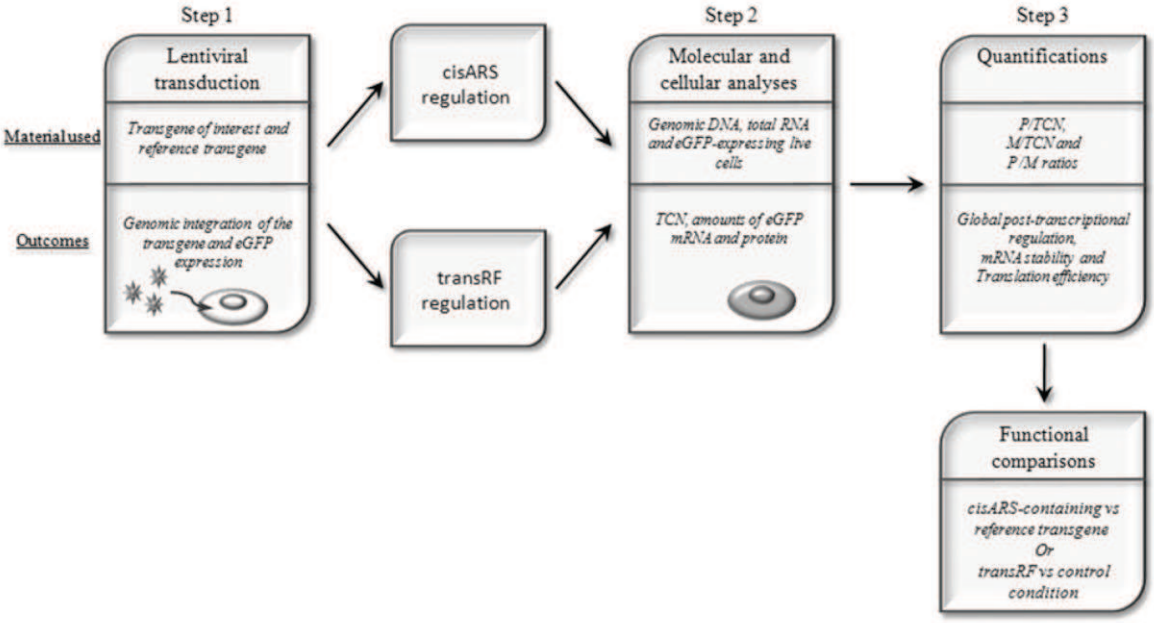


Figure 2 | Schematic representation of FunREG experimental pipeline

This research was originally published in Molecular and Cellular Proteomics. Laloo, B., Simon, D., Veilla, V., Lauzel, D., Guyonnet-Duperat, V., Moreau-Gaudry, F., Sagliocco, F. and Grosset, C., Analysis of post-transcriptional regulations by a functional, integrated, and quantitative method. *Mol. Cell. Proteomics* (2009) **8**: 1777–1788. © the American Society for Biochemistry and Molecular Biology.



efficiency in the observed post-transcriptional control had to be established, if possible at once.

As a lentivirus-production platform was available locally [33], and as we had already dealt with a fluorescent reporter system before [34], a strategy was elaborated based on the efficient delivery of transgenes by lentiviruses into a broad range of human cells, including non-dividing ones [33]. Having assessed in different cell types that the expression of an eGFP (enhanced green fluorescent protein) transgene was directly proportional to the number of integrated lentiviral transgene copies per cell (see Figure 2 in [32]), we speculated that any difference in the expression of a given transgene reporter subsequent to insertion of a definite regulatory sequence (i.e. 5′- or 3′-UTR) upstream or downstream of the open reading frame might exclusively originate from a post-transcriptional event. Therefore, by comparing, in a given cell type, the expression of an eGFP transgene bearing a regulatory RNA sequence (test transgene, Figure 1) and that of the same transgene devoid of it (reference transgene, as it serves as the reference for eGFP expression in this cell type, Figure 1), we should be able to measure the post-transcriptional control mediated by this sequence. It was on the basis of this idea that the FunREG experimental pipeline was built [32] (Figure 2). Following lentiviral infections,

three parameters were determined from each transduced cell population (reference and test). The amount of eGFP (P) was measured by flow cytometry using living cells. The TCN (transgene copy number per cell), and the amount of the corresponding mRNA (M) were measured by real-time qPCR (quantitative PCR) using genomic DNA and reverse-transcribed total RNA respectively [32] (Figure 2). Finally, these three parameters produced three ratios (Figure 2). In the transduced cell population, P/TCN represents the quantity of protein produced per transgene, M/TCN represents the quantity of mRNA produced per transgene (or the steady-state level of mRNA), and P/M represents the quantity of protein produced per mRNA. Now, in comparing, in a given cell type, the expression of the post-transcriptional-regulated eGFP test transgene with that of the reference, we assumed that P/TCN, M/TCN and P/M are indicative of the global post-transcriptional regulation, relative mRNA stability and relative translation efficiency respectively (see [32] for more details).

Validation of FunREG and applications in laboratory routine

To test FunREG function, we first measured post-transcriptional regulation mediated by a prototype ARE, or selected 3′-UTRs deriving from oncogenes in HCC cells (HuH7) by following the '*cis*ARS-regulation' pipeline [32] (Figure 2). Results clearly established the efficiency of FunREG for measuring post-transcriptional events mediated by the various regulatory sequences. As expected, results directly reported the contribution of either mRNA stability or translation efficiency in the observed regulation [32]. In a second set of experiments (depicted as the 'transRF-regulation' pipeline in Figure 2), FunREG was tested for its ability to sense regulation mediated by competent siRNA (small interfering RNA) and miRNAs, and determine the molecular process involved. The reporter expression of reference was given here by the cell population expressing the test transgene, and then transfected with an irrelevant small RNA (Figure 4 in [32]). Results revealed that the anti-eGFP siRNA not only induced mRNA decay, but also repressed translation. Although less potent than the siRNA, but in agreement with the literature [14,15], *miR-98* and *Let-7a* efficiently reduced eGFP expression through c-*myc* 3′-UTR by destabilizing the mRNA, with limited effects on translation efficiency [35]. Finally, we assessed the capacity of FunREG to reveal post-transcriptional changes from one cell type to another in different pathological contexts (see [19,32]). We first compared *cis*ARS function in cancerous and normal hepatocytes. In order to do so, HuH7, HepG2 (HB cells) and normal hepatocytes were transduced by different eGFP reporter transgenes under the regulation of potent *cis*ARSs. The FunREG pipeline was followed in each case, and the resulting ratios were compared. Results not only confirmed that *cis*ARS-mediated regulation is cell-specific, but also showed that some oncogenic genes are post-transcriptionally deregulated in tumour cells. Results showed further that this deregulation is in accordance with an overexpression of the corresponding genes in HCC tissues, and that they concern variations of either mRNA stability or translational capacity. In a second study, FunREG was used to assess the functional consequence of a variant (c.*281A>T [19]) found in HDAC6 3′-UTR. This variant was located in the *miR-433* 'seed' sequence, and fully segregated with a familial dominant X-linked chondrodysplasia (disease caused by anomalies in chondrocyte differentiation and proliferation [19]). Using MG63 osteosarcoma cells, we demonstrated that this single base change was sufficient to completely abrogate the post-transcriptional regulation of HDAC6 by *miR-433*, therefore justifying the HDAC6 overexpression in tissues of affected patients [19].

Advantages of infectious lentiviruses for delivering a transgene into host cells

Experimental transduction using lentiviruses has many advantages over classical delivery methods using either chemical reagents or electroporation: it is quick, simple and minimally stressful for cells and for the biological process to be studied. Transduction has three more advantages over transfection: first, because the transgene integrates into the host cell genome, it is stably found in cell progenies. Secondly, as this integration occurs at random sites in the transduced cell population, the transgene expression becomes independent of its insertional position. Thirdly, the average number of integrated transgene copies per cell can be precisely defined, by using an adequate amount of infectious lentiviruses [32]. Therefore the investigator can design his experimentation in such a way that the mechanism being studied is not subjected to saturation or to non-specific biases. Finally, results obtained are guaranteed to be physiologically relevant. For these reasons, we generally work with cellular populations whose transduced cells contain an average of one transgene copy integrated per cell.

Concluding remarks and perspectives

As described above, FunREG is a unique, versatile and easy-to-handle method, allowing the measurement of post-transcriptional regulation mediated by *cis*- and *trans*-RFs in a broad range of experimental conditions and human cells, including non-dividing cells. FunREG connects transgene transfer efficiency and stable expression with laboratory routine methodologies for gene reporter detection (qPCR and flow cytometry). Results are achieved in a 'one time point' experiment, which avoids kinetic studies, but permits wide functional analyses and screening. No other methods so far provide, with such accuracy, both a global functional picture of a given post-transcriptional element or factor, and information about the contribution of mRNA stability or translation efficiency in the observed regulation. With the expanding utilization of lentiviruses in laboratories,

we believe that FunREG may be of particular help for numerous investigators familiar or not with post-transcriptional mechanisms. Initially, FunREG could be used as a prospective tool and help investigators to manage their experimentations and future projects. As described in Figure 2 and [32], FunREG generates two transduced cell populations and necessitates a double technical handling of cells and materials. Soon, the FunREG pipeline will integrate a fluorescent dual-reporter system that overcomes the above constraints. In its new format, FunREG should be even more convenient for investigations regarding post-transcriptional analyses in laboratory routines, and should open the way to new areas of investigation in functional screening, combined -omics and human pathology.

Acknowledgement

We warmly thank Ray Godfrey for his critical reading of the paper.

Funding

This work was supported by grants from the Ligue Régionale contre le Cancer-Comités Aquitaine et Dordogne, and the Agence Nationale pour la Recherche (ANR) Young Researcher Programme [grant number JC07_184264 to C.F.G.].

References

1 Lopez de Silanes, I., Quesada, M.P. and Esteller, M. (2007) Aberrant regulation of messenger RNA 3′-untranslated region in human cancer. Cell. Oncol. **29**, 1–17

2 Glisovic, T., Bachorik, J.L., Yong, J. and Dreyfuss, G. (2008) RNA-binding proteins and post-transcriptional gene regulation. FEBS Lett. **582**, 1977–1986

3 Bakheet, T., Frevel, M., Williams, B.R., Greer, W. and Khabar, K.S. (2001) ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. Nucleic Acids Res. **29**, 246–254

4 Guhaniyogi, J. and Brewer, G. (2001) Regulation of mRNA stability in mammalian cells. Gene **265**, 11–23

5 Ross, J. (1995) mRNA stability in mammalian cells. Microbiol. Rev. **59**, 423–450

6 Grosset, C., Chen, C.Y., Xu, N., Sonenberg, N., Jacquemin-Sablon, H. and Shyu, A.B. (2000) A mechanism for translationally coupled mRNA turnover: interaction between the poly(A) tail and a c-fos RNA coding determinant via a protein complex. Cell **103**, 29–40

7 Brodersen, P. and Voinnet, O. (2009) Revisiting the principles of microRNA target recognition and mode of action. Nat. Rev. Mol. Cell Biol. **10**, 141–148

8 Meng, Z., King, P.H., Nabors, L.B., Jackson, N.L., Chen, C.Y., Emanuel, P.D. and Blume, S.W. (2005) The ELAV RNA-stability factor HuR binds the 5′-untranslated region of the human IGF-IR transcript and differentially represses cap-dependent and IRES-mediated translation. Nucleic Acids Res. **33**, 2962–2979

9 Houseley, J. and Tollervey, D. (2009) The many pathways of RNA degradation. Cell **136**, 763–776

10 Sotiropoulou, G., Pampalakis, G., Lianidou, E. and Mourelatos, Z. (2009) Emerging roles of microRNAs as molecular switches in the integrated circuit of the cancer cell. RNA **15**, 1443–1461

11 Hogan, D.J., Riordan, D.P., Gerber, A.P., Herschlag, D. and Brown, P.O. (2008) Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system. PLoS Biol. **6**, e255

12 Vázquez-Chantada, M., Fernández-Ramos, D., Embade, N., Martinez-Lopez, N., Varela-Rey, M., Woodhoo, A., Luka, Z., Wagner, C., Anglim, P.P., Finnell, R.H. et al. (2010) HuR/methyl-HuR and AU-rich RNA binding factor 1 regulate the methionine adenosyltransferase expressed during liver proliferation, differentiation, and carcinogenesis. Gastroenterology **138**, 1943–1953

13 Doller, A., Pfeilschifter, J. and Eberhardt, W. (2008) Signalling pathways regulating nucleo-cytoplasmic shuttling of the mRNA-binding protein HuR. Cell. Signalling **20**, 2165–2173

14 Fabian, M.R., Mathonnet, G., Sundermeier, T., Mathys, H., Zipprich, J.T., Svitkin, Y.V., Rivas, F., Jinek, M., Wohlschlegel, J., Doudna, J.A. et al. (2009) Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol. Cell **35**, 868–880

15 Chen, C.Y., Zheng, D., Xia, Z. and Shyu, A.B. (2009) Ago-TNRC6 triggers microRNA-mediated decay by promoting two deadenylation steps. Nat. Struct. Mol. Biol. **16**, 1160–1166

16 Tong, A.W. and Nemunaitis, J. (2008) Modulation of miRNA activity in human cancer: a new paradigm for cancer gene therapy? Cancer Gene Ther. **15**, 341–355

17 Jiang, Q., Wang, Y., Hao, Y., Juan, L., Teng, M., Zhang, X., Li, M., Wang, G. and Liu, Y. (2009) miR2Disease: a manually curated database for microRNA deregulation in human disease. Nucleic Acids Res. **37**, D98–D104

18 Audic, Y. and Hartley, R.S. (2004) Post-transcriptional regulation in cancer. Biol. Cell **96**, 479–498

19 Simon, D., Laloo, B., Barillot, M., Barnetche, T., Blanchard, C., Rooryck, C., Marche, M., Burgelin, I., Coupry, I., Chassaing, N. et al. (2010) A mutation in the 3′-UTR of the *HDAC6* gene abolishing the post-transcriptional regulation mediated by hsa-miR-433 is linked to a new form of dominant X-linked chondrodysplasia. Hum. Mol. Genet. **19**, 2015–2027

20 Sohn, B.H., Park, I.Y., Lee, J.J., Yang, S.J., Jang, Y.J., Park, K.C., Kim, D.J., Lee, D.C., Sohn, H.A., Kim, T.W. et al. (2010) Functional switching of TGF-β1 signaling in liver cancer via epigenetic modulation of a single CpG site in TTP promoter. Gastroenterology **138**, 1898–1908

21 Sawicka, K., Bushell, M., Spriggs, K.A. and Willis, A.E. (2008) Polypyrimidine-tract-binding protein: a multifunctional RNA-binding protein. Biochem. Soc. Trans. **36**, 641–647

22 Imbeaud, S., Ladeiro, Y. and Zucman-Rossi, J. (2010) Identification of novel oncogenes and tumor suppressors in hepatocellular carcinoma. Semin. Liver Dis. **30**, 75–86

23 Sherman, M. (2005) Hepatocellular carcinoma: epidemiology, risk factors, and screening. Semin. Liver Dis. **25**, 143–154

24 Bosch, F.X., Ribes, J., Diaz, M. and Cleries, R. (2004) Primary liver cancer: worldwide incidence and trends. Gastroenterology **127**, S5–S16

25 Avila, M.A., Berasain, C., Sangro, B. and Prieto, J. (2006) New therapies for hepatocellular carcinoma. Oncogene **25**, 3866–3884

26 Boyault, S., Rickman, D.S., de Reynies, A., Balabaud, C., Rebouissou, S., Jeannot, E., Herault, A., Saric, J., Belghiti, J., Franco, D. et al. (2007) Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. Hepatology **45**, 42–52

27 Acevedo, L.G., Bieda, M., Green, R. and Farnham, P.J. (2008) Analysis of the mechanisms mediating tumor-specific changes in gene expression in human liver tumors. Cancer Res. **68**, 2641–2651

28 Minagawa, H., Honda, M., Miyazaki, K., Tabuse, Y., Teramoto, R., Yamashita, T., Nishino, R., Takatori, H., Ueda, T., Kamijo, K. and Kaneko, S. (2008) Comparative proteomic and transcriptomic profiling of the human hepatocellular carcinoma. Biochem. Biophys. Res. Commun. **366**, 186–192

29 Chen, C.Y., Ezzeddine, N. and Shyu, A.B. (2008) Messenger RNA half-life measurements in mammalian cells. Methods Enzymol. **448**, 335–357

30 Foster, D., Strong, R. and Morgan, W.W. (2001) A tetracycline-repressible transactivator approach suggests a shorter half-life for tyrosine hydroxylase mRNA. Brain Res. Protoc. **7**, 137–146

31 Fritz, D.T., Ford, L.P. and Wilusz, J. (2000) An *in vitro* assay to study regulated mRNA stability. Sci. STKE **2000**, pl1

32 Laloo, B., Simon, D., Veilla, V., Lauzel, D., Guyonnet-Duperat, V., Moreau-Gaudry, F., Sagliocco, F. and Grosset, C. (2009) Analysis of post-transcriptional regulations by a functional, integrated, and quantitative method. Mol. Cell. Proteomics **8**, 1777–1788

33 Geronimi, F., Richard, E., Lamrissi-Garcia, I., Lalanne, M., Ged, C., Redonnet-Vernhet, I., Moreau-Gaudry, F. and de Verneuil, H. (2003) Lentivirus-mediated gene transfer of uroporphyrinogen III synthase fully corrects the porphyric phenotype in human cells. J. Mol. Med. **81**, 310–320
34 Grosset, C., Boniface, R., Duchez, P., Solanilla, A., Cosson, B. and Ripoche, J. (2004) *In vivo* studies of translational repression mediated by the granulocyte-macrophage colony-stimulating factor AU-rich element. J. Biol. Chem. **279**, 13354–13362
35 Sampson, V.B., Rong, N.H., Han, J., Yang, Q., Aris, V., Soteropoulos, P., Petrelli, N.J., Dunn, S.P. and Krueger, L.J. (2007) MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. Cancer Res. **67**, 9762–9770
36 Brooks, S.A., Connolly, J.E., Diegel, R.J., Fava, R.A. and Rigby, W.F. (2002) Analysis of the function, expression, and subcellular distribution of human tristetraprolin. Arthritis Rheum. **46**, 1362–1370
37 Hitti, E., Al-Yahya, S., Al-Saif, M., Mohideen, P., Mahmoud, L., Polyak, S.J. and Khabar, K.S. (2010) A versatile ribosomal protein promoter-based reporter system for selective assessment of RNA stability and post-transcriptional control. RNA **16**, 1245–1255

Received 6 February 2010
doi:10.1042/BST0381608

ANNEXE 3: Molecular basis of differential target regulation by miR-96 and miR-182: the Glypican-3 as a model

1356–1365 Nucleic Acids Research, 2012, Vol. 40, No. 3 Published online 18 October 2011
doi:10.1093/nar/gkr843

Molecular basis of differential target regulation by miR-96 and miR-182: the Glypican-3 as a model

Sandra Jalvy-Delvaille[1,2], Marion Maurel[1,2], Vanessa Majo[1,2], Nathalie Pierre[1,3], Sandrine Chabas[1,3], Chantal Combe[1,2], Jean Rosenbaum[1,2], Francis Sagliocco[1,2] and Christophe F. Grosset[1,2,*]

[1]Université Bordeaux Segalen, [2]INSERM, U1053 and [3]INSERM, U869, Bordeaux, F-33076 Bordeaux, France

Received June 10, 2011; Revised September 20, 2011; Accepted September 21, 2011

ABSTRACT

Besides the fact that miR-96 and miR-182 belong to the miR-182/183 cluster, their seed region (UUGGCA, nucleotides 2–7) is identical suggesting potential common properties in mRNA target recognition and cellular functions. Here, we used the mRNA encoding Glypican-3, a heparan-sulfate proteoglycan, as a model target as its short 3′ untranslated region is predicted to contain one miR-96/182 site, and assessed whether it is post-transcriptionally regulated by these two microRNAs. We found that miR-96 downregulated GPC3 expression by targeting its mRNA 3′-untranslated region and interacting with the predicted site. This downregulatory effect was due to an increased mRNA degradation and depended on Argonaute-2. Despite its seed similarity with miR-96, miR-182 was unable to regulate GPC3. This differential regulation was confirmed on two other targets, FOXO1 and FN1. By site-directed mutagenesis, we demonstrated that the miRNA nucleotide 8, immediately downstream the UUGGCA seed, plays a critical role in target recognition by miR-96 and miR-182. Our data suggest that because of a base difference at miRNA position 8, these two microRNAs control a completely different set of genes and therefore are functionally independent.

INTRODUCTION

Post-transcriptional regulations are complex cellular mechanisms involving *cis*-acting RNA sequences located throughout the messenger RNA, and their associated *trans*-regulatory factors (1,2). Typical *cis*-acting RNA sequences are microRNA sites, the AU-rich element (ARE) or the major protein-coding region determinant of instability (1,3–5). AREs are mainly located in 3′-untranslated regions (UTR) and predicted to control 5–8% of cellular mRNAs (6). By comparison microRNA sites are particular in the sense that they are found in both untranslated and coding regions, and in many organisms, microRNAs (miRNAs) are predicted to exert their regulatory effects on at least 50% of cellular mRNAs (5,7).

MiRNAs are ~22-nt-long non-coding RNAs, originally discovered in worms and plants, which control expression of a plethora of genes involved in transient and adaptable cellular processes, such as induced proliferation, metabolism or stress. Mainly described as translation regulators in earlier studies (8), more recent reports show that some miRNAs downregulate protein output at the mRNA level by inducing mRNA decay (7,9–12). Many mammalian miRNAs are organized in miRNA families (13) or in gene clusters transcribed as a long polycistronic primary miRNA (e.g. miR182-183 cluster or miR-17-92 cluster) (14). Clustered miRNAs are under intense post-transcriptional controls which fine tune their cellular abundance and biological functions (15). Finally, although double-stranded once matured, miRNAs are loaded into an Argonaute (AGO) family protein as single-guide strand, this association forming the active miRNA-induced silencing complex (miRISC) (7,12).

At a molecular level, miRNAs control gene expression by annealing to their mRNA targets through perfect or imperfect matching following base-pairing rules (5,12). However this interaction depends on many other molecular features amongst which the miRNA-site neighborhood, proximity of poly(A) tail or of termination codon, proximal AU-richness, the number of miRNA sites, as well as protein *trans*-regulatory factors [Argonautes, GW182/Trinucleotide repeat-containing gene 6A protein (TNRC6) and their accessory proteins] (5,7,12,13). Target recognition is mainly based on conserved and continuous Watson–Crick pairing centered on miRNA positions 2–7, the so-called seed sequence (5,7). Sometimes seed pairing can tolerate wobble (i.e. Let-7a:MYC recognition) or requires an extra match at position 8 (5,9). In other cases,

*To whom correspondence should be addressed. Tel: +33 557 57 46 30; Fax: +33 556 51 40 77; Email: christophe.grosset@u-bordeaux2.fr

miRNAs can recognize their targets through contiguous base pairing to their central region (16). A match at miRNA position 1 seems not necessary for miRNA: target recognition, nor miRNA function. In fact the miRNA 5′-end nucleotide is hidden in AGO and not accessible for target recognition (5,7). Involvement of 3′-compensatory pairing in miRNA:target recognition is still a matter of debate as functional data failed to demonstrate its general requirement for miRNA function (5). Intriguingly some mammalian miRNAs have multiple isoforms (paralogues) with the same seed region but a variable remaining sequence. Based on this feature, those were classified in families and are predicted to target the same genes (5,13). However questions about the role of such a functional redundancy at the level of a cell or a whole organism mostly remain unanswered.

Here, we evaluated the regulatory potential of specific miRNAs selected by *in silico* approaches on the Glypican-3 (GPC3), a gene encoding a cell-membrane-embedded glycosyl-phosphatidylinositol-anchored extracellular glycoprotein which belongs to the heparan-sulfate proteoglycan family. GPC3 was chosen as model because of its involvement in various human pathologies (17,18). We found that miR-96 post-transcriptionally controls GPC3 whereas miR-182, a miRNA bearing a 2–7 seed region identical to that of miR-96, had no effect on GPC3 expression. Using the lentiviral- and fluorescent reporter-based method named FunREG method (9,10,19) and molecular approaches, we deciphered the differential mechanism governing the regulation of GPC3 by miR-96 and miR-182.

MATERIAL AND METHODS

Plasmid constructs

The pTRIP-eGFP plasmid has been described previously (9). pTRIP-eGFP-GPC3 was constructed as follows. The GPC3 3′-UTR was amplified with the primers 5′-CAGAC TCGAGCTGCCTGGTGCCCAGC-3′ and 5′-GAGAGG TACCCAAAGAAATCCATGCAAAGAG-3′ using normal liver cDNA. The PCR product was cloned into the pGEM-T plasmid (Promega) creating the pGEM-GPC3 plasmid and integrity of the insert was controlled by DNA sequencing. The pGEM-GPC3 plasmid was digested by XhoI and KpnI, and the resulting insert was gel purified and cloned into the pTRIP-eGFP. The plasmids pGEM-GPC3ΔA was constructed by PCR-site directed deletion using the pGEM-GPC3 plasmid as template, two primers spanning the sequence to delete (5′-CATATAGATTGTC CCCATCAAGTTGTGCC-3′ and 5′-GGCACAACTTG ATGGGGACAATCTATATGC-3′) as well as the primers spanning the GPC3 3′-UTR (see above). The final PCR product was cloned into the pTRIP-eGFP plasmid as described above. Similarly the pTRIP-eGFP-mutGPC3 and pTRIP-eGFP-G>U GPC3 plasmids were constructed by PCR-site directed mutagenesis using the pTRIP-eGFP-GPC3 plasmid as template. The miRNA site was mutated using either the primers 5′-CCATCAAGT TGTCCGATATTATTCTCCTATG-3′ and 5′-CATAGG AGAATAATATCGGACAACTTGATGG-3′ or the primers 5′-CCATCAAGTTTTGCCAAATTAT-3′ and 5′–ATAATTTGGCAAAACTTGATGG-3′. Each PCR product was digested by XhoI and KpnI, cloned into pTRIP-eGFP and DNA sequenced as described above.

Cultures of cell lines and primary hepatocytes, small RNA synthesis and transfection

The hepatocellular carcinoma (HCC)-derived HuH7 and SNU398 cell lines were grown in D-MEM medium (Invitrogen) containing 10% FCS and penicillin/streptomycin antibiotics. The Luciferase small interfering RNA (siLuc, sense 5′-CGUACGCGGAAUACUUCGA-3′) was from Eurofin MWG Operon. The miRNA mimics and hairpin inhibitors, as well as Argonaute protein siRNAs were from Thermo Scientific Dharmacon Products. When mentioned, artificial double-strand wild type or mutated miRNAs (bearing a RNA backbone) were chemically synthesized and purified (Supplementary Table S2). Then the corresponding strands were annealed. Small RNAs were transferred into the target cells by reverse-transfection using Lipofectamine RNAi Max (Invitrogen) following manufacturer's instructions at a final concentration of 12 nM.

Lentiviral production, titration and cell transduction

Production and titration of infectious lentiviral particles, as well as biosafety considerations, procedures and policies have been described previously (9). Lentiviral particles were added to the target cells and incubated for 24 h. Then the cells were washed twice in PBS and grown in the presence of medium for 6 days before experimental use.

FunREG analysis

Flow cytometry. One week after transduction, cells were washed in PBS, detached with trypsin/EDTA, collected and analyzed by FACS using a BD FACSCanto II (BD Biosciences, San Jose, CA, USA) and the BD FACSDiva software as described previously (9).

Real-time quantitative PCR and RT–PCR. Genomic DNA and total RNA were respectively isolated with the Nucleospin Tissue kit (Macherey-Nagel) and the TRI Reagent (Sigma) following manufacturer's instructions. Complementary DNA was synthesized with the AMV Reverse Transcriptase (Promega). Real-time quantitative PCR (QPCR) amplifications were performed in 12-µl multiplex PCR reactions containing 1× SYBR® Green Supermix (Quanta Biosciences). The primers used were as described previously (9). TaqMan microRNA assays (Applied Biosystems) were used to quantify the expression levels of mature miRNAs. The Albumin gene and 18s ribosomal RNA served as internal controls for normalization when using, respectively, genomic DNA or cDNA as template (9). Subsequent data analyses were performed using the Step One Plus Quantitative PCR System (Applied Biosystems).

Antibodies and western blot analyses

Whole cell extracts were prepared by treating cells with RIPA buffer (Sigma). Proteins were separated by 10% SDS–PAGE and blotted onto nitrocellulose membrane (Protran, Whatman). After blotting, total loaded proteins were quantified with SYPRO Ruby following manufacturer's instructions (Invitrogen). Stained membranes were imaged with the Molecular Imager PharosFX Plus System (Biorad) and proteins were analyzed with the Quantity One (Biorad) basic software. Then membranes were saturated in Odyssey Blocker and successively incubated with the indicated primary antibodies and adequate InfraRed-labeled secondary antibody (either IRDye-680 or -800 conjugated secondary antibodies) following manufacturer's instructions. Fluorescence signals were detected and quantified using the Odyssey infrared imaging system. Blocker and Odyssey infrared imaging system were from LI-COR Biosciences (ScienceTec, Les Ulis, France). Specific protein staining was normalized to the quantity of total proteins. Anti-GPC3 was from Biomosaics. Anti-FOXO1 (C-20) was from Santa Cruz Biotechnology, anti-FN1 was from BD Biosciences and anti-ADCY6 (SAB2100054) was from Sigma.

Statistical analyses

All analyses were done using GraphPad Prism 5.0 software. Data are represented as mean with standard deviation (SD) from the indicated number of independent experiments. When experiment contained three groups of values or more, regular one-way analysis of variance (ANOVA) was used for the comparison of multiple means. Means were considered significantly different if the $P < 0.05$. NS means 'not significant'. The ANOVA test was followed by a Bonferroni's multiple-comparison post-test and selected pairs of data were compared. Significant variations were represented by asterisks above the corresponding bar when comparing the test with the control condition or above the line when comparing the two indicated conditions.

RESULTS

miR-96, but not miR-182, controls Glypican-3 expression

To find miRNAs involved in GPC3 post-transcriptional regulation, we submitted the GPC3 mRNA to 10 established prediction programs gathered on miRWalk [miRWalk—A Database on Predicted and Validated microRNA Targets, (http://mirwalk.uni-hd.de)] and selected those targeting the 3′-UTR. As shown in Figure 1A, miR-96 appeared in top position with 6 positive predictions over 10. MiR-182, which displays the same 5′-UUGGCA-3′ seed sequence to positions 2–7 (Figure 1B) than miR-96, appeared just below miR-96 among the four top-ranked miRNAs with 5 positive predictions over 10 (Figure 1A). By individually testing different programs, miR-96 and miR-182 were predicted to target GPC3 using Targetscan (Figure 1B and Supplementary Figure S1A) (13), Diana microT v.4 (Supplementary Figure S1B) (20), miRanda/mirSVR (Supplementary Figure S1C) (21) and Pictar (Supplementary Figure S1E) (22), whereas only miR-96 was found amongst the miRNAs predicted using PITA (23) and miRDB (24,25) (Supplementary Figure S1D and F, respectively). The unique 5′-UGCCAA-3′ miRNA site located in the GPC3 3′-UTR and predicted to pair with the seed of miR-96 or miR-182 is very conserved amongst species (Supplementary Figure S2A) suggesting an apparent evolutionary-conserved partnership between GPC3 and these two miRNAs. *In silico*, miR-96:GPC3 3′-UTR pairing is defined as an 8-mer site by Targetscan, whereas miR-182:GPC3 3′-UTR interaction is defined as 7-mer-1A (13). The only difference between these two types of sites resides in the fact that in miR-96, the nucleotide at position 8 immediately downstream the seed can pair the GPC3 3′-UTR (G–C pair), whereas that of miR-182 cannot (Figure 1B). Finally miR-96 and miR-182, together with miR-183, belong to the same intergenic miRNA cluster (miR-182-183, miRBase (26)).

Based on these observations, experimental analyses were undertaken. Expression of miR-96 and miR-182 in HuH7 and SNU398 cells, two human HCC-derived GPC3-expressing cell lines, was first confirmed by RT-qPCR (Supplementary Figure S2B). Results showed that miR-182 was apparently more abundant in both HCC cell lines than miR-96 (compare ΔCt values in Supplementary Figure S2B) suggesting the existence of post-transcriptional regulations in the course of miR-182-183 cluster biogenesis. Then we tested the ability of the two miRNAs to control GPC3 expression in HCC-derived HuH7 cells by over-expressing each miRNA using cell transfection. As shown in Figure 1C, miR-96 significantly decreased GPC3-protein amount by more than half. As expected, a specific hairpin inhibitor, AM96, counterbalanced the negative effect induced by miR-96. However when used alone, AM96 slightly, but significantly, increased GPC3-protein expression suggesting that it efficiently interacted with the endogenous miR-96 and inhibited its function. Comparable results were obtained when monitoring GPC3-transcript amounts (Figure 1D) suggesting that miR-96 downregulated GPC3 expression at the mRNA level. Surprisingly, although miR-96 and miR-182 carry the same seed (Figure 1B), miR-182 had no effect on GPC3 protein expression, nor mRNA level (Figure 1C and D). This absence of effect could not originate from a poor cell-transfection efficiency as miR-182 overexpression was observed in the corresponding transfected HuH7 cells (Supplementary Figure S2C). To assess miR-182 functioning, we studied its regulatory effect on Adenylate cyclase type 6 (ADCY6), one of its validated target (27). Noticeably ADCY6 3′-UTR is predicted to contain one 8-mer site for miR-182 and three 8-mer sites for miR-96 (Figure 4A). As shown in Supplementary Figure S2D, both miR-96 and miR-182 downregulated expression of ADCY6 mRNA and protein, despite the fact positions of their predicted sites into the ADCY6 3′-UTR remain to be confirmed. Together these results showed that (i) miR-96 specifically controls GPC3 expression at the mRNA level, and (ii) miR-182 is functionally inefficient on GPC3.

A

Gene Name	MicroRNA	StemLoop ID	DIANAmT	miRanda	miRDB	miRWalk	PICTAR4	PICTAR5	PITA	RNA22	RNAhybrid	Targetscan	SUM
GPC3	hsa-miR-96	hsa-mir-96	1	1	1	0	1	0	1	0	0	1	6
GPC3	hsa-miR-182	hsa-mir-182	1	1	0	0	1	0	1	0	0	1	5
GPC3	hsa-miR-450b-5p	hsa-mir-450b	1	1	1	0	0	0	1	0	0	1	5
GPC3	hsa-miR-570	hsa-mir-570	1	1	1	0	0	0	1	0	0	1	5
GPC3	hsa-miR-518a-5p	hsa-mir-518a-2	1	1	0	0	0	0	1	0	0	1	4
GPC3	hsa-miR-7	hsa-mir-7-3	1	1	0	0	0	0	1	0	0	1	4
GPC3	hsa-miR-765	hsa-mir-765	1	1	0	0	0	0	1	0	0	1	4
GPC3	hsa-miR-140-5p	hsa-mir-140	1	1	0	0	0	0	1	0	0	1	4
GPC3	hsa-miR-140-3p	hsa-mir-140	1	1	0	0	0	0	1	0	0	1	4
GPC3	hsa-miR-503	hsa-mir-503	1	1	0	0	0	0	1	0	0	1	4

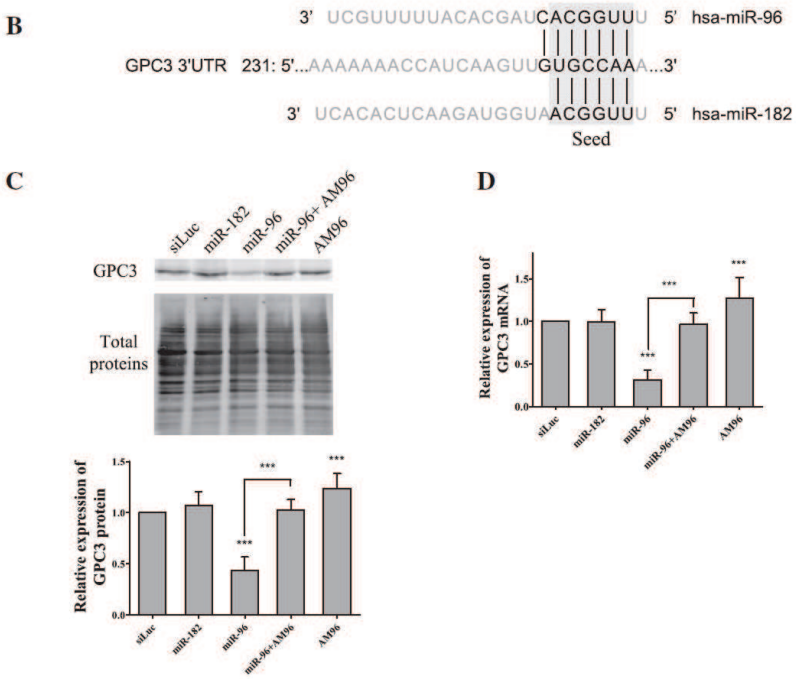


Figure 1. GPC3 is a target of miR-96, but not of miR-182. (**A**) The 10 highest-ranking miRNA:GPC3-3′-UTR pairings as predicted by miRWalk using the indicated algorithms (1/grey: pairing predicted; 0/empty: pairing not predicted). (**B**) Schematic representation of the pairing of GPC3 3′-UTR with miR-96 or miR-182 as predicted by TargetScan. In this figure and the following ones, base pairings between indicated mRNAs and miRNAs are represented by vertical lines. (**C** and **D**): HuH7 cells were transfected with small RNAs as indicated. Three days later, the amounts of GPC3 protein (C) and mRNA (D) were quantified (ANOVA: $P < 0.0001$; $n = 6$). In Panel C, a representative western blot experiment is shown on top and a bar graph recapitulating means with standard deviation (SD) of six experiments is shown on bottom. In this figure and the following ones, bars represent means, error bars indicate SD and the ANOVA test was followed by a Bonferroni's multiple comparison post-test. $^{***}P < 0.001$.

miR-96 post-transcriptionally controls GPC3 expression through the 3′-UTR of its transcript

To assess the regulatory role of miR-96 on GPC3 and identify the molecular process, we followed the FunREG experimental pipeline (9,19). Infectious lentiviral particles were used to deliver an eGFP-reporter transgene bearing the GPC3 3′-UTR (Figure 2A) into HuH7 cells (and SNU398 cells, Supplementary Figure S3A). A multiplicity of infection (moi) of 0.5 was used to generate a cell population with no more than one transgene copy per cell (9). One week later the average number of lentiviral transgene copies per cell ('transgene copy number', TCN) was assessed by quantitative PCR. Then cells expressing the eGFP-GPC3 transgene were transfected with the above-mentioned small RNAs and 3 days later, the eGFP protein (P) and mRNA (M) expressions were determined by FACS and RT-qPCR, respectively. Finally the three ratios (P/TCN, M/TCN and P/M) were calculated (9). With a fluorescent reporter transgene bearing the GPC3 3′-UTR, results obtained with the FunREG method fully recapitulated the ones obtained with endogenous GPC3. Indeed expression of the eGFP-GPC3 transgene significantly decreased by 47% in HuH7 cells transfected with miR-96 (Figure 2B). A similar but less profound effect was observed with miR-96 when using another HCC cell line (SNU398 cells, Supplementary Figure S3A). This difference could be due to the fact that SNU398 cells are generally more refractory to cell transfection than HuH7 cells. Whereas the specific AM96 neutralized miR-96 effect, it led to a slight but significant increase in eGFP expression when used alone (Figure 2B). All these results demonstrated that in HuH7 cells, the GPC3 3′-UTR is targeted by

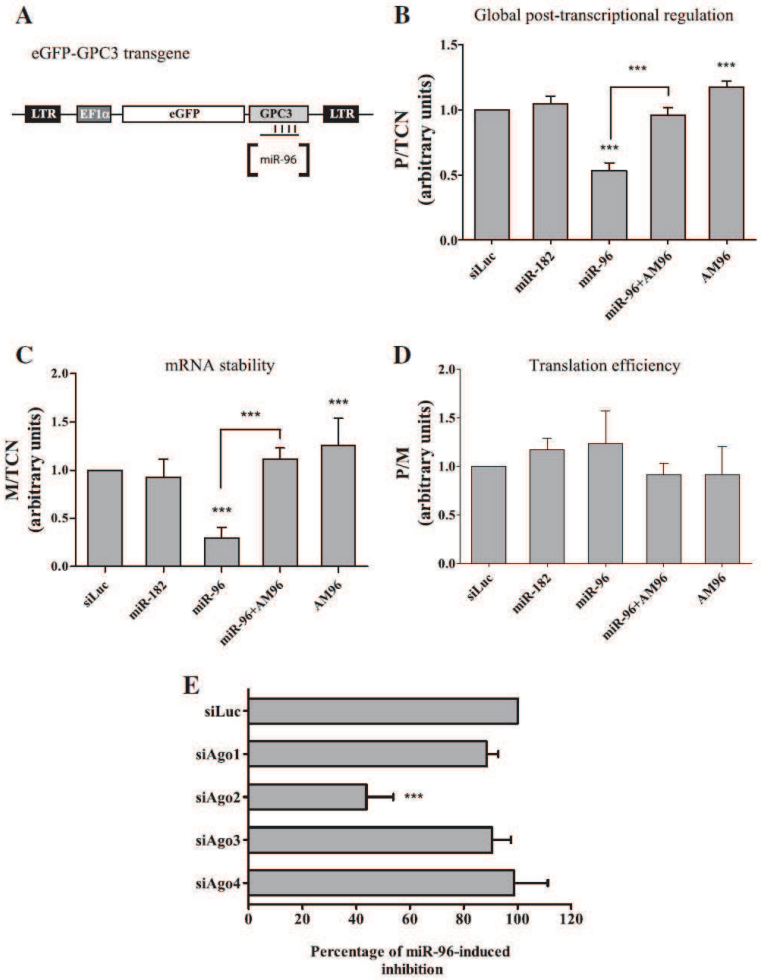


Figure 2. Molecular basis of the post-transcriptional regulation mediated by miR-96 on GPC3. (**A**) Schematic representation of the eGFP-GPC3 transgene with the 3′-UTR being targeted by miR-96. (**B–D**) HuH7 cells were transduced once with lentiviral particles expressing the transgene. After one week, the TCN was calculated using genomic DNA extracted from the eGFP-GPC3-expressing HuH7 cell population. Then cells were transfected with the indicated small RNAs. Three days later, the eGFP protein expression and mRNA amount were measured and data were analyzed following the FunREG experimental pipeline (9). (B) Global post-transcriptional regulation (ANOVA: $P < 0.0001$; $n = 6$). (C) mRNA stability (ANOVA: $P < 0.0001$; $n = 6$). (**D**) Translation efficiency (ANOVA: P = NS; $n = 6$). Panel (**E**): EGFP-GPC3-expressing HuH7 cells were first transfected with the indicated siRNA. Twenty-four hours later, cells were transfected with miR-96. Forty-eight hours later, eGFP protein expression was analyzed by FACS and data normalized to that of siLuc (ANOVA: $P < 0.0001$; $n = 3$). ***$P < 0.001$.

miR-96 in either its mimic or endogenous form. Moreover M/TCN (Figure 2C) and P/M (Figure 2D) ratios, which are respectively indicative of mRNA stability and translation efficiency (9), showed that miR-96 induced destabilisation of the chimeric eGFP-GPC3 mRNA with minor apparent effect on its translation. Finally as AM96 suppressed miR-96-induced mRNA decay, it led to a significant increase in eGFP-GPC3 mRNA stability. In the same conditions, miR-182 had no effect on eGFP-GPC3 expression in either HuH7 or SNU398 cells (Figure 2B–D, Supplementary Figure S3A). To further substantiate our findings, eGFP-GPC3-expressing HuH7 cells were transfected with increasing amounts of miR-96 (0–16 nM). As shown in the Supplementary Figure S3B, the downregulation mediated by miR-96 on eGFP-GPC3 expression was dose-dependent and reached its maximal effect when using miR-96 at a concentration of 12 nM or more.

As miRNA-induced effect depends on miRISC and its core protein Argonaute (Ago), we looked for the isoform(s) of this protein that was involved in the regulation of GPC3 by miR-96. We reasoned that the negative effect of miR-96 could not take place in absence of the corresponding Ago protein. We therefore pre-depleted

HuH7 cells of each Argonaute isoform with specific siRNAs (Supplementary Figure S3C–F). Then depleted cells were transfected with miR-96. Results in Figure 2E clearly showed that miR-96 mainly required Argonaute 2, as its inhibitory effect (shown at its maximum in the siLuc control condition) was decreased by 60% in absence of Ago2. It should be noted that depletion of Ago2 in HuH7 cells was compensated by an increase of Ago1 and Ago3 mRNAs (Supplementary Figure S3D). Altogether these results showed that miR-96 negatively controls GPC3 expression at a post-transcriptional level by targeting the 3′-UTR of its transcript and by inducing its degradation by an Ago-2-dependent mechanism.

Molecular basis of miR-96-target recognition

A set of experiments was performed to decipher the mechanism of miR-96-target recognition. First we confirmed the predicted miR-96 site using eGFP transgenes bearing mutated GPC3 3′-UTRs (Figure 3A). The functional consequences of these mutations were studied by FunREG, as described above, using HuH7 cells expressing the corresponding transgene (Figure 3A) and transfected with siLuc, miR-96 or miR-182 (Figure 3B). Results in terms of global post-transcriptional regulation were compared with those obtained with the wild-type 3′-UTR (Figure 3B, 'wt GPC3' transgene). As shown, three-point mutations (r.250G>C, r.252C>G and r.254A>U) inserted at the positions predicted to pair with miR-96 seed ('mut GPC3' transgene, Figure 3B) fully abrogated miR-96 effect validating the bioinformatic predictions. As miR-96 displays a uridine (U)-rich stretch in its 3′-end (5 consecutive Us at positions 16–20) and the GPC3 3′-UTR contains an adenosine (A)-rich sequence just upstream miR-96 site (Figure 3A), we assessed whether this potential A/U pairing could play a role in miR-96:GPC3 mRNA interaction. As shown in Figure 3B, absence of the A-rich sequence ('ΔA GPC3' transgene) did not hamper the targeting of GPC3 3′-UTR by miR-96. Similar results were obtained with miR-1271, the paralog of miR-96, and the different transgenes presented in Figure 3A (data not shown). Together these results suggested that the seed is important in GPC3 mRNA recognition by miR-96 and that a 3′-end A/U pairing mechanism does not seem necessary to make this miRNA functional (5). Based on these evidences, we tried to explain why miR-182 was unable to control GPC3 expression. Contrarily to miR-182, miR-96 pairs the GPC3 3′-UTR through its seed incremented of nucleotide 8 (a cytidine, C; Figure 1B). Therefore we hypothesized that pairing of the target with the seed + nucleotide 8 might be a prerequisite to yield a stable miRNA:mRNA complex. Consequently the guanosine (G) at position 248 of GPC3 3′-UTR, which normally matches a C at position 8 of miR-96, was mutated in U ('G>U GPC3' transgene, Figure 3A). Results in Figure 3B ('G>U GPC3' transgene) showed that as expected, the r.248G>U mutation abrogated the regulation of eGFP-GPC3 by miR-96. In addition it allowed miR-182 to target the GPC3 3′-UTR and control eGFP-GPC3 expression. To confirm these findings, we synthesised wild type and mutated

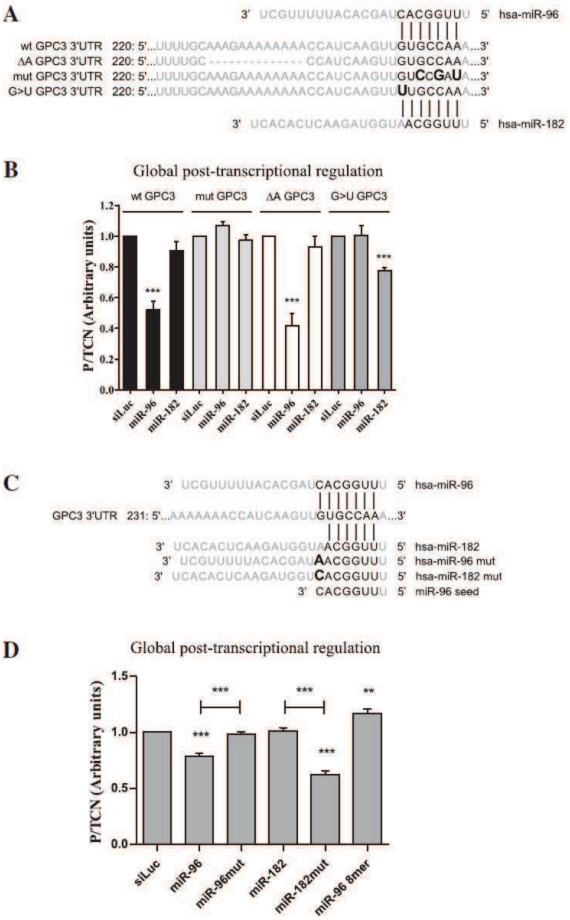


Figure 3. Base requirements for miR-96:GPC3 3′-UTR recognition and miR-96 function. (**A**) Schematic representation of miR-96 or miR-182 pairing with the GPC3 3′-UTR sequence in its wild-type, deleted or mutated versions. (**B**) HuH7 cells expressing the indicated transgene (shown above the bars; TCN value measured) were transfected with the indicated small RNAs. Three days later, eGFP protein expression was analyzed by FACS as described in Figure 2B (ANOVA: $P < 0.0001$; $n = 3$). (**C**) Schematic representation of the wild type GPC3 3′-UTR sequence and the various synthetic double-strand small RNAs used (only the guide strand is shown). (**D**) eGFP-GPC3-expressing HuH7 cells were transfected with the indicated small RNAs. Three days later, eGFP protein expression was analyzed as described in panel B (ANOVA: $P < 0.0001$; $n = 5$). $^{**}P < 0.01$; $^{***}P < 0.001$.

double-stranded miRNAs (Figure 3C and Supplementary Table S2). miRNAs were mutated as follows: a C>A mutation was created at position 8 of miR-96 to specifically mimic the seed sequence plus nucleotide 8 of miR-182. Conversely, a A>C mutation was created at position 8 of miR-182 to mimic the seed + position 8 of miR-96 (Figure 3C). We then tested the efficiency of these miRNAs to control expression of the eGFP-GPC3 transgene in HuH7 cells. As expected, miR-96 downregulated the expression of the eGFP-GPC3 transgene in HuH7 cells (Figure 3D) whereas its

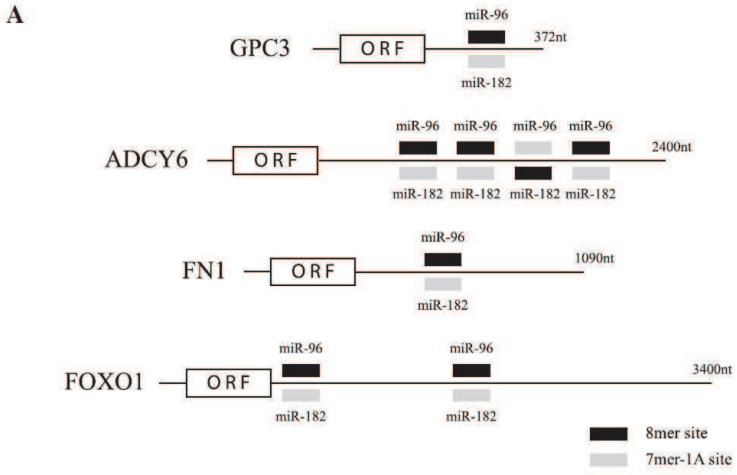
mutated counterpart (miR-96mut) was inefficient. It should be noted however that the synthetic miR-96 that we produced was less efficient than its commercial mimic form. Although miR-182 had still no effect, its mutated counterpart (miR-182mut) was fully functional and decreased eGFP expression by 47%. Because of the central role of the first 8 nt of miR-96 in its functioning, we also tested the impact of transfecting cells with the 8-mer oligonucleotide ('miR-96 8-mer', Figure 3C) on eGFP-GPC3 expression. Unexpectedly miR-96 8-mer did not reduce, but rather slightly increased eGFP-GPC3 expression (Figure 3D). This increase was similar to that obtained with AM96 in Figure 2B suggesting that the 8-mer nucleotide cannot recapitulate miR-96 activity, but rather acts as a specific competitive inhibitor.

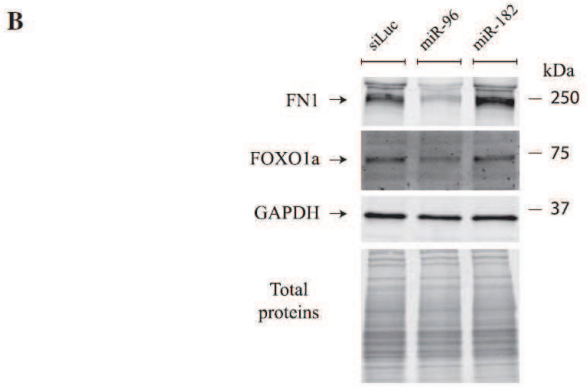
Finally we evaluated whether the differential regulation of GPC3 expression observed with miR-96 and miR-182 was transposable to other genes. Forkhead box protein O1 (FOXO1) was first chosen as a model, as its 3′-UTR was predicted to contain two miR-96/182 sites (two 8-mer sites for miR-96 and consequently, two 7-mer-1A sites for miR-182, see Figure 4A) (13). Moreover FOXO1 was described as a target of miR-96 and miR-182 (28–30). As expected from our data using GPC3 as target, transfection of HuH7 cells with miR-96 led to a slight decrease of FOXO1 protein, whereas transfection with miR-182 had no effect (Figure 4B). The differential effect of these two miRNAs on FOXO1 expression was confirmed at the mRNA level as only miR-96 significantly decreased FOXO1 mRNA expression (Supplementary Figure S4). To further support these results, we assessed our findings using a second predicted miR-96/182 target. Fibronectin-1 (FN1) was chosen as it is predicted to contain one 8-mer site for miR-96 and consequently one 7-mer-1A sites for miR-182 (Figure 4A) (13). As shown in Figure 4B, miR-96 very efficiently down-regulated FN1 expression whereas miR-182 was still non-functional. Altogether these results demonstrated that despite sharing similarities in their seeding region, miR-96 and miR-182 do not control the same genes. The differential regulation depicted by these two miRNAs depends on the presence of a site containing a seed match augmented by a match at position 8 in targeted 3′-UTRs.

DISCUSSION

Here, we reported that GPC3 is post-transcriptionally regulated by miR-96. We also confirmed the regulation of ADCY6 and FOXO1 by this microRNA and reported for the first time the downregulation of FN1 by miR-96. Using FunREG, we showed that miR-96 induces GPC3 mRNA degradation by targeting its 3′-UTR through a mechanism requiring the RISC-core protein Ago2 (Figure 2). Similar results were obtained with miR-1271 (31), a miR-96-paralog which bears the same seed + 1 region but a different base at position 1 (data not shown). The mRNA destabilizing effect mediated by miR-96 is in agreement with current findings showing that some miRNAs induce mRNA decay rather than repressing translation (7,9–11,31,32). Using silencing of individual Ago proteins, we found that miR-96 effect depended exclusively on Ago2 but not on other Ago proteins. As all four Ago proteins can mediate miRNA repression and bind to a nearly similar set of mRNAs (33,34), it is not clear why the down-regulation of GPC3 by miR-96 specifically requires Ago2. However, a specific requirement for Ago2 in miRNA-mediated gene repression has already been reported for Let7-a (35). It is possible that during miRNA biogenesis, factors associating with the pri- and/or the pre-RNAs direct the mature miR-96 to the Ago2-associated RISC. Alternatively, the cellular concentration of each individual Ago protein, their competition for the mRNAs or their post-translational status might determine the sorting of miR-96 to Ago2 (12,32).

As predicted by several bioinformatic programs, miR-96 recognized a single 8-mer site located at positions 248–254 downstream the stop codon of the GPC3 3′-UTR (370 nt long) (Figure 3). Intriguingly we found that miR-182 was inefficient on GPC3, despite the presence of one 7-mer-1A site in its 3′-UTR (Figure 4A) and the fact the seed borne by miR-182 was identical to that of miR-96 (Figure 1B). However miR-182 functioned on its validated target ADCY6 (Supplementary Figure S2D), the 3′-UTR of which contains an 8-mer site for miR-182 (Figure 4A) (27). It should be reminded that an 8-mer site for miR-182 constitutes a 7-mer-1A site for miR-96 and reciprocally. By studying in more details these paradoxical findings, we demonstrated that miR-182 functioning on GPC3 depended on an extra nucleotide match associating its nucleotide 8 and the mRNA target. Indeed by either introducing the mutation r.248G>U in GPC3 3′-UTR (Figure 3A) or changing the A>C at miRNA position 8 (Figure 3C), miR-182 became functional on GPC3 (Figure 3C and D). Conversely the mutation r.248G>U in GPC3 3′-UTR or r.8C>A in miR-96 abrogated the regulatory effect of this miRNA on GPC3. We therefore concluded that the 'seed-dependent target recognition' hypothesis *per se* is not applicable to miR-96 and miR-182 sites (5). Indeed target recognition by miR-96/182 5′-UUGGCA-3′ seed is not sufficient to make the miRNA functional *in cellulo* and this required an additional nucleotide match at position 8 in order to generate a stable miRNA:target complex. This assumption was supported by (i) the differential effects of miR-96 and miR-182 on FOXO1, FN1 and GPC3, and (ii) the regulatory effect of miR-96 and miR-182 on ADCY6 (27). Indeed both miRNAs recognized their targets only when they contained at least one 8-mer site in their 3′-UTR (seed + 1 match with the target, Figure 4A). Our findings are also in agreement with the regulatory role of miR-182 on Cortactin and on Regulator of G-protein signaling 17 (RGS17), two genes which contain two miR-182 8-mer sites in their 3′-UTR (13) and which are regulated by miR-182 at the mRNA level (36,37). However the work reported by Moskwa *et al.* (38) suggests that the 'seed+1-matched site recognition' model is not a general rule for miR-182. Moreover our data apparently disagree with other reports that suggested a regulation of FOXO1 by miR-182 (28–30). But the demonstration was partly based on the mutation of the





C

Gene Name	MicroRNA	StemLoop ID	DIANAmT	miRanda	miRDB	miRWalk	PICTAR4	PICTAR5	PITA	RNA22	RNAhybrid	Targetscan	SUM
GPC3	hsa-miR-96	hsa-mir-96	1	1	1	0	1	0	1	0	0	1	6
GPC3	hsa-miR-182	hsa-mir-182	1	1	0	0	1	0	1	0	0	1	5

Gene Name	MicroRNA	StemLoop ID	DIANAmT	miRanda	miRDB	miRWalk	PICTAR4	PICTAR5	PITA	RNA22	RNAhybrid	Targetscan	SUM
FOXO1	hsa-miR-96	hsa-mir-96	1	1	1	1	1	1	1	0	0	1	8
FOXO1	hsa-miR-182	hsa-mir-182	1	1	0	1	1	1	1	0	0	1	7

Gene Name	MicroRNA	StemLoop ID	DIANAmT	miRanda	miRDB	miRWalk	PICTAR4	PICTAR5	PITA	RNA22	RNAhybrid	Targetscan	SUM
ADCY6	hsa-miR-96	hsa-mir-96	1	1	1	1	1	0	1	1	0	1	8
ADCY6	hsa-miR-182	hsa-mir-182	1	1	0	1	1	0	1	1	0	1	7

Gene Name	MicroRNA	StemLoop ID	DIANAmT	miRanda	miRDB	miRWalk	PICTAR4	PICTAR5	PITA	RNA22	RNAhybrid	Targetscan	SUM
FN1	hsa-miR-96	hsa-mir-96	1	1	1	1	0	0	1	1	0	1	7
FN1	hsa-miR-182	hsa-mir-182	1	1	0	1	0	0	1	0	1	1	6

Figure 4. Target preference of miR-96 and miR-182 depends on presence of a 8-mer site. (**A**) Schematic representation of the indicated mRNAs with their miR-96/182 sites. The type of site as defined by Targetscan is as indicated (see the legend at the bottom-right). (**B**) HuH7 cells were transfected with small RNAs as indicated. Three days later, the amounts of FN1, FOXO1 and GAPDH proteins were measured. Representative of three independent experiments. (C) miRNA:target 3′-UTR pairings as predicted by miRWalk using the indicated programs.

miR-182 site in the region matching the seed (and not the nucleotide matching the miRNA position 8) (29,30), therefore leading to a loss of target recognition not only by miR-182, but also by miR-96 and its paralog, miR-1271. In addition, the use of different experimental conditions or cell types may also affect the results. Indeed, miR-182 site accessibility in FOXO1 mRNA may be different from a cell type to another depending on specific 3′-UTR secondary structures, 3′-UTR contexts, associated RNA-binding proteins or site multiplicity which are all determinants influencing miRNA activity (13,39). Interestingly a small RNA corresponding to the first 8 nt of miR-96 functionally acted as a competitive inhibitor of the endogenous miR-96/RISC complex (Figure 3D). We therefore concluded that an 8-mer RNA is sufficient to recognize its intracellular target but is unable to support miRISC loading, probably because the miRNA 3′-end region is lacking. These data are consistent with a recent report

showing that small locked nucleic acids (LNA) targeting miRNA seed can abrogate miRNA function (40). Therefore any interference in miRNA:target recognition by using either seed complementary or 8-mer mimic (comprising the seed flanked by a base at positions 1 and 8) LNAs should specifically block miRNA action, a property which could lead to therapeutic effects (40). At last we showed that miR-96:target pairing does not require a 3′-compensatory mechanism. Indeed both miR-96 and miR-182mut (as well as miR-1271, data not shown) controlled GPC3 expression, although the remaining sequences downstream nucleotide 8 are completely different (Figure 3). Together these observations are in accordance with the proposed 'seed nucleation' model where the miRNA 3′-end is necessary for the biological function, but does not participate in target recognition (5). Therefore such a 3′-compensatory mechanism seems not to be required in target recognition by miR-96 nor miR-182. Because of the central role played by the miRNA seed+1 region in our model, we used the RNAhybrid software to estimate the strength of these miRNA-seed:target hybridisations (41). The analyses showed that the free energy hybridisations of miR-96:GPC3 (nucleotides 2–8) and miR-182:GPC3 (nucleotides 2–7) complexes were −14.8 and −12.8 kCal/mol, respectively. In the same condition, the free energy hybridisation of the miR-182mut:GPC3 (nucleotides 2–8) complex was −13.7 kCal/mol. Therefore in our experimental conditions, a minimum free energy of at least −13.7 kCal/mol is apparently required to make these miRNAs functional. Together our data demonstrated that the nucleotide 8 of miR-96 and miR-182 is as critical as nucleotides 2–7 for target recognition and miRNA:mRNA complex stabilization. Our results are in accordance with other reports showing that introducing single nucleotide changes in the target site complementary to nucleotides 2–8 of *Human* miR-96/1271 or *Drosophila* miR-7 abrogates gene regulation (31,42). Therefore although dissimilar (Figure 1B), the seeds of miR-96 and miR-182 comprise nucleotides 2–8 (31,32,43) rather than 2–7 (5) and their matching site can be classified as 5′ dominant seed site (42) or 8-mer site (5). However we cannot exclude the possibility that these miRNA could act differently under specific circumstances as previously reported for miR-182 (38).

By taking into account all these findings, we can speculate that the sets of cellular genes regulated by miR-96 and by miR-182 are profoundly different with the exception of those carrying at least one 8-mer site for both miRNAs (e.g. ADCY6, Figure 4A). Moreover it can be proposed that any C>A variations at position 8 of miR-96 (or conversely A>C change in miR-182) or any G>U changes in the target at the corresponding matching position might lead to a gene reprogramming with deep cellular consequences. Indeed such miRNA modifications might take place in physiological conditions (i.e. edition (12)) and play a role in cell plasticity especially during organism development or cell differentiation. They may also be linked to evolutionary mechanisms such as single nucleotide polymorphisms or to post-transcriptional RNA modifications. Finally they may also be linked to human illness since several single nucleotide mutations involving miRNA:target recognition have been reported lately, some involving miR-96 or miR-433 (10,44,45). With the advent of genomic deep sequencing programs and the recent discovery of gene deregulations associated with 3′-UTR mutations, maybe some of these questions will find answers.

Our data also point to the lack of accuracy of the bioinformatic prediction tools gathered on miRWalk (http://mirwalk.uni-hd.de). Indeed none of the algorithms in Figure 4C were in complete accordance with our functional data: the regulatory effect of miR-96 on GPC3, ADCY6, FOXO1 and FN1, and that of miR-182 on ADCY6. It should be specified however that miRDB (24,25) gave the best prognostication as it efficiently predicted a regulation of GPC3, FN1 and FOXO1 by miR-96, and not by miR-182. However it missed the regulation of ADCY6 by miR-182. Because the number of cell types used in our work was limited, we cannot pretend that our results reflect a general mechanism. However it is clear that room remains for bioinformatic tool improvement and that functional studies, associated with new valuable experimental and screening tools, should greatly contribute to their enhancement.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2, Supplementary Figures 1–4, Supplementary References (13,20–25).

ACKNOWLEDGEMENTS

Hélène Jacquemin-Sablon, Violaine Moreau, Frédéric Saltel, Patrick Lestienne and Eric Chevet are warmly acknowledged for their critical comments of the manuscript.

FUNDING

This work was supported by the Agence Nationale pour la Recherche (ANR)—Programme Jeunes Chercheurs (Paris, France) (Grant no: JC07_184264 to C.G.) and by La Ligue Nationale Contre le Cancer. M.M. and V.M. were recipients of fellowships from the Ministère de l'Enseignement Supérieur et de la Recherche (MESR). Funding for open access charge: INSERM.

Conflict of interest statement. None declared.

REFERENCES

1. Barreau,C., Paillard,L. and Osborne,H.B. (2005) AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res.*, **33**, 7138–7150.
2. Garneau,N.L., Wilusz,J. and Wilusz,C.J. (2007) The highways and byways of mRNA decay. *Nat. Rev. Mol. Cell Biol.*, **8**, 113–126.
3. Grosset,C., Chen,C.Y., Xu,N., Sonenberg,N., Jacquemin-Sablon,H. and Shyu,A.B. (2000) A mechanism for translationally coupled mRNA turnover: interaction between the poly(A) tail and a c-fos RNA coding determinant via a protein complex. *Cell*, **103**, 29–40.

4. Grosset,C., Boniface,R., Duchez,P., Solanilla,A., Cosson,B. and Ripoche,J. (2004) In vivo studies of translational repression mediated by the granulocyte-macrophage colony-stimulating factor AU-rich element. *J. Biol. Chem.*, **279**, 13354–13362.
5. Bartel,D.P. (2009) MicroRNAs: target recognition and regulatory functions. *Cell*, **136**, 215–233.
6. Bakheet,T., Williams,B.R. and Khabar,K.S. (2006) ARED 3.0: the large and diverse AU-rich transcriptome. *Nucleic Acids Res.*, **34**, D111–D114.
7. Huntzinger,E. and Izaurralde,E. (2011) Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat. Rev. Genet.*, **12**, 99–110.
8. Bartel,D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281–297.
9. Laloo,B., Simon,D., Veillat,V., Lauzel,D., Guyonnet-Duperat,V., Moreau-Gaudry,F., Sagliocco,F. and Grosset,C. (2009) Analysis of post-transcriptional regulations by a functional, integrated, and quantitative method. *Mol. Cell Proteomics*, **8**, 1777–1788.
10. Simon,D., Laloo,B., Barillot,M., Barnetche,T., Blanchard,C., Rooryck,C., Marche,M., Burgelin,I., Coupry,I., Chassaing,N. *et al.* (2010) A mutation in the 3′-UTR of the HDAC6 gene abolishing the post-transcriptional regulation mediated by hsa-miR-433 is linked to a new form of dominant X-linked chondrodysplasia. *Hum Mol Genet.*, **19**, 2015–2027.
11. Guo,H., Ingolia,N.T., Weissman,J.S. and Bartel,D.P. (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*, **466**, 835–840.
12. Krol,J., Loedige,I. and Filipowicz,W. (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nat. Rev. Genet.*, **11**, 597–610.
13. Grimson,A., Farh,K.K., Johnston,W.K., Garrett-Engele,P., Lim,L.P. and Bartel,D.P. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol. Cell*, **27**, 91–105.
14. Kim,V.N., Han,J. and Siomi,M.C. (2009) Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell Biol.*, **10**, 126–139.
15. Slezak-Prochazka,I., Durmus,S., Kroesen,B.J. and van den Berg,A. (2010) MicroRNAs, macrocontrol: regulation of miRNA processing. *RNA*, **16**, 1087–1095.
16. Shin,C., Nam,J.W., Farh,K.K., Chiang,H.R., Shkumatava,A. and Bartel,D.P. (2010) Expanding the microRNA targeting code: functional sites with centered pairing. *Mol. Cell*, **38**, 789–802.
17. Filmus,J. and Capurro,M. (2008) The role of glypican-3 in the regulation of body size and cancer. *Cell Cycle*, **7**, 2787–2790.
18. Jakubovic,B.D. and Jothy,S. (2007) Glypican-3: From the mutations of Simpson-Golabi-Behmel genetic syndrome to a tumor marker for hepatocellular carcinoma. *Exp. Mol. Pathol.*, **82**, 184–189.
19. Laloo,B., Maurel,M., Jalvy-Delvaille,S., Sagliocco,F. and Grosset,C.F. (2010) Analysis of post-transcriptional regulation using the FunREG method. *Biochem. Soc. Trans.*, **38**, 1608–1614.
20. Maragkakis,M., Reczko,M., Simossis,V.A., Alexiou,P., Papadopoulos,G.L., Dalamagas,T., Giannopoulos,G., Goumas,G., Koukis,E., Kourtis,K. *et al.* (2009) DIANA-microT web server: elucidating microRNA functions through target prediction. *Nucleic Acids Res.*, **37**, W273–W276.
21. Betel,D., Koppal,A., Agius,P., Sander,C. and Leslie,C. (2010) Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol.*, **11**, R90.
22. Krek,A., Grun,D., Poy,M.N., Wolf,R., Rosenberg,L., Epstein,E.J., MacMenamin,P., da Piedade,I., Gunsalus,K.C., Stoffel,M. *et al.* (2005) Combinatorial microRNA target predictions. *Nat. Genet.*, **37**, 495–500.
23. Kertesz,M., Iovino,N., Unnerstall,U., Gaul,U. and Segal,E. (2007) The role of site accessibility in microRNA target recognition. *Nat. Genet.*, **39**, 1278–1284.
24. Wang,X. (2008) miRDB: a microRNA target prediction and functional annotation database with a wiki interface. *RNA*, **14**, 1012–1017.
25. Wang,X. and El Naqa,I.M. (2008) Prediction of both conserved and nonconserved microRNA targets in animals. *Bioinformatics*, **24**, 325–332.
26. Griffiths-Jones,S., Saini,H.K., van Dongen,S. and Enright,A.J. (2008) miRBase: tools for microRNA genomics. *Nucleic Acids Res.*, **36**, D154–D158.
27. Xu,S., Witmer,P.D., Lumayag,S., Kovacs,B. and Valle,D. (2007) MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. *J. Biol. Chem.*, **282**, 25053–25066.
28. Myatt,S.S., Wang,J., Monteiro,L.J., Christian,M., Ho,K.K., Fusi,L., Dina,R.E., Brosens,J.J., Ghaem-Maghami,S. and Lam,E.W. (2010) Definition of microRNAs that repress expression of the tumor suppressor gene FOXO1 in endometrial cancer. *Cancer Res.*, **70**, 367–377.
29. Guttilla,I.K. and White,B.A. (2009) Coordinate regulation of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells. *J. Biol. Chem.*, **284**, 23204–23216.
30. Stittrich,A.B., Haftmann,C., Sgouroudis,E., Kuhl,A.A., Hegazy,A.N., Panse,I., Riedel,R., Flossdorf,M., Dong,J., Fuhrmann,F. *et al.* (2010) The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. *Nat. Immunol.*, **11**, 1057–1062.
31. Jensen,K.P. and Covault,J. (2011) Human miR-1271 is a miR-96 paralog with distinct non-conserved brain expression pattern. *Nucleic Acids Res.*, **39**, 701–711.
32. Fabian,M.R., Sonenberg,N. and Filipowicz,W. (2010) Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.*, **79**, 351–379.
33. Su,H., Trombly,M.I., Chen,J. and Wang,X. (2009) Essential and overlapping functions for mammalian Argonautes in microRNA silencing. *Genes Dev.*, **23**, 304–317.
34. Landthaler,M., Gaidatzis,D., Rothballer,A., Chen,P.Y., Soll,S.J., Dinic,L., Ojo,T., Hafner,M., Zavolan,M. and Tuschl,T. (2008) Molecular characterization of human Argonaute-containing ribonucleoprotein complexes and their bound target mRNAs. *RNA*, **14**, 2580–2596.
35. Schmitter,D., Filkowski,J., Sewer,A., Pillai,R.S., Oakeley,E.J., Zavolan,M., Svoboda,P. and Filipowicz,W. (2006) Effects of Dicer and Argonaute down-regulation on mRNA levels in human HEK293 cells. *Nucleic Acids Res.*, **34**, 4801–4815.
36. Zhang,L., Liu,T., Huang,Y. and Liu,J. (2011) microRNA-182 inhibits the proliferation and invasion of human lung adenocarcinoma cells through its effect on human cortical actin-associated protein. *Int. J. Mol. Med.*, **28**, 381–388.
37. Sun,Y., Fang,R., Li,C., Li,L., Li,F., Ye,X. and Chen,H. (2010) Hsa-mir-182 suppresses lung tumorigenesis through down regulation of RGS17 expression in vitro. *Biochem. Biophys. Res. Commun.*, **396**, 501–507.
38. Moskwa,P., Buffa,F.M., Pan,Y., Panchakshari,R., Gottipati,P., Muschel,R.J., Beech,J., Kulshrestha,R., Abdelmohsen,K., Weinstock,D.M. *et al.* (2011) miR-182-mediated downregulation of BRCA1 impacts DNA repair and sensitivity to PARP inhibitors. *Mol. Cell*, **41**, 210–220.
39. Brodersen,P. and Voinnet,O. (2009) Revisiting the principles of microRNA target recognition and mode of action. *Nat. Rev. Mol. Cell Biol.*, **10**, 141–148.
40. Obad,S., dos Santos,C.O., Petri,A., Heidenblad,M., Broom,O., Ruse,C., Fu,C., Lindow,M., Stenvang,J., Straarup,E.M. *et al.* (2011) Silencing of microRNA families by seed-targeting tiny LNAs. *Nat. Genet.*, **43**, 371–378.
41. Rehmsmeier,M., Steffen,P., Hochsmann,M. and Giegerich,R. (2004) Fast and effective prediction of microRNA/target duplexes. *RNA*, **10**, 1507–1517.
42. Brennecke,J., Stark,A., Russell,R.B. and Cohen,S.M. (2005) Principles of microRNA-target recognition. *PLoS Biol.*, **3**, e85.
43. Filipowicz,W., Bhattacharyya,S.N. and Sonenberg,N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.*, **9**, 102–114.
44. Mencia,A., Modamio-Hoybjor,S., Redshaw,N., Morin,M., Mayo-Merino,F., Olavarrieta,L., Aguirre,L.A., del Castillo,I., Steel,K.P., Dalmay,T. *et al.* (2009) Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss. *Nat. Genet.*, **41**, 609–613.
45. Lewis,M.A., Quint,E., Glazier,A.M., Fuchs,H., De Angelis,M.H., Langford,C., van Dongen,S., Abreu-Goodger,C., Piipari,M., Redshaw,N. *et al.* (2009) An ENU-induced mutation of miR-96 associated with progressive hearing loss in mice. *Nat. Genet.*, **41**, 614–618.

Résumé

Le Glypican-3 (GPC3) est surexprimé dans 72% des carcinomes hépatocellulaire (CHC). C'est un co-récepteur membranaire du récepteur WNT, qui appartient à la famille des protéoglycanes à sulfates d'héparane. L'objectif général de ma thèse vise à étudier les mécanismes de régulation post-transcriptionnelle de l'expression du GPC3 dans le CHC. Pour cela, j'ai développé un test fonctionnel qui m'a permis de cribler une bibliothèque de 876 microARNs humains. Ceci a conduit à l'identification de 5 microARNs régulateurs de l'expression de l'ARNm codant pour le *GPC3 via* sa région 3' non traduite (NT). Mon travail de thèse porte plus particulièrement sur le miR-1271 et le miR-1291 car ils sont dérégulés dans le CHC et sont respectivement inhibiteur et inducteur de l'expression du *GPC3*. Dans un premier projet, j'ai démontré que le miR-1271 cible directement la région 3'NT du *GPC3* et diminue la stabilité de son ARNm. Ce microARN est sous-exprimé dans le CHC et son expression corrèle négativement avec celle de l'ARNm du *GPC3* dans les CHC associés à une infection par le virus de l'hépatite B. Dans un deuxième projet, j'ai démontré que le miR-1291 régule positivement l'expression du *GPC3* en inhibant un facteur intermédiaire. Une analyse *in silico* a permis d'identifier IRE1α comme candidat. IRE1α est une protéine transmembranaire du réticulum endoplasmique (RE) qui participe à « l'Unfolded Protein Response », une réponse adaptative activée lors de l'accumulation de protéines mal conformées dans le RE. J'ai démontré qu'IRE1α clive l'ARNm codant pour le *GPC3* grâce à son activité endoribonucléase. D'autre part, le miR-1291 cible directement l'ARNm codant pour *IRE1α* dans sa région 5'NT ce qui inhibe son expression et induit une surexpression du GPC3. Le miR-1291 est surexprimé dans le CHC et son expression corrèle positivement avec celle de l'ARNm du *GPC3*.
En conclusion, mon travail de thèse m'a permis de mettre en évidence et de caractériser deux nouveaux microARNs (miR-1271 et miR-1291) contrôlant l'expression du *GPC3* par des mécanismes directs ou indirects. La pertinence physiopathologique de ces régulations dans le CHC est en accord avec les niveaux d'expression respectifs de ces microARNs, qui pourraient contribuer à la surexpression du GPC3 dans ces tumeurs.

Summary

Glypican-3 (GPC3) is overexpressed in 72% of hepatocellular carcinoma (HCC). It is a co-receptor for WNT receptor and belongs to the heparan sulfate proteoglycans family. The general objective of my PhD thesis was to study the mechanisms by which GPC3 is post-transcriptionnally regulated in HCC. To this end, I developed a functional test that allowed me to screen a library of 876 human microRNAs. This led me to identify 5 microRNAs that regulate the expression of *GPC3* mRNA through its 3'Untranslated Region (UTR). The work presented in this thesis particulary focuses on miR-1271 and miR-1291 as both microRNAs present a deregulated expression in HCC and are respectively inhibitor and activator of *GPC3* mRNA expression. In a first project, I demonstrated that miR-1271 directly binds to *GPC3* mRNA 3'UTR and affects its stability. This microRNA is underexpressed in HCC and its expression negatively correlates with that of *GPC3* mRNA in a subgroup of HCC corresponding to those associated with hepatitis B virus infection. In a second project, I demonstrated that miR-1291 postively regulates the expression of *GPC3* mRNA by targeting an intermediate factor. An *in silico* analysis led to the identification of the Inositol Requiring Enzyme 1 alpha (IRE1α) as a potential candidate. IRE1α is an endoplasmic reticulum (ER) resident type I transmembrane protein and contributes to the signaling of the Unfolded Protein Response (UPR). The UPR is an adaptive response activated upon accumulation of improperly folded proteins in the ER. I showed that IRE1α cleaves *GPC3* mRNA through its endoribonuclease activity. Moreover I demonstrated that miR-1291 directly targets *IRE1α* mRNA through its 5'UTR, thereby decreasing its expression and contributing to *GPC3* mRNA overexpression. MiR-1291 is overexpressed in HCC and its expression positively correlates with that of *GPC3* mRNA.
In summary, the work carried out during my PhD allowed the identification and the characterization of two new microRNAs (miR-1271 and miR-1291) that control the expression of *GPC3* mRNA through direct or indirect mechanisms. The pathophysiological relevance of these regulatory mechanisms is in agreement with the respective expression levels of these microRNAs in HCC, which could therefore contribute to the overexpression of GPC3 in those tumors.