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Activité anti-tumorale de la voie XBP1 dans les leucémies aiguës myéloïdes

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Table des abréviations

1,25D : α1,25-dihydroxyvitamin D3

2-HG :2-hydroxyglutarate

ABL : Abelson

ADN : Acide désoxyribonucléique

AGT : angiotensinogen

AMM : Autorisation de Mise sur le Marché

ANGII : Angiotensin II

AP : Arrested Peptide

ARN : Acide ribonucléique

ASK1 : Apoptosis Signal-Regulating Kinase 1

ATF4 : pour Activating Transcription Factor 4

ATF6 : Activating Transcription Factor-6

ATO : Arsenic Tri Oxide

ATRA : All Trans Retinoic Acid

ATRA : All-Trans-Retinoic Acid

BACH2 : BTB Domain And CNC Homolog 2

BAK : Bcl-2 homologous antagonist/killer

BAX : Bcl-2–associated X

Bcl-2 : B-cell lymphoma 2

Bcl-xL : B-cell lymphoma-extra large

BCR : Breakpoint Cluster Region

Bid : BH3 interacting-domain Death

Bim/BCL2L11 : Bcl-2-like protein 11

BiP : Binding Immunoglobulin Protein

BRCA1 : Breast Cancer 1

BSAP/ PAX5 : B cell lineage-specific activator protein/PAired boX gene 5

bZIP : basic leucine ZIPper

C/EBP : CCAAT enhancer binding protein

CCR4-NOT : Carbon Catabolite Repression—Negative On TATA-less

CCTα : choline cytidylyltransferase α

cDC : conventional Dendritic Cell

CDK5 : Cyclin-dependent kinase 5

CHIP : carboxyl terminus of HSC70-interacting protein

ChIP : Chromatin Immunoprecipitation

CHOP : C/EBP Homologous Protein

CIL : Cellule Initiatrice de Leucémie

CLP : Common Lymphoid Progenitor

CMH : Complexe Majeur d’Histocompatibilité

CMP : Common Myeloid Progenitor

CSH : Cellule Souche Hématopoïétique

CXCL12 : C-X-C Ligand 12

DDR : DNA Damage Response

DDX6 : DEAD-Box Helicase 6

DGAT1 : Diacylglycérol O-Acyltransférase 1

DGCR8 : DiGeorge Syndrome Critical Region 8

DLI : Donor Lymphocyte Infusions

DMSO : diméthylsulfoxyde

DNAJB9 : DnaJ Heat Shock Protein Family (Hsp40) Member B9

DNMT : DNA methyl-transferase

EDEM : ERAD enhancing mannonidase-like

EGCG : l'épigallocatechingallate

EIF2AK3 : Eucaryotic Initiation Factor 2 Activating Kinase 3

ERAD : ER Associated Degradation

ERdj4 : Endoplasmic reticulum–localized DnaJ 4

ERN1/2 : Endoplasmic Reticulum To Nucleus Signaling 1/2

FAB : classification Franco-Américain Britannique

FDA : Food and Drug Administration

FGF-1 : Fibroblast Growth Factor-1

FLT3 : Fms-Like Tyrosine Kinase

FT : facteur de Transcription

GADD34 : Growth arrest and DNA damage-Inducible protein 34

GCN5 : General Control Non-repressed 5 protein

G-CSF : Granulocytes Colony Stimulating Factor

GM-CSF : Granulocytes-Macrophages Colony Stimulating Factor

GMP : Granulo-Monocytic Progenitor

GO : Gemtuzumab Ozogamicine

HAC1 : hyperpolarization-activated cation channel

HCV : Hepatitis C virus

HDAC : Histone Désacétylase

HEX : Hematopoietically Expressed Homeobox

HIF : Hypoxia-Induced Factor

HMA : hypomethylating Agent

Hrd1 : HMG-CoA reductase degradation protein 1

Hsp47 : Heat Shock Protein 47

IDH1/2 : Isocitrate Déshydrogénase 1 / 2

IGF-1 : Insulin-Like Growth Factor-1

IKK : Inhibitor κB Kinase

IL-1α / lL-1β : Interleukin 1 α et β

IL-7 : Interleukine 7

IRE-1α : Inositol Requiring Enzyme-1

IRES : Internal Ribosome Entry Sites

IRF8 : Interferon regulatory factor 8

ITD : Internal Tandem Duplication

JAG1 : Jagged-1

JAK : Janus Kinase 2

JNK : c-Jun-N-terminal Kinase

LAL : Leucémies Aigües Lymphoïdes

LAM : Leucémie Aigüe Myéloïde

LAP : Leucémie Aigüe Promyélocytaire

LMC : Leucémies Myéloïdes Chroniques

LSC : Leukemia Stem Cells

LT-HSC : Long-Term Hematopoietic Stem Cell

MAMs : Mitochondrial-Associated Membrane

MAPK : Mitogen-Activated Protein Kinase

Mcl-1 : Myeloid Leukemia Cell

MCL1 : Myeloid Cell Leukemia 1

Mdm1 : Mitochondrial Distribution and Morphology 1

MDM2 : Murine Double Minute 2

MEF : Mouse Embryonic Fibroblast

MEP : Megakaryo-Erythrocytic Progenitor

Mfn2 : mitofusine-2

MIR22HG : miR-22 Host Gene

miRISC : miRNA-induced silencing complex

miRNA : micro-RNA

Mitf : Microphthalmia-associated transcription factor

MLL : Mixed-Lineage Leukemia

MM : Myélome Mutliple

MPP : Multipotent Progenitor

MSC : Mesenchymal Stem Cells

NAD(P)H : Nicotinamide Adénine Dinucléotide Phosphate

N-CoR : Nuclear hormone receptor CoRepressor

NDRG2 : N-myc downstream-regulated gene 2

NF-κB : Nuclear Factor-kappa B

NHEJ : Non Homologous End Joining

NK : Natural Killer

NLS : Nuclear Localization Sequence

NPM1 : Nucléophosmine 1

Nrf2 : Nuclear factor erythroid-2-related Factor 2

NS4B : Nonstructural protein 4B

NSG : NOD scid gamma

NUP98 : Nucleoporine 98

OBs : ostéoblastes

PABPC : poly(A) binding protein C

PCA3 : Prostate Cancer Antigen 3

PD1 : Programmed cell death 1

pDC : plasmacytoid Dendritic Cell

PDI : Protein Disulfide Isomérase

PDIA6 : Protein Disulfide Isomerase A6

PDI-P5 : Protein Disulfide Isomerase P5

PDX : Patients-Derived Xenograft

PERK : PKR-like ER-associated protein Kinase)

PI3k : Phosphoinositide 3-kinase

PIAS : Protein Inhibitor of activated STAT2

Plk4 : Polo-like Kinase 4

PML : Promyelocytic Leukemia

PTEN : Phosphatase and Tensin homolog

PTH : Parathyroid Hormone

qPCR : quantitative Polymerase Chain Reaction

RARα : Récepteur à l'Acide Rétinoïque

RARα : Retinoic Acid Receptor α

RE : Réticulum Endoplasmique

REL : Réticulum Endoplasmique Lisse

RER : Réticulum Endoplasmique Rugueux

RIDD : Regulated IRE1-Dependent Decay

ROS : Reactive Oxigen Species

RT : Reverse Transcription

RUNX-1 : Runt-related transcription factor 1

S1P / S2P : Site-1 Protease / Site-2 Protease

SENP1 : Sentrin/SUMO-specifique

SERCA : Sarco/Endoplasmic Reticulum Ca2+-ATPase

Sig-1R : Sigma-1 Receptor

SNS : Sympathetic Nervous System

SPP : Signal Peptide Peptidase

STARD3 : Steroidogenic Acute Regulatory (StAR) Related lipid Transfer

STARD3NL : STARD3 N-Terminal Like

STAT5 : Signal Transducer and Activator of Transcription 5

ST-HSC : Short-Term Hematopoietic Stem Cell

STIM1 : Stromal interaction molecule 1

TAD : Transcriptional Activation Domain

TAP1 : Transporter associated with Antigen Processing 1

TCGA : The Cancer Genome Atlas

TET2 : Ten-eleven-translocation 2

TGF-β : Transforming Growth Factor beta

TKD : Tyrosine Kinase Domain

TLR : Toll-Like Receptor

Tmp21 : Transmembrane Trafficking Protein 21

TNFR1 : TNF Receptor 1

TNF-α / TNF- β : Tumor Necrosis Factor α et β

TNFα : Tumor Necrosis Factor α

TNRC6 : Trinucleotide Repeat Containing 6 protein A

TRAF2 : TNFR-associated factor 2

TRBP : Transactivation Response Element RNA-Binding Protein

TRC8 : Translocation In Renal Carcinoma On Chromosome 8

ProteinTSLP : Thymic Stromal Cell–derived Lymphopoietin

TUDCA : Tauroursodeoxycholic acid

Tv-6 : Tenovin 6

uORFs : upstream open reading frames

UPR : Unfolded Protein Response

UTR : Untranslated Region

VAMP7 : Vesicle Associated Membrane Protein 7

VDP : Vesicle Docking Protein

VEGF : Vascular endothelial growth factor

WT1 : Wilms Tumor antigen 1

XBP1 : X box-binding protein 1

XIAP : X-linked inhibitor of apoptosis

XPO5 : exportine 5

α-KG : α-cétogluratate

Liste des publications

- **ARTICLES SCIENTIFIQUES**

The PERK Branch of the Unfolded Protein Response Promotes DLL4 Expression by Activating an Alternative Translation Mechanism.

Manon Jaud*, Céline Philippe*, Loic Van Den Berghe, Christèle Ségura, Laurent Mazzolini, Stéphane Pyronnet, Henrik Laurell, Christian Touriol

- **REVUES**

Translational Regulations in Response to Endoplasmic Reticulum Stress in Cancers

Manon Jaud*, Céline Philippe*, Doriana Di Bella, Weiwei Tang, Stéphane Pyronnet, Henrik Laurell, Laurent Mazzolini, Kevin Rouault-Pierre, Christian Touriol

ER Stress and Unfolded Protein Response in Leukemia: Friend, Foe, or Both?

Kelly Féral*, Manon Jaud*, Céline Philippe*, Doriana Di Bella, Stéphane Pyronnet, Kevin Rouault-Pierre, Laurent Mazzolini, Christian Touriol

INTRODUCTION

I. L'Hématopoïèse saine

A. Définition générale

L'hématopoïèse (du grec ancien αἷμα, αἵματος, *haíma, haímatos* « sang » et ποίησις, *poíêsis* « création ») est le processus de production de l'ensemble des cellules matures et fonctionnelles qui constituent le tissu sanguin. Elle est décrite classiquement comme un mécanisme pyramidal, dominée par une population rare de cellules hématopoïétiques multipotentes, les Cellules Souches Hématopoïétiques (CSH), qui résident majoritairement dans la moelle osseuse. Elles sont à elles seules capables de produire tous les lignages hématopoïétiques grâce à une balance entre auto-renouvèlement, qui permet le maintien du pool multipotent de CSH, et la différenciation cellulaire, qui engendre un engagement dans un lignage spécifique par l'activation de programme d'expression génique [1] (Figure 1).

B. La différenciation hématopoïétique

1. Hétérogénéité des Cellules Souches Hématopoïétiques

On distingue deux sous-types de CSH :

- Les Long-Term Hematopoietic Stem Cell (LT-HSC) : Ces cellules possèdent de grandes capacités d'auto-renouvellement. Elles résident dans un état quiescent, caractérisé par de faibles capacités de divisions, une activité métabolique ainsi qu'une synthèse protéique basse [2,3,4]. Les LT-HCS sont une « réserve » de cellules multipotentes, à la fois nécessaires et suffisantes à la reconstitution et au maintien à long terme de l'intégralité des différents lignages [5,6].

- Les Short-Term Hematopoietic Stem Cell (ST-HSC) : Sous l'effet de variations dans leur environnement, les LT-HSC sortent de leur quiescence et entre dans le cycle cellulaire pour donner les ST-HSC [7]. Ces cellules acquièrent des capacités prolifératives plus importantes mais perdent en auto-renouvèlement. En effet, elles ne sont plus capables de reconstituer l'intégralité des lignages hématopoïétiques à long terme, et sont moins efficaces après des transplantations en série [5,8].

Une fois entrées dans le cycle cellulaire, les ST-HSC vont continuer à proliférer, tout en réduisant encore leurs capacités d'auto-renouvèlement. On ne parle alors plus de ST-

HSC, mais de Progéniteur Multipotents (ou MPP pour Multipotent Progenitor)[5,9,10]. Cette catégorie de cellules est très hétérogène et reste assez mal caractérisée [11,12]. Cependant, elles conservent une certaine multipotentialité [12] et constituent le pénultième état de différenciation avant un engagement dans un lignage spécifique.

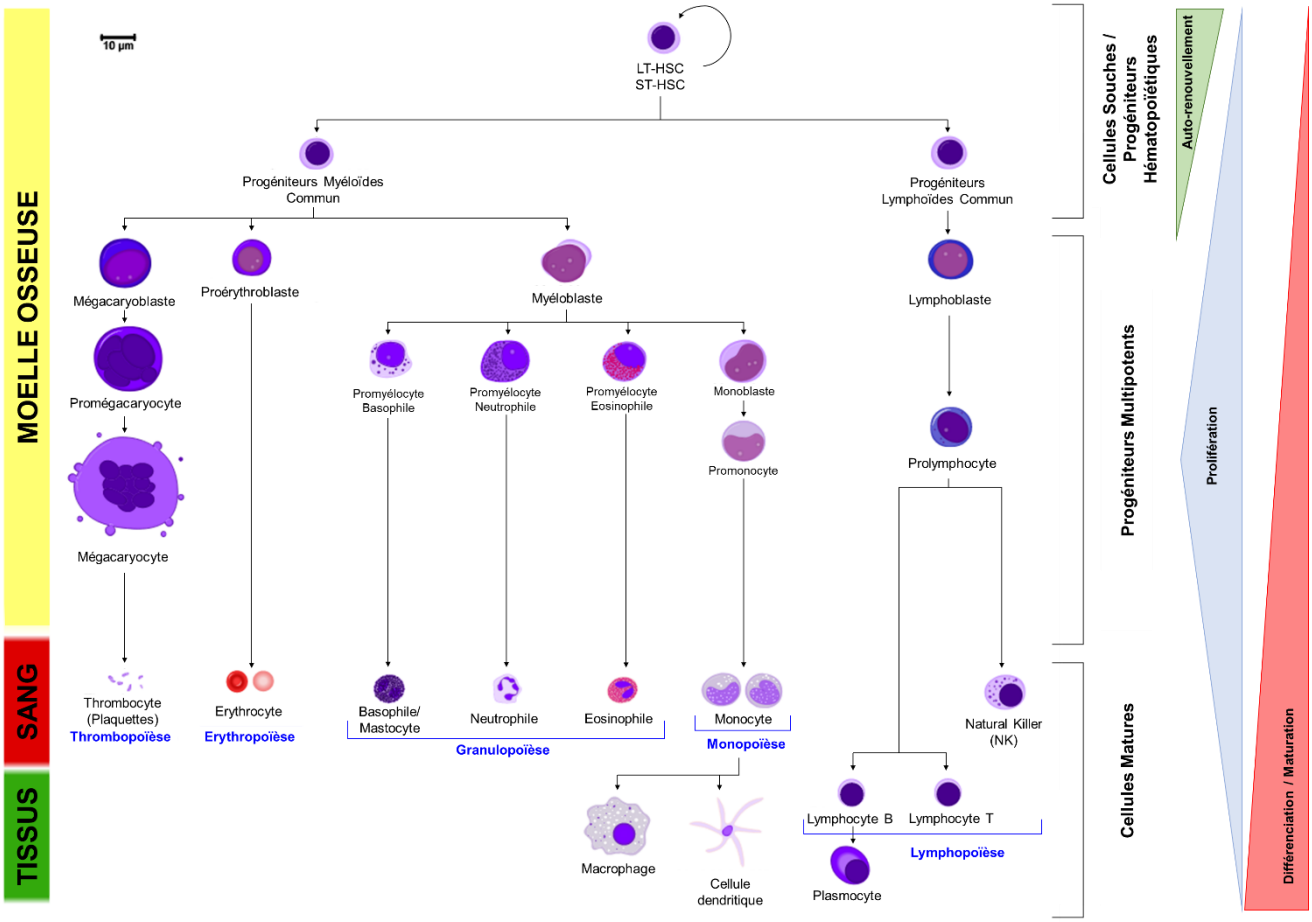

Figure 1: Hématopoïèse normale

L'hématopoïèse est un processus pyramidale dominé par des cellules souches hématopoïétiques multipotentes, à partir desquelles sont générés des progéniteurs myéloïdes et lymphoïdes. Grâce à des processus de différenciation et de maturation, ces progéniteurs engendrent l'ensemble des cellules immunitaires et sanguines. Le lignage lymphoïde regroupe 3 types de cellules immunitaires : les lymphocytes B et T, issus d'un même précurseur, et les cellules NK (Natural Killer). Les progéniteurs communs myéloïdes permettent notamment la production des érythrocytes, principaux constituants sanguins. Ils permettent également la production des granulocytes par le processus de granulopoïèse, ainsi que de monocytes grâce à la monopoïèse : après les érythrocytes, ce sont des les principales cellules circulantes. Enfin, la thrombopoïèse permet de générer des mégacaryocytes, qui restent dans la moelle osseuse mais qui sécrètent les plaquettes..

2. Devenir des MPP

Les MPPs progressent ensuite vers un stade de progéniteurs oligopotents, engagés dans une voie de différenciation (Figure 1) [13]. On distingue deux lignages différents :

- Les cellules de la lignée lymphoïde, dominées par les Progéniteurs Communs Lymphoïdes (ou CLP pour Common Lymphoid Progenitor)[14]. Une fois mature, les CLP produisent les lymphocytes B, les lymphocytes T et les Natural Killer (NK).
- Les cellules de la lignée myéloïde, dominées par les Progéniteurs Communs Myéloïdes (ou CMP pour Common Myeloid Progenitor)[15] qui produisent les érythrocytes, les plaquettes, les granulocytes et les monocytes.

Les processus amenant à la production des cellules matures sont explicités en partie I.C et I.D.

3. Evolution du dogme

De récentes analyses ont néanmoins permis de concevoir une nouvelle représentation de l'hématopoïèse. En effet, il est maintenant largement accepté que la différenciation hématopoïétique est moins un processus pyramidal qu'un continuum, composé de sous-populations très hétérogènes au sein même des CSH et des progéniteurs. Des analyses de dilution-limite et de « single-cell transplantation » de CSH chez la souris ont permis de démontrer des capacités de repeuplement différentes, aussi bien en termes d'efficacité de reconstitution de la moelle osseuse qu'en termes de proportions des différents lignages produits après la greffe [8,16]. De plus, le groupe de Andreas Trumpp recense en 2008 quatre populations de MPP, de MPP1 à MPP4 [17] : les MPP1 sont phénotypiquement et fonctionnellement les plus proches des CSH. Les MPP2-3 sont plutôt orientés dans le lignage myéloïde et enfin, les MPP4 sont plutôt engagés dans le lignage lymphoïde. Ces sous-populations de MPP sont *a priori* produit indépendamment par les CSH, mais gardent une certaine plasticité dans leur différenciation, influencée par l'homéostasie sanguine [16,18,19]. L'arrivée de techniques de séquençage à haut-débit type « single-cell RNA sequencing » n'ont fait que confirmer l'existence de cette hétérogénéité des populations hématopoïétiques, au-delà même du stade de progéniteurs multipotents [20,21].

L'ensemble des précédents résultats réfutent également l'idée d'une hématopoïèse unidirectionnelle, dans laquelle l'engagement dans un lignage serait un processus irrévocable. Déjà, en 1995, Kulessa *et al*. montre que l'expression du facteur de transcription GATA-1 dans des monocytes aviaires est suffisant pour induire un changement de lignage et orienter ces cellules vers une différenciation granulocytaire et érythrocytaire, suggérant l'idée d'une possible reprogrammation cellulaire [22]. Cette reprogrammation du devenir cellulaire est indépendante du lignage. Ainsi, des précurseurs lymphocytaires T et B peuvent

encore être reprogrammés pour donner des macrophages mais aussi des granulocytes complètement fonctionnels [23,24].

L'hétérogénéité des populations hématopoïétiques ainsi que leur grande plasticité suggèrent donc une régulation très fine du processus de différenciation, nécessaire au maintien de l'homéostasie ; cette régulation est influencée par de nombreux facteurs, tels que le contexte cellulaire et chimique dans lequel les futures cellules sanguines évoluent, mais aussi l'activation de programmes d'expressions géniques permettant la spécification et la maturation de ces cellules.

C. Régulations extrinsèques de la différenciation hématopoïétique

L'environnement médullaire fourni un ensemble d'éléments nécessaire au maintien des capacités souches des CSH. Ce microenvironnement local est appelé « niche hématopoïétique » [25].

1. Protection des CSH par la niche hématopoïétique

La moelle osseuse est un tissu complexe, avec une forte densité cellulaire, majoritairement constituée de cellules hématopoïétiques. On distingue malgré tout d'autres types cellulaires comme des ostéoblastes, précurseurs osseux, des cellules endothéliales et des cellules souches mésenchymateuses périvasculaires (ou MSCs pour Mesenchymal Stem Cells), qui composent les parois des vaisseaux sanguins, ou encore des fibres nerveuses.

De nombreuses analyses soulignent l'importance de la fraction vasculaire et périvasculaire (cellules endothéliales et MSC) au sein de la niche. La proximité des vaisseaux permet le contrôle du trafic leucocytaire ainsi que l'export des érythrocytes vers la circulation générale et les CSH sont retrouvées préférentiellement associées aux capillaires sanguins veineux sinusoïdaux [26,27] ; ces capillaires particuliers ont une parois fines, parfois inexistante du fait de cellules endothéliales non jointives. Le tissu médullaire environnant et sa forte densité cellulaire assurent le maintien et la cohésion de ces vaisseaux, qui sont parmi les plus perméables. Par ailleurs, la perméabilité des vaisseaux médullaires a été montrée comme étant un facteur d'activation ou de maintien en quiescence des CSH : les vaisseaux les plus perméables sont associés à une mobilisation des CSH, c'est-à-dire à une activation de leur prolifération et leur différenciation suivi de

l'export des cellules générées vers la circulation générale ; à l'inverse, une diminution de la perméabilité vasculaire est associée à une diminution du métabolisme des CSH, marquant leur état quiescent [26,27,28]. Ces effets sont médiés par la protéine Jagged-1 (JAG-1), un ligand de Notch, agissant de façon paracrine. La délétion de JAG-1 spécifiquement dans les cellules endothéliales provoque une diminution de la voie de signalisation Notch au sein des CSH, entraînant une entrée dans le cycle. En résulte un défaut d'autorenouvèlement des CSH, marqué par une diminution du nombre de CSH médullaires ainsi qu'un affaiblissement de leurs capacités de reconstitution après greffe [29].

Les cellules endothéliales sont largement soutenues par les MSC périvasculaires et le système nerveux sympathique (ou SNS pour Sympathetic Nervous System). Après transplantation, les CSH sont retrouvées à proximité des MSC périvasculaires, et une déplétion de ces dernières entraîne une forte diminution des progéniteurs multipotents. De plus, les MSC périvasculaires expriment un ensemble de gènes impliqués dans le maintien et la rétention des CSH dans la moelle osseuse, comme C-X-C Ligand 12 (ou CXCL12, molécule chimioattractante) et Kitl (ligand de c-Kit, nécessaire à la quiescence) [30,31,32]. Les fibres nerveuses, quant à elles, agissent sur les MSC périvasculaires et contrôlent la production de CXCL12 [30,33]. Mais elles peuvent aussi agir directement sur les CSH par la sécrétion de facteurs paracrines : c'est le cas des cellules de Schwann, qui sont retrouvées pour la plupart, au contact direct des CSH et qui produisent du Transforming Growth Factor beta (TGF-β), facteur inhibiteur du cycle cellulaire [34,35].

Enfin, les cellules hématopoïétiques elles-mêmes peuvent influencer le devenir des CSH. C'est le cas des macrophages résidant dans la moelle osseuse, qui permettent la rétention des CSH au sein de la niche. Ces macrophages agissent sur les MSC et induisent la production de CXCL-12. Leur déplétion par un traitement au clodronate de disodium entraîne une diminution de l'expression de l'ARNm de CXCL-12 au sein des MSC, et de fait, une augmentation des LT-HSC et des MPPs circulants [36,37]. Les mégacaryocytes, population hématopoïétique résidente de la moelle osseuse et fortement associée aux capillaires veineux sinusoïdes, sont également impliqués dans ces processus. Leur rôle est binaire : ils sécrètent des facteurs paracrines impliqués dans la prolifération des CSH, comme le Insulin-Like Growth Factor-1 (IGF-1) ou le Fibroblast Growth Factor-1 (FGF-1) en réponse à un stress myéloablatif type chimiothérapie. A l'inverse, ils participent à l'inhibition de la prolifération des CSH et au maintien en quiescence par la sécrétion de C-X-C Ligand 4 (ou CXCL-4) et de TGF-β [38,39].

2. Niche hématopoïétique et différenciation

L'environnement périvasculaire permet donc le contrôle des CSH, par la régulation de la quiescence et la protection de leur caractère multipotent, tout en induisant leur expansion si nécessaire. *A contrario*, plusieurs études ont mis en évidence l'importance de la fraction ostéoblastiques dans les processus de régulation de la différenciation.

En effet, des marquages immunohistochimiques montrent un enrichissement des CSH dans des régions ostéoblastiques [40] et les variations au sein de la population des ostéoblastes (ou OBs) sont positivement corrélées aux variations de la population des CSH, dues à une activation de leur prolifération [36,40,41,42]. De plus, la déplétion transitoire des ostéoblastes entraîne une accumulation des progéniteurs, ainsi qu'une perte totale de la production de tous les lignages hématopoïétiques [41,43].

Les ostéoblastes expriment un ensemble de cytokines liées à la différenciation et à l'acquisition des fonctionnalités des différents lignages. Ainsi, des analyses montrent que les ostéoblastes expriment et sécrètent des cytokines telles que le GM-CSF (Granulocytes-Macrophages Colony Stimulating Factor) et le G-CSF (Granulocytes Colony Stimulating Factor), toutes deux impliquées dans la différenciation myéloïde [44]. La lymphopoïèse B est également régulée par les ostéoblastes : des expériences de co-culture entre des ostéoblastes et des progéniteurs multipotents montrent une stimulation de la différenciation lymphocytaire B ; la stimulation des OBs par un traitement à la PTH (Parathyroid Hormone) entraîne la sécrétion d'IL-7 (Interleukine 7) et de TSLP (Thymic Stromal Cell–derived Lymphopoietin), entres autres, et orientent la différenciation des précurseurs lymphocytaire B [41,45].

Enfin, les OBs sécrètent des cytokines inflammatoires et favorisent l'efflux et l'activation des leucocytes vers la circulation générale. Ils libèrent du TNF-α et TNF-β (Tumor Necrosis Factor α et β), qui participent à la maturation des lymphocytes T et des macrophages [46,47] , mais aussi de l'IL-1α et β, qui augmentent l'expression de facteurs d'adhésions membranaires au niveau des cellules endothéliales afin de favoriser l'export des cellules matures [44,48].

D. Régulations intrinsèques de la différenciation hématopoïétique

1. Les Facteurs de Transcription : acteurs clés de l'orientation du lignage hématopoïétique

Combiné à l'impact de la niche hématopoïétique, le devenir des cellules est également déterminé par l'expression de facteurs de transcription spécifiques (FT) ; ils permettent d'établir de nouveaux programmes d'expression géniques et d'orienter la différenciation, tout en restreignant le potentiel des cellules [7]. L'exemple le plus classique est la régulation négative réciproque des FT GATA-1 et PU.1, qui détermine le devenir érythroïde ou myéloïde des Progéniteurs Communs Myéloïdes (ou CMP pour Common Myeloid Progenitor) : ainsi l'expression de GATA-1 est nécessaire à la différenciation érythrocytaire, mais induit également une suppression de la différenciation granulocytaire et monocytaire [49]. A l'inverse, l'expression de PU.1 induit une différenciation myélo-monocytaire, et supprime le développement érythrocytaire [49,50].

Etonnamment, ces FT fondamentaux sont assez peu nombreux et se retrouvent différentiellement exprimés tout au long de la différenciation, suggérant la nécessité d'une combinaison spatiale et temporelle de leur expression (Figure 2). Ainsi, PU.1 est exprimé par les cellules monocytaires mais est également nécessaire à la maturation des lymphocytes B [15,50]. De même, GATA-2 est retrouvé exprimé dans les CSH, mais aussi au niveau des progéniteurs mégacaryocytaires et granulocytaires [15,51,52].

L'activité « contexte-dépendante » et les antagonismes directs entre ces régulateurs transcriptionnels cruciaux expliquent aussi la plasticité des cellules hématopoïétiques et leur capacité de trans-différenciation. Comme développé ultérieurement (cf. Partie I ; B ; 3), l'expression *de novo* de certains de ces facteurs est suffisante pour engendrer un changement de lignage. C'est le cas du FT C/EBPα, un facteur nécessaire à la myélopoïèse. La réexpression de C/EBPα dans des progéniteurs lymphocytaire T induit leur différenciation en macrophages et en cellules dendritiques. Cet effet est contrecarré par l'expression de GATA-3, qui antagonise complètement C/EBPα, au profit d'une différenciation lymphocytaire [23,53].

J'ai souhaité par la suite illustrer les variations d'expressions de ces FT fondamentaux à travers l'exemple de la myélopoïèse, dont une des contreparties pathologiques, la leucémie aiguë myéloïde, constitue notre pathologie d'étude. L'ensemble de FT ainsi que leur variation au cours de ce processus sont résumés dans la Figure 2.

2. Exemple de la myélopoïèse

La myélopoïèse est le processus de production des cellules myéloïde matures, parmi lesquelles les érythrocytes, les plaquettes, les granulocytes et les monocytes.

Toutes les cellules myéloïdes dérivent d'un progéniteur myéloïde commun (CMP, cf. partie I ; B ; 2). Etant donné l'expression mutuellement exclusive de GATA-1 et PU.1, le réseau d'activation transcriptionnelle de ces 2 FTs pourrait déterminer l'embranchement entre la formation des progéniteurs mégacaryo-érythrocytaires (ou MEP pour Megakaryo-Erythrocytic Progenitor) et celle des progéniteurs Granulo-Monocytaires (ou GMP pour Granulo-Monocytic Progenitor) [54].

a) Différenciation des MEP

Les MEP se subdivisent eux-mêmes en deux sous-populations matures distinctes : les mégacaryocytes et les érythrocytes. Si les deux populations expriment chacune GATA-1, l'engagement dans la voie mégacaryocytaire est marquée par la réexpression de GATA-2. En effet, l'expression de GATA-2 inhibe la différenciation terminale érythrocytaire grâce à la régulation transcriptionnelle des gènes de l'identité mégacaryocytaire [21,55,56,57]. L'orientation des MEP est également tributaire de la régulation de c-MYB. Ce FT a été montré comme crucial pour la production d'érythrocytes matures, la perte d'expression de c-MYB conduisant à de sévères anémies [58]. Il est fortement exprimé par les progéniteurs érythrocytaires, puis diminue afin d'induire leur différenciation terminale [59,60,61]. De plus, c-MYB réprime des gènes de l'identité mégacaryocytaire et la diminution précoce de son expression oriente les MEP vers ce lignage [62].

b) Différenciation des Granulocytes

L'initiation du réseau transcriptionnel granulo-monocytaire dépend, quant à lui, de l'expression de PU.1. Ainsi, la perte de l'expression de PU.1 conduit à une accumulation des progéniteurs au stade CMP sans affecter la production de MEP [50,63]. La progression des CMP en GMP est également associée à une répression de c-MYB, qui marque le passage d'un état prolifératif du progéniteur à une phase de maturation [60,61].

Les GMP se subdivisent à nouveau en deux sous-populations de progéniteurs : les progéniteurs granulocytaires, qui maturent en mastocytes/basophiles, éosinophiles ou neutrophiles, et les progéniteurs monocytaires, qui maturent en monocytes/macrophages et en cellules dendritiques. L'induction de la granulopoïèse est marquée par l'expression du FT C/EBPα, mis en évidence comme le « master gene » de ce lignage. Historiquement, C/EBPα a été montré comme induisant la différenciation granulocytaire et plus

particulièrement la différenciation neutrophilique, à partir des GMP [53]. En outre, la perte de C/EBPα à ce stade de maturation engendre un arrêt de la granulopoïèse, sans affecter la monopoïèse [64,65,66]. A l'exception des neutrophiles, l'expression de ce FT est transitoire et diminue au cours de la différenciation terminale des basophiles et des éosinophiles [67]. Plus récemment, il a été montré que l'activation transcriptionnelle de C/EBPα dépend directement du FT RUNX-1 (aussi appelé AML-1, CBFA-2, et PEPB2A), confirmant l'implication de RUNX-1 dans la granulopoïèse [64]. Ce dernier a d'abord été mis en évidence comme crucial dans l'émergence et la maintenance des CSH [68,69]. Cependant, l'utilisation de techniques « gène rapporteur » couplées à l'expression de RUNX-1 ont révélées une réexpression dans les cellules myéloïdes [70]. La perte de RUNX-1 est par ailleurs associée à une accumulation de GMP et un défaut de production des basophiles et des éosinophiles, sans altération de la production des neutrophiles et des monocytes [64,71].

La différenciation terminale neutrophilique est marquée par l'expression persistante de RUNX-1, accompagnée, entres autres, par l'augmentation de C/EBPε. L'absence de C/EBPε n'impacte pas directement la différenciation mais provoque un déficit de neutrophiles fonctionnels [72,73]. Phénotypiquement, l'absence de C/EBPε chez la souris conduit à une neutropénie sévère, marquée par une absence de formation des granules cytoplasmiques nécessaires à la fonction bactéricide de ces cellules, et conduisant ainsi au développement d'infections bactériennes opportunistes [74,75].

La différenciation terminale des basophiles/mastocytes et des éosinophiles, quant à elle, est marquée par l'expression de GATA-1 [76,77] ; les éosinophiles expriment eux aussi C/EBPε, puisque, tout comme les neutrophiles, ils produisent des granules cytoplasmiques qui leurs permettent de réaliser leur rôle antiparasitaire et cytotoxique. C/EBPε permettant la formation de ces granules, il est absolument nécessaire à l'acquisition d'éosinophiles fonctionnels [73,78]. Dans le cas des basophiles/mastocytes, GATA-2 est essentiel à la différenciation terminale, ainsi qu'à l'acquisition et à la maintenance des gènes impliqués dans leur fonction, comme la production de l'histamine [79].

c) La différenciation des Monocytes

Si l'orientation granulocytaire est marquée par l'expression de C/EBPα, le « master gene » de la monopoïèse reste PU.1, qui est exprimé depuis l'étape de GMP jusqu'à la production de monocytes/macrophages et de cellules dendritiques pleinement fonctionnels. La perte d'un allèle de PU.1 est suffisante à orienter les GMP vers une différenciation granulocytaire [80]. En effet, des progéniteurs myéloïdes *PU.1 +/–* n'expriment aucun gène spécifique des macrophages, malgré la production de granulocytes pleinement fonctionnels

Cependant, des expériences de « sauvetage phénotypique » montrent que ces mêmes progéniteurs transduits par un lentivecteur codant pour PU.1 acquièrent des caractéristiques morphologiques spécifiques aux macrophages et expriment les gènes spécifiques de l'identité monocytaire [63,81]. Dans ce cas, PU.1 coopère avec le FT IRF8 pour promouvoir la différenciation monocytaire. Des expériences d'immunoprécipitation de chromatine suivi d'un séquençage (ChIP sequencing) ont montré une colocalisation de PU.1 et IRF8 au niveau des promoteurs de gènes spécifiques des monocytes [82]. L'induction du phénotype monocytaire par IRF8 passe aussi par une répression de l'action de C/EBPα ; IRF8 inhibe la différenciation granulocytaire en interagissant physiquement avec C/EBPα, l'empêchant de se fixer sur ses promoteurs cibles [83].

Il est également démontré que l'expression de IRF8 est nécessaire à la production de cellules dendritiques fonctionnelles. L'utilisation de systèmes « perte de fonction » conditionnelle ont montré que, non seulement IRF8 est indispensable à l'acquisition et au maintien de la fonction des cellules dendritiques plasmacytoïdes (ou pDC pour plasmacytoid Dendritic Cells), mais il est requis au cours de la différenciation terminale des cellules dendritiques conventionnelles (cDC pour conventional Dendritic Cells) [84].

L'hématopoïèse est donc un processus fondamental, qui met en jeu un grand nombre d'intermédiaires afin de maintenir l'homéostasie sanguine. Il n'est alors pas surprenant que la moindre dérégulation d'un de ces maillons engendre le développement de maladies, anémies ou neutropénie comme évoqué plus tôt, ou encore des hémopathies malignes telles que les Leucémies Aigües Myéloïdes.

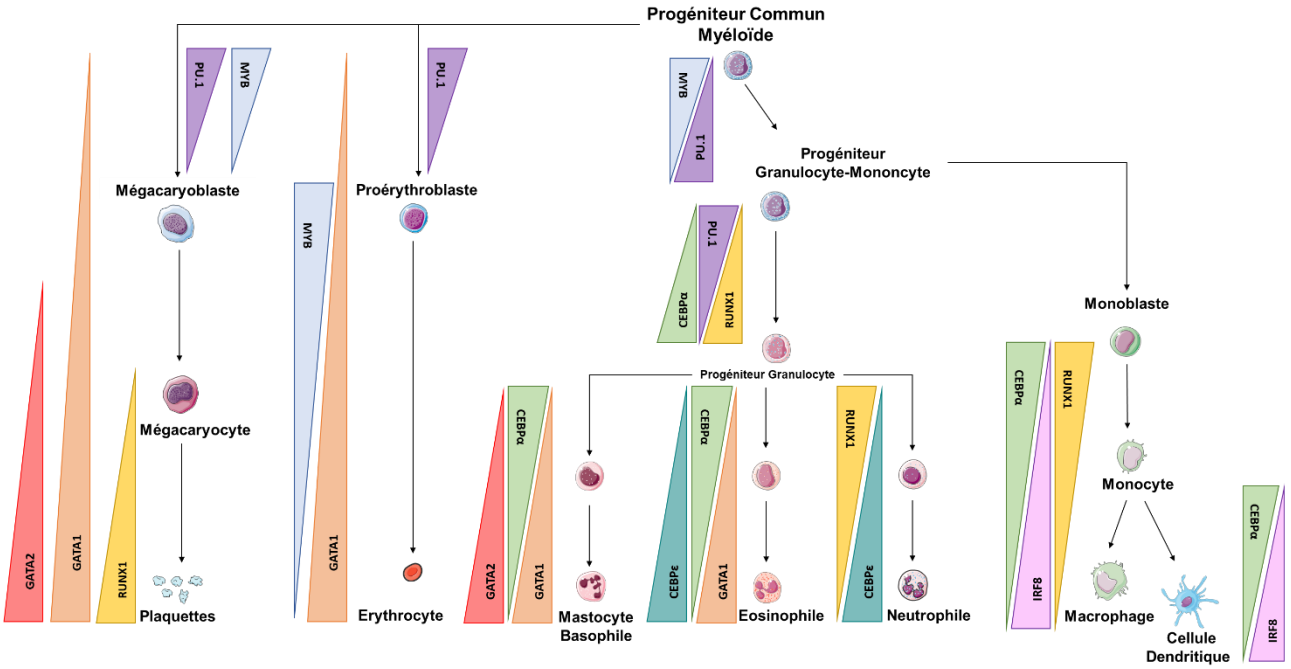

Figure 2 : Variations de l'expression de FT clé au cours de la myélopoïèse

II. Les Leucémies Aigües Myéloïdes

A. Définition

Une hémopathie maligne est un cancer des tissus hématopoïétiques (moelle osseuse, rate ou encore thymus), caractérisée par un défaut de synthèse d'une ou plusieurs lignées hématopoïétiques. Ces cancers sont classés selon deux catégories :

-les lymphomes, qui se développent dans les organes hématopoïétiques secondaires comme les nœuds lymphatiques ou la rate.

-les leucémies, qui prennent leur source dans la moelle osseuse et se propagent dans le sang.

Dans le cas des leucémies, on distingue une forme chronique, à développement lent, qui touche des cellules différenciées pouvant assurer une partie de leurs fonctions, et une forme aigüe, à évolution rapide.

Les Leucémies Aigües Myéloïdes (ou LAM) sont donc des hémopathies malignes à évolution rapide, caractérisées par un défaut de la myélopoïèse médullaire : les blastes leucémiques, des progéniteurs myéloïdes bloqués à différents stades de leur différenciation, s'expandent et colonisent la moelle osseuse, puis le sang pour enfin s'infiltrer dans différents tissus et former des métastases [85].

B. Généralités

1. Epidémiologie

La LAM est la forme de leucémie aigüe la plus commune, et avec le taux de mortalité le plus élevé (50% en 2019). Elle reste relativement rare, avec un taux d'incidence annuel de 3/100 000 personnes. L'incidence augmente néanmoins rapidement avec l'âge, pour atteindre 26/100 000 à partir de 70 ans, voir quasiment 40/100 000 au-delà de 80 ans, en fonction du sexe. En effet, les hommes ont 1,2 à 1,6 fois plus de chance de développer une LAM que les femmes, avec une incidence respective de 5,42 et 3,47/100 000 personnes-années (Figure 3).

La LAM est une pathologie de très mauvais pronostic, pour laquelle le taux de mortalité n'a pas connu d'évolution majeure depuis 2005 ; en comparaison, d'autres leucémies, comme les leucémies lymphoïdes aigües (LAL) ou les leucémies myéloïdes chroniques (LMC), ont connues un déclin de la mortalité des patients sur ce même intervalle de temps. De plus, avec un âge médian au diagnostic de 68 ans, la LAM présente la plus mauvaise survie globale tous cancers confondus, environ 3 mois, si le sujet est âgé de plus de 65 ans ; le pronostic s'assombrie encore pour les tranches d'âge plus élevées. Enfin, après traitement, la survie à 5 ans est de 24%, avec une médiane de survie estimée à 46 mois, et ce, malgré de nouvelles thérapeutiques mises en place [86,87].

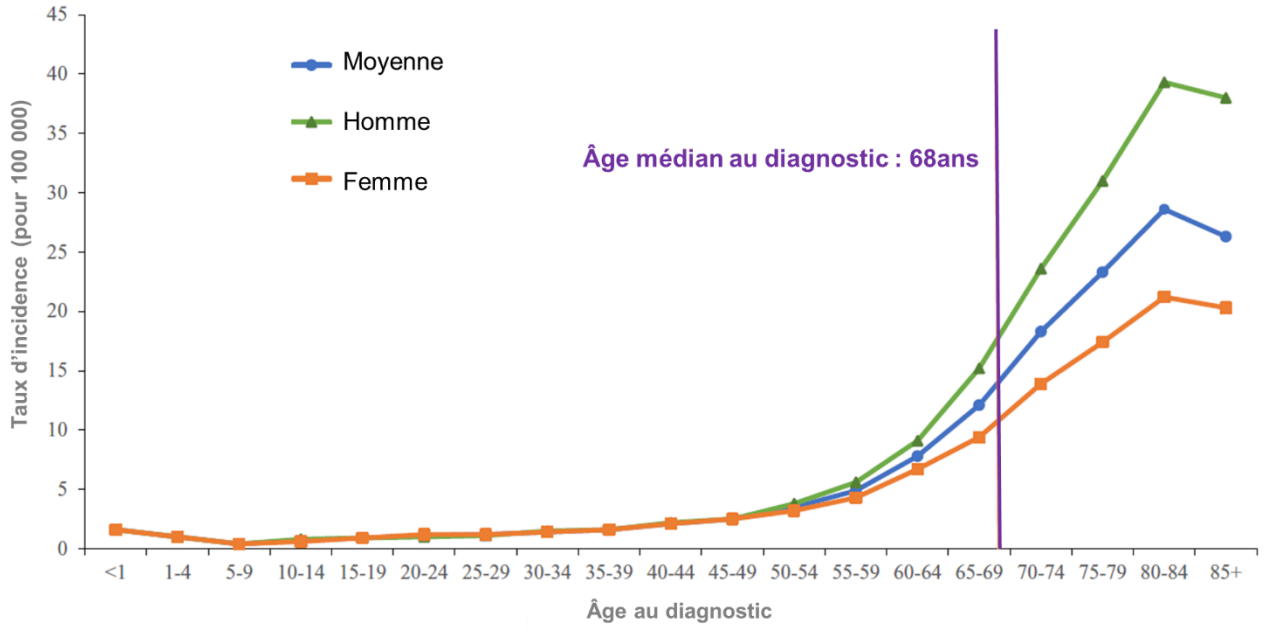

Figure 3 : Taux d'incidence des Leucémies Aigües Myéloïdes par catégorie d'âge

Estimation de l'incidence des LAM sur la période 2011-2016 aux Etats-Unis

D'après Deschler & Lübbert, 2006

2. Etiologie et diagnostic

Les facteurs étiologiques de la LAM sont assez peu caractérisés ; la majorité des cas sont des LAM *de novo*, néanmoins, dans 10 à 30%, on parle de LAM secondaires. Elles font suite à différents facteurs préalables qui fragilisent l'hématopoïèse et favorisent l'expansion clonale comme :

-des facteurs environnementaux tels que l'exposition au benzène,
-des facteurs sociaux tels que l'obésité ou le tabagisme,
-l'évolution d'hémopathie maligne telle que les syndromes myéloprolifératifs,
-la présence de maladies congénitales telles que la trisomie 21 ou l'anémie de Fanconi,
-l'exposition à des traitements génotoxiques tels que la radiothérapie ou la chimiothérapie [88].

Le développement d'une LAM entraîne une insuffisance médullaire et, de fait, l'apparition de symptômes tels que des hémorragies cutanées, une fatigue chronique ou encore le développement d'infections opportunistes. Loin d'être spécifiques à la LAM, ces symptômes ne permettent pas à eux seuls de poser un diagnostic. En premier lieu, un myélogramme permet d'établir la présence de blastes dans le sang et la moelle par une analyse morphologique. Le diagnostic est avéré lorsque la proportion de blastes est supérieure ou égale à 20% dans chacun des compartiments (pourcentage calculé sur 200 leucocytes comptés dans le sang, et sur 500 cellules nucléées comptées dans la moelle).

L'analyse moléculaire des blastes permet ensuite d'affiner le diagnostic ; les cellules cancéreuses sont immunophénotypées, afin de déterminer des marqueurs spécifiques d'un lignage.

3. Classification des LAM

Le blocage des blastes dans des stades plus ou moins tardifs dans la différentiation rend ces cellules immunophénotypiquement et morphologiquement identifiables, permettant ainsi une première classification des LAM. Un premier système de classification a donc été créé en 1976, puis révisé en 1985, en se basant sur ces caractéristiques : la classification FAB (pour Franco-Américain Britannique) subdivise les LAM en 8 sous-types, de M0, blastes les plus indifférenciés, jusqu'au stade M8, blocage de différenciation le plus tardif [89] (Figure 4).

Cette classification est encore utilisée de nos jours, mais elle reste cependant très incomplète. En effet, elle ne prend aucunement en compte le statut mutationnel des patients. Afin de proposer un diagnostic plus complet, un criblage des anomalies

cytogénétiques récurrentes est maintenant réalisé systématiquement, permettant ainsi d'évaluer le pronostic vital et d'améliorer la prise en charge du patient en aval [90].

FAB	Description
M0	Blastes indifférenciés
M1	Sans maturation ; majorité de myéloblastes ; différenciation granulocytaire < 10%
M2	Avec maturation ; myéloblastes > 30% ; différenciation granulocytaire > 10% ; différenciation monocytaire < 20%
M3	Translocation t(15 ; 17)(q22 ; q12)
M4	LAM myélomonocytaire ; monocytes différenciés > 30 %
M5	LAM monocytaire ; monocytes différenciés > 80%
M6	LAM érythroleucémique ; érythroblastes > 50% ; myéloblastes non érythroïdes > 30%
M7	LAM mégacaryoblastique ; blastes vacuolés, pouvant présenter des expansions cytoplasmiques

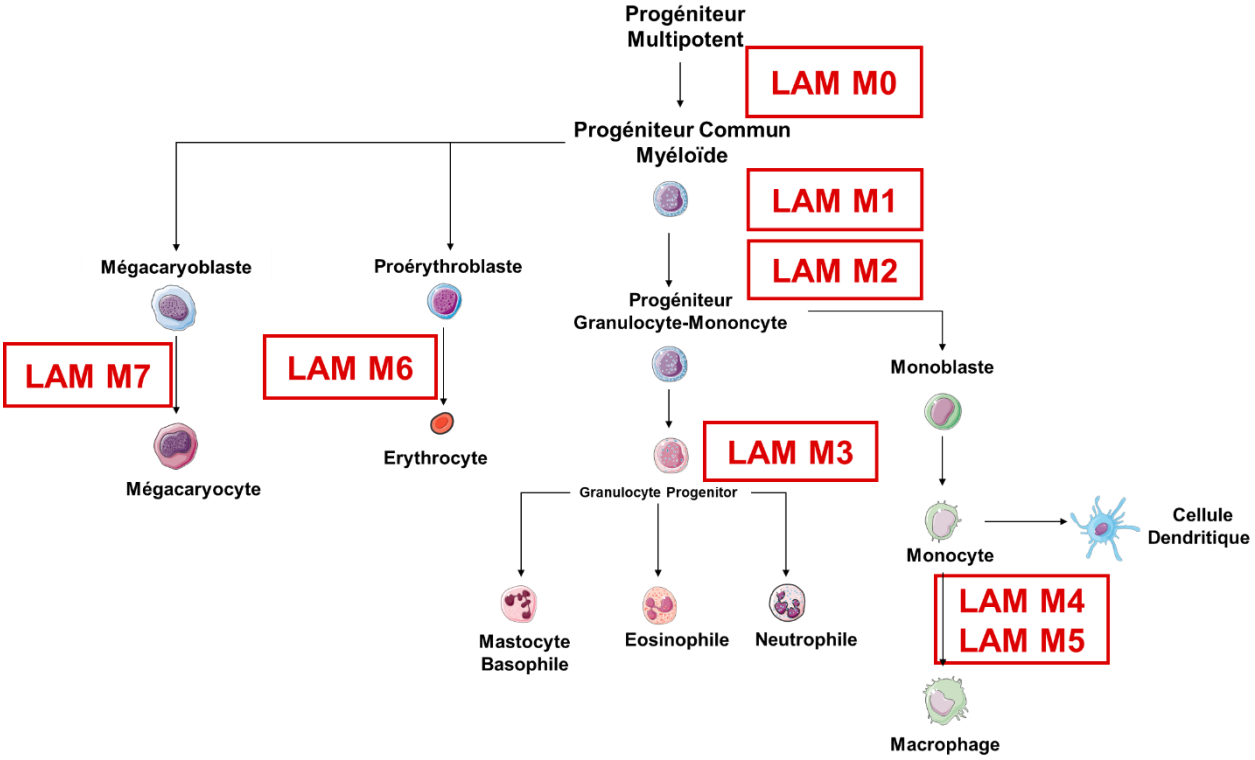

Figure 4 : Classification FAB des LAM

C. Les anomalies cytogénétiques

1. Anomalies récurrentes

En 2013, une étude réalisée à partir des cohortes du TCGA (The Cancer Genome Atlas, 2013) rapporte que les LAM présentent un nombre de mutations récurrentes parmi les plus faibles, environ une trentaine de gènes mutés en tout, et ceci tous cancers confondus [91]. Ces anomalies sont classées dans 9 groupes, prenant en compte la fonction ou la voie dans lesquelles ces gènes sont impliqués. Ainsi, on retrouve des mutations

classiquement impliquées dans le développement de cancer, comme la perte de p53, des mutations activatrices de KRAS ou des dérégulation de l'épigénome, mais aussi des mutations plus spécifiques au développement hématopoïétique, comme l'activation constitutive du récepteur FLT3, la perte de fonction de CEBPα, ou encore des translocations chromosomiques impliquant RUNX-1 (Figure 5) [92,93].

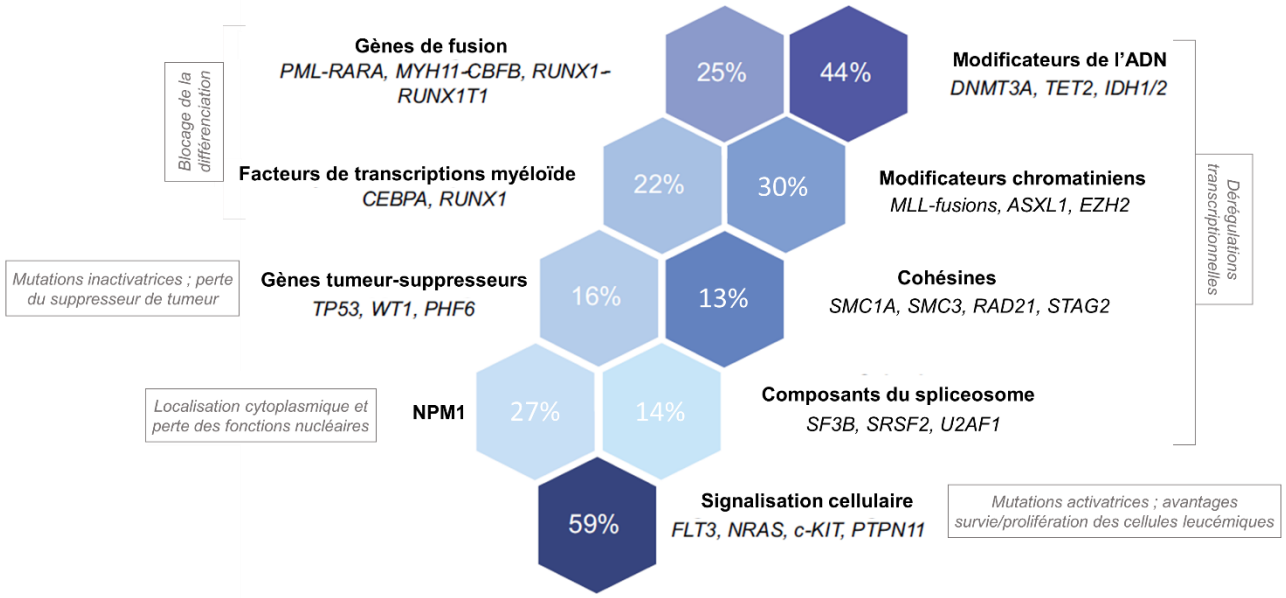

Figure 5 : Figure 5 : Mutations récurrentes dans les LAM de novo
D'après Döhner et al. 2017

De plus, l'analyse systématique du statut moléculaire des patients a permis d'observer un nombre de mutations par cas encore plus restreint, pas plus de 5 mutations pour un patient, ainsi que des schémas de co-occurrence mutationnelle [90,94,95]. L'ensemble de ces configurations d'occurrence est résumé dans la Figure 6.

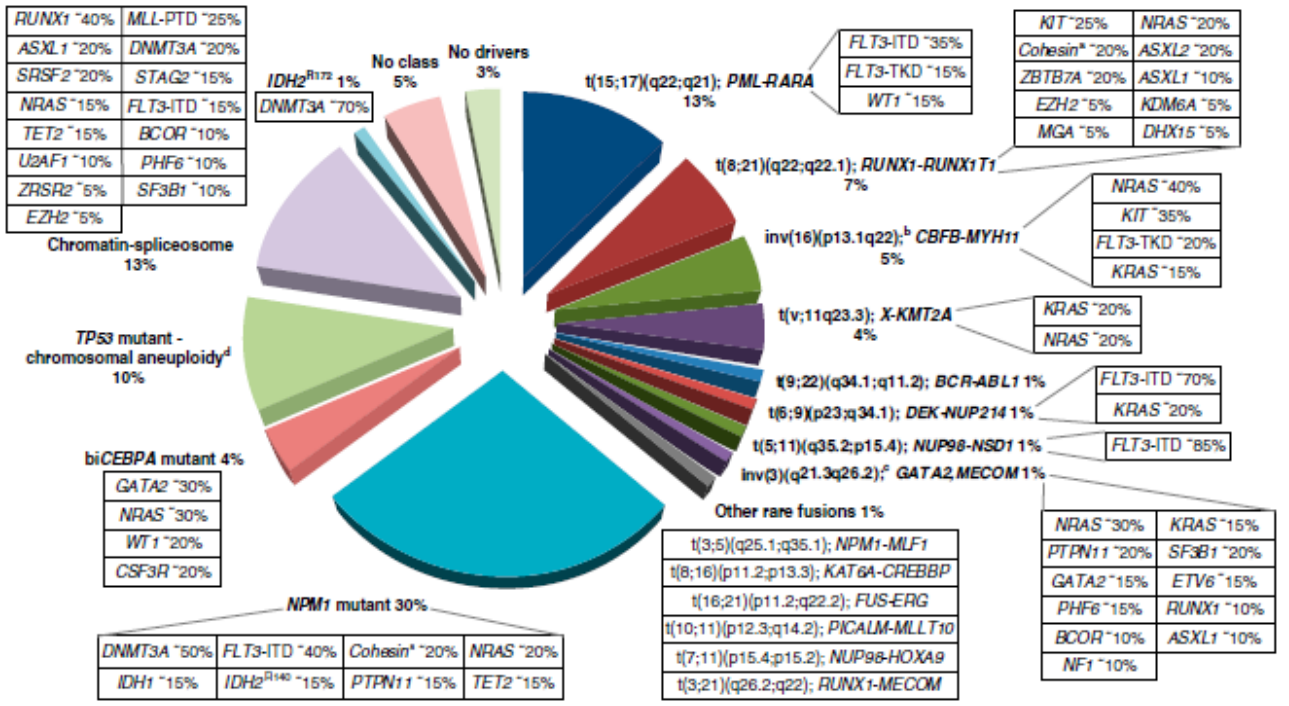

Figure 6 : Incidence et association des anomalies récurrentes dans la LAM

D'après Döhner et al., 2017

2. Impact sur le pronostic

L'identification de ces altérations récurrentes représente un puissant outil diagnostique. Basée sur des analyses rétrospectives, l'ELN (European LeukemiaNet) propose dès 2010 une classification du pronostic des patients en 3 groupes de risques, favorable, intermédiaire et défavorable, en fonction du statut génétique et mutationnel des blastes (Figure 7). Cette classification est révisée en fonction de l'évolution des connaissances et de l'identification de nouvelles anomalies, comme l'ajout des mutations de RUNX-1, ASXL1 ou TP53 en 2017 [96].

De plus, cette nouvelle stratification des risques pronostiques prend désormais en compte la cooccurrence de ces facteurs : c'est le cas de la mutation NPM1 (Nucléophosmine 1) et de son association ou non avec la mutation FLT3-ITD. La mutation NPM1, mutation très fréquente et qui représente 30% des patients, est associée à un bon pronostic. Cependant, la combinaison avec la mutation FLT3-ITD, mutation elle-aussi très fréquente et retrouvée dans 25% des cas, est associée à un risque intermédiaire. Plus encore, le ratio allèle muté/sauvage de FLT3-ITD influence la classification : NPM1 muté associé à deux allèles FLT3-ITD sera plus défavorable que NPM1 muté/FLT3-ITD mono-allélique [96].

La stratification du pronostic des patients a permis de faire évoluer leur prise en charge : plus que l'identification du stade de blocage, l'analyse moléculaire des cellules cancéreuses permet d'affiner le diagnostic et de proposer le meilleur traitement possible.

Catégorie de risque	Anomalie génétique
Favorable	t(8;21)(q22;q22.1); RUNX1-RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 Mutation NPM1 sans FLT3-ITD ou FLT3-ITD^{low} Mutation biallélique de CEBPA
Intermédiaire	t(9;11)(p21.3;q23.3); MLLT3-KMT2A Mutation NPM1 avec mutation FLT3-ITD^{high} NPM1 sauvage sans mutation FLT3-ITD ou avec mutation FLT3-ITD^{low} Toutes les autres anomalies non classes comme favorable ou défavorables
Défavorable	t(6;9)(p23;q34.1); DEK-NUP214 t(v;11q23.3); KMT2A réarrangé t(9;22)(q34.1;q11.2); BCR-ABL inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1) 25 or del(5q) ; 27 ; 217/abn(17p) Caryotype complexe (au moins 3 translocations), caryotype monosomal NPM1 sauvage avec mutation FLT3-ITD^{high} Mutation RUNX1 Mutation ASXL1 Mutation TP53

Figure 7: Stratification pronostique des patients selon L'European Leukemia Net, 2017

D'après Döhner et al., 2017

D. Prise en charge des patients

1. Traitement standard

Malgré les avancées dans la stratification des patients, notamment au niveau du pronostic, le traitement de référence n'a pas connu d'évolution majeure depuis les années 70.

a) Chimiothérapie d'induction

La première ligne de traitement, ou phase d'induction, a pour but de diminuer suffisamment la proportion de cellules leucémiques afin de restaurer une hématopoïèse normale, et procéder ensuite à une phase de consolidation. Cette première ligne de traitement reste basée sur une chimiothérapie classique : la cytarabine ou aracytine, un

analogue de la cytosine, est utilisée comme molécule de base dans les différents protocoles en combinaison avec une anthracycline, généralement la daunorubicine. Le protocole de chimiothérapie d'induction se fait sur une semaine : la cytarabine est administrée durant les 7 jours, associée à l'anthracycline durant les 3 premiers jours (protocole 3 + 7). La rémission complète des patients (proportions de blastes résiduels dans la moelle < 5%) est atteinte dans 60 à 80% des cas chez les jeunes adultes, et dans 40 à 60% des cas pour les patients de plus de 60 ans [97].

b) Stratégies de consolidation

La seconde ligne de traitement varie selon le groupe de stratification des risques :

-les patients du groupe « favorable » et pour lesquels la rémission complète a été observée, continuent d'être traités par chimiothérapie. Le protocole est cette fois à base de forte de dose de cytarabine seule.

-les patients « intermédiaires » ou « défavorables » sont orientés vers une allogreffe de cellules souches voire une autogreffe pour les plus fragiles [90].

Chez 30% des patients toutefois, la chimiothérapie d'induction ainsi que les protocoles de consolidation ne sont pas envisageables. C'est le cas des patients âgés (plus de 60 ans) ou présentant des comorbidités. On préfèrera des protocoles utilisant des doses plus faibles de cytarabine ou alors des agents hypométhylants (ou HMAs comme l'azacitidine ou la décitabine), ces protocoles ayant montrés un taux de mortalité moindre que la chimiothérapie classique. Néanmoins, la médiane de survie pour ces patients ne dépasse pas 10 mois, avec un taux de rémission complète inférieure à 20% [95], et sans réelles autres options thérapeutiques pour cette catégorie.

2. Le cas particulier des Leucémies Aigües Promyélocytaires (LAP)

La prise en charge est différente dans le cas des leucémies aiguës promyélocytaires (LAP), un sous-type de LAM de bon pronostic. Le diagnostic des LAP repose sur la détection d'une translocation chromosomique impliquant la fusion du gène PML (Promyelocytic Leukemia) avec le gène codant pour le récepteur à l'acide rétinoïque RARα. Dans 95% des cas de LAP, c'est la translocation t (15 ; 17)(q24 ; q21) qui est retrouvée, et qui donne lieu l'expression de la protéine de fusion PML- RARα [98]. Cette protéine chimère agit comme un dominant négatif de RARα, et inhibe sa fonction de facteur de transcription. En résulte un

blocage la différenciation des progéniteurs myéloïdes, une activation de la prolifération ainsi qu'une inhibition de l'apoptose [99,100].

Le traitement de la LAP est basé sur une chimiothérapie d'induction classique, associée à l'utilisation de l'ATRA (All Trans Retinoic Acid) et de l'ATO (Arsenic Tri Oxide). Ces deux molécules engendrent la dégradation de la protéine de fusion, et de fait, une levée d'inhibition de la différenciation des progéniteurs myéloïdes [101]. Cet exemple unique de thérapie par différenciation a drastiquement amélioré la survie des patients, et permet une rémission dans 75% des cas. Cependant, des mécanismes de résistances à l'ATRA et à l'ATO ont été observés, menant à des rechutes dans 10 à 15% des cas [102].

3. Nouvelles thérapies

Les fréquentes rechutes, mais aussi l'absence de réponse à la chimiothérapie observée chez certains patients soulignent le besoin de développer de nouvelles thérapeutiques. De nombreux essais cliniques ont été initiés et certains sont encore en cours. Ces recherches ont tout de même permis à la FDA (Food and Drug Administration) d'approuver 8 nouvelles molécules dans le traitement des LAM entre 2017 et 2019 ; le spectre d'action de ces molécules est très large : on retrouve des molécules de thérapies ciblées, mais aussi des immunothérapies ou encore des inhibiteurs de protéines anti-apoptotiques.

a) Amélioration de la chimiothérapie

Le CPX-351 est une encapsulation liposomale d'un mélange de cytarabine et de daunorubicine à un ratio molaire de 5:1 [103,104]. En 2017, la FDA approuve l'utilisation de ce protocole dans 2 cas : LAM secondaire due à un syndrome myélodysplasique et LAM secondaire provoquée par un traitement préalable, qui sont toutes 2 des LAM de mauvais pronostic [105].

De plus, en 2018, un essai clinique confirme que l'utilisation du CPX-351 en première ligne de traitement pour des patients entre 60 et 75 ans améliore significativement la survie globale, ainsi que la survie sans progression [106].

b) Les thérapies ciblées

-Inhibition de FLT3 :

FLT3 (Fms-Like Tyrosine Kinase) est un récepteur à activité tyrosine kinase exprimé notamment par les progéniteurs multipotents. L'activation de FLT3 est reliée à des processus tels que la prolifération ou la survie [11,107]. Les mutations du gène FLT3 représentent environ un tiers des patients atteints de LAM nouvellement diagnostiquée : environ 20 à 25% de ces mutations sont dites ITD (Internal Tandem Duplication) et 5 à 10% sont dites TKD (Tyrosine Kinase Domain), les deux conduisant à une activation constitutive du récepteur. Ce sont des LAM de mauvais pronostique, principalement en cas de combinaison avec les mutations NPM1 et DNMT3A [108,109].

On dénombre trois inhibiteurs de FLT3 qui ont montré une amélioration de la survie globale : la midostaurine, le quizartinib et le giltertinib. Seuls la midostaurine et le giltertinib ont été approuvés par la FDA, respectivement en 2017 et en 2018, le quizartinib n'ayant pas montrés des résultats suffisamment robustes concernant l'amélioration de la survie globale en phase II d'essai clinique. Cependant, il a été approuvé dans le traitement des LAM au Japon en 2019, et est en cours d'autorisation dans d'autres pays [95].

La midostaurine est administrée en combinaison avec la chimiothérapie en phase d'induction et en phase de consolidation. Cependant, des résistances ont rapidement été observées, provoquées par des mutations secondaires du gène FLT3. Le giltertinib, un inhibiteur de seconde génération, a donc été approuvés dans le cas de LAM en rechute associée à une persistance de FLT3 muté [110,111].

Enfin, plusieurs essais cliniques sont en cours afin de statuer sur l'importance de la spécificité des inhibiteurs utilisés. En effet, la midostaurine cible différents récepteurs à activité tyrosine kinase, comme c-kit ou PDGFR. Le giltertinib est lui plus spécifique de FLT3. Des études de phase III sont par conséquent actuellement en cours, et comparent l'association chimiothérapie/midostaurine avec l'association chimiothérapie/giltertinib (références essais cliniques : NCT03836209, NCT03836209).

-Inhibition de c-Kit :

L'utilisation des inhibiteurs de tyrosine kinases s'est étendue à d'autres mutations que celles de FLT3. Bien que n'ayant pas encore été approuvés, des protocoles de phases I et II utilisent la midostaurine ou le dasatinib, tous deux des inhibiteurs multi-kinases, dans le cas de patients porteur de mutations de c-Kit.

C-Kit est également un récepteur à activité tyrosine kinase, exprimé par les CSH et les progéniteurs multipotents. Il est associé aux LAM porteuses des translocations CBFB-MYH11 et RUNX1/RUNX1T1 dans 25% des cas ; chez ces patients, la présence de la mutation est de moins bon pronostic que chez les patients porteurs de la forme sauvage de c-Kit [112].

Dans le cas de patients nouvellement diagnostiqués et porteurs d'une translocation CBFB-MYH11, des essais de phase II ont combiné le dasatinib avec le protocole classique de chimiothérapie d'induction (régime 7+3), suivi d'un protocole de consolidation à base de cytarabine à haute dose, associée là encore au dasatinib. Ces essais ont montré une amélioration de la survie sans progression ainsi qu'un retard de la rechute des patients [113]. Actuellement, une phase III est en cours afin de valider les précédents résultats (référence essai clinique : NCT02013648).

-Inhibition des isocitrate-déshydrogénases (IDH) :

IDH1 et IDH2 (Isocitrate Déshydrogénase 1 ou 2) sont des enzymes responsables de la production d'α-cétogluratate (α-KG) à partir de l'isocitrate. Des mutations peuvent affecter leur fonction, et conduisent à la production de 2-hydroxyglutarate (2-HG), un oncométabolite, qui provoque une inhibition compétitive des enzymes dépendantes de l'α-KG. Ces enzymes sont pour la plupart impliquées dans les régulations épigénétiques, comme TET2 (Ten-eleven-translocation 2), une méthylcytosine dioxygénase impliquée dans la déméthylation de l'ADN, particulièrement impliquée dans la différentiation des CSH. De fait, l'inhibition de leur fonction par le 2-HG conduit à un blocage de la différenciation hématopoïétique [114]. Ces mutations se retrouvent dans 5 à 15% (IDH1) et 10 à 15% (IDH2) des patients nouvellement diagnostiqués.

En 2018, la FDA approuve deux molécules, l'ivosidenib et l'enasidenib, dans le cas des patients en rechute ou porteur de mutations réfractaires d'IDH1 ou d'IDH2. L'ivosidenib est également approuvé en 2019 en tant qu'agent seul, dans le cas de patients nouvellement diagnostiqués et non éligible à la chimiothérapie intensive [115,116].

Cependant, des résistances sont d'ores et déjà apparues, notamment un échange de l'isoforme muté ou encore le développement de nouveaux sites de mutation [117,118].

-Réactivation de p53 :

Des mutations du gène TP53 sont également détectées dans 5 à 20% des patients nouvellement diagnostiqués. Cette incidence augmente encore chez des patients âgés ou

dans le cas de LAM secondaires [94]. La seule présence d'une mutation de TP53 est associée à un mauvais pronostic [119].

L'APR-246 est une molécule qui permet de restaurer l'activité transcriptionnelle des mutants p53, conduisant à la réactivation de sa signalisation jusqu'à l'apoptose des cellules cancéreuses [120,121]. Des essais de phase Ib et II ont utilisés l'APR-246 en combinaison avec l'azacitidine sur des patients porteurs de mutation de TP53 et non éligible à la chimiothérapie intensive. Cette combinaison a montré une rémission complète des patients dans 82% des cas, et 72% des patients répondeurs ne présentaient aucune trace de mutation de TP53 après séquençage [122]. Actuellement, une phase III est en cours afin de confirmer les précédents résultats (référence essai clinique : NCT03745716).

c) *Activation de la voie apoptotique*

La dérégulation de l'apoptose est une des « hallmarks » des cellules cancéreuses et la LAM ne fait pas exception. Comme exposé ci-dessus, restaurer l'activité de p53 semble être une approche prometteuse. Cependant, il est fréquent de retrouver des protéines anti-apoptotiques, comme BCL-2 ou BCL-XL, surexprimées dans les LAM, menant notamment à une résistance à la chimiothérapie. C'est pourquoi plusieurs études proposent de cibler directement les composantes de la voie apoptotique.

-Inhibition de BCL-2 :

En 2018, la FDA approuve l'utilisation du vénétoclax, un inhibiteur de BCL-2, dans le traitement des LAM, en combinaison avec de l'azacitidine ou de la décitabine, pour des patients non éligibles à la chimiothérapie intensive. Cependant, les résultats de phase III de ces protocoles sont encore en cours (référence essai clinique : NCT03586609).

Plusieurs essais de phase III sont également encore en cours afin de comparer les effets du vénétoclax en combinaison avec les protocoles classiques de première ligne : la chimiothérapie d'induction classique (référence essai clinique : NCT03709758) ou le CPX-351 (référence essai clinique : NCT03629171). Toutefois, des rechutes associées à des résistances au vénétoclax sont déjà observées.

-Inhibition de MCL-1 :

Le mécanisme de résistance au vénétoclax le mieux décrit actuellement est l'augmentation de MCL1 (Myeloid Cell Leukemia 1), qui est également une protéine anti-apoptotique. Des données précliniques ont permis d'identifier des inhibiteurs de MCL1 (comme l'AMG-176) qui montrent une synergie avec le vénétoclax [123], allant même jusqu'à

inverser la résistance au vénétoclax [124,125,126]. Cependant, les essais cliniques visant à étudier les bénéfices de ces molécules en sont encore à leur début [127].

-Inhibition de MDM2 :

Dans le cas de l'absence de mutation de p53, une altération dans le processus apoptotique peut être due à une surexpression de MDM2 (Murine Double Minute 2). MDM2 forme un complexe avec p53, et conduit à l'inhibition de son activité transcriptionnelle, couplée à une dégradation de ce dernier [128].

Différents inhibiteurs de MDM2, comme l'idasanutlin ou le milademetan, ont été étudiés en phase Ib et II dans le cadre de la LAM [129]. Seul l'idasanutlin est actuellement en phase III en association avec la chimiothérapie, dans le cas de patients en première rechute (référence essai clinique : NCT02545283).

De plus, de récentes études de phase Ib montre une synergie entre l'idasanutlin et différents autres agents comme le vénétoclax dans le cas de patient de plus de 60 ans en rechute ou inéligible à la chimiothérapie [130].

d) Les immunothérapies

Le développement d'immunothérapie dans le cadre des LAM est étudié depuis un certain temps, étant donné les résultats bénéfiques observés dans le cadre de greffe allogénique et de DLI (Donor Lymphocyte Infusions), qui consiste en une injection de lymphocyte T activés du donneur faisant suite à une greffe allogénique.

-Les anticorps monoclonaux :

Le Gemtuzumab Ozogamicine (GO) est le premier exemple d'anticorps à avoir été développé. Le GO est un anticorps monoclonal, ciblant le CD33 et couplé à une molécule de chimiothérapie, la calicheamicine [131]. Le CD33 est exprimé par les progéniteurs myéloïdes et des cellules plus différenciées, mais n'est pas retrouvé dans le compartiment souche. Le ciblage grâce au CD33 permet ainsi d'intensifier les doses de chimiothérapie, tout en réduisant sa toxicité [132], mais les effets bénéfiques sont toutefois plus manifestes dans le cas de LAM de bon pronostic [133]. Il est approuvé en 2017 par la FDA en première ligne de traitement et associé à la chimiothérapie d'induction classique, ou en tant qu'agent seul pour les patients non-éligible ou en rechute et présentant une résistance à la chimiothérapie [134,135].

D'autres types d'anticorps anti-CD33 ont ensuite été développé, conjugués à différents types de molécules, comme le dimère de pyrrolobenzodiazepine (molécule de

chimiothérapie), le DGN462 (agent alkylant), mais aussi des molécules de radiothérapie [136], et sont encore en phase I ou II d'essai clinique [137,138].

D'autres études mettent en évidence l'intérêt de cibler le CD45, une phosphatase membranaire exprimée par l'ensemble des cellules immunitaires, souches ou différenciées. Les anticorps anti-CD45 menant à une myéloablation très efficace, ils ont été proposés en tant que pré-traitement avant une allogreffe. Une étude de phase III est actuellement en cours et compare les effets du Iomab-B, un anti-CD45 couplé à une molécule de radiothérapie, chez des patients éligibles à une allogreffe (référence essai clinique : NCT02665065).

-Les inhibiteurs des « immune checkpoint » :

Les checkpoints immunitaires ont été retrouvés augmentés après la chimiothérapie et, dans une moindre mesure, après exposition aux HMAs [139,140], suggérant qu'ils pourraient représenter un mécanisme de résistance à ces thérapies conventionnelles. En effet, ces traitements sont connus pour faciliter la destruction des cellules leucémiques par les cellules immunitaires : la chimiothérapie provoque l'expression de néo-antigènes, menant l'activation des macrophages et des cellules dendritiques [141]. De même, les HMAs conduisent à la réexpression de néo-antigènes leucémiques préalablement réprimés, ou de rétrovirus endogènes qui activent l'expansion des lymphocytes T cytotoxiques [142].

Un essai en phase III est actuellement en cours, qui compare l'effet de l'azacitidine en présence ou non de nivolumab (anti-PD1) dans le cas de patients nouvellement diagnostiqués et non-éligible à la chimiothérapie (référence essai clinique : NCT03092674). L'utilisation du nivolumab en agent seul, comme thérapie de maintenance est également étudié en phase II dans le cas de patient en rémission ayant terminé leur phase de consolidation (référence essai clinique : NCT02275533).

-Les vaccins :

Malgré l'absence d'antigènes de surfaces spécifiques aux LAM, le développement de vaccins a été exploré en tant que thérapie de maintenance post-chimiothérapie ou post-greffe allogénique.

L'antigène WT1 (Wilms Tumor antigen 1), retrouvé surexprimé par les cellules leucémiques, a été étudié dans les LAM en rémission complète, en phase I et II [143,144,145]. Les patients vaccinés avec les peptides WT1 montrent une amélioration de la survie globale, mais une phase III est maintenant nécessaire pour confirmer ces résultats.

Les alternatives thérapeutiques commencent donc à se développer dans le cas des LAM en rechute ou contractées par des sujets fragiles. Cependant, ces traitements permettent d'améliorer sporadiquement la survie, et les rechutes dues à des résistances sont encore nombreuses.

4. Les micro-ARNs comme outil diagnostic et thérapeutique dans LAM

a) Biogénèse et mécanismes d'action des micro-ARN

Les micro-ARNs (ou miRNA) sont de petits ARNs non codant d'une vingtaine de nucléotides, impliqués dans la régulation et la stabilité d'ARNm cibles.

Ces petits ARNs régulateurs sont majoritairement transcrits par l'ARN-polymérase II, à partir de séquences introniques (30% environ), ou d'unités transcriptionnelles propres. Le produit de transcription est appelé micro-ARN primaire (ou pri-miRNA), et possède une structure secondaire en tige-boucle, contenant la séquence du miRNA. Le pri-miRNA est rapidement pris en charge par le complexe nucléaire microprocesseur, un complexe comprenant un hétérodimère de la ribonucléase III Drosha et de DGCR8 (DiGeorge Syndrome Critical Region 8) [146,147]. Le pri-miRNA est clivé au niveau de la structure tige-boucle en un pré-miRNA, exporté dans le cytoplasme *via* la protéine XPO5 [148,149]. A nouveau, le pré-miRNA est clivé par la ribonucléase cytoplasmique de type III DICER, au niveau de la structure boucle, en un miRNA duplex d'une vingtaine de nucléotides. L'interaction de DICER avec TRBP permet l'incorporation du miRNA duplex dans le complexe Argonaute, afin de former le miRISC (miRNA-induced silencing complex) [150,151,152] (Figure 8).

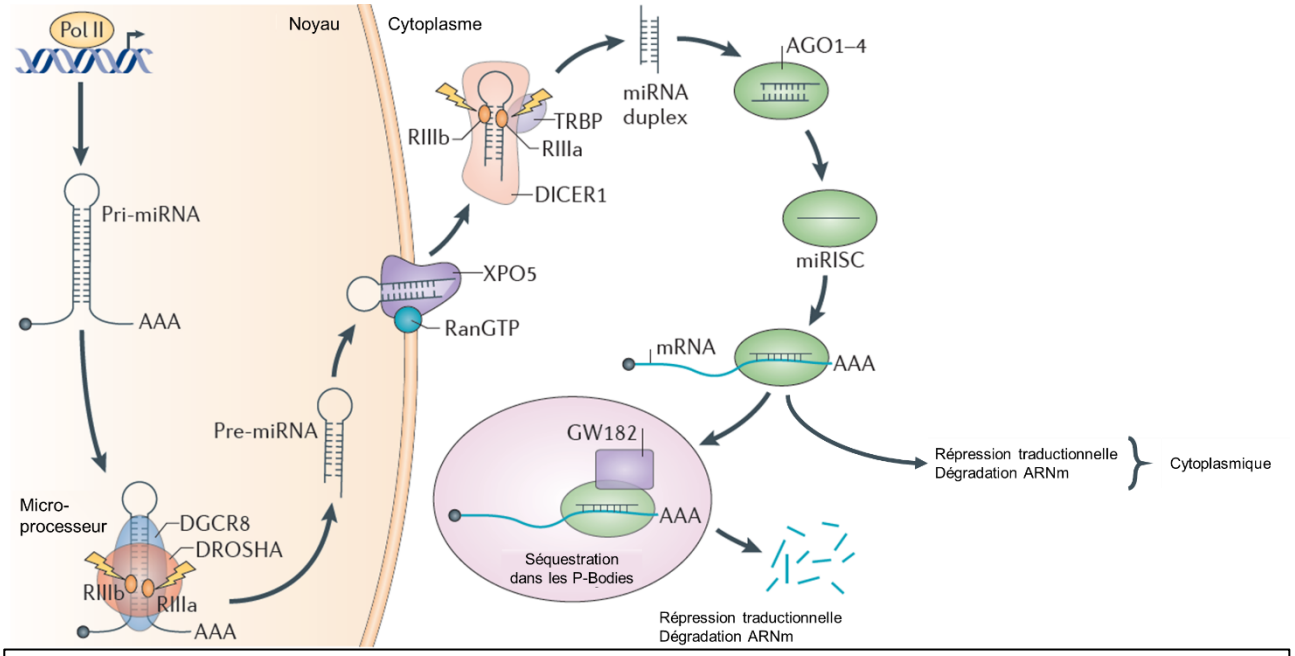

Figure 8 : Biogénèse des micro-ARNs

La majorité des microARNs sont transcrits par l'ARN polymérase II qui génère un pri-miRNA. Le complexe nucléaire DROSHA/DGCR8 réalise une première étape de maturation et produit un pré-miRNA qui est ensuite exporté dans le cytoplasme par XPO5. Une seconde étape de maturation est réalisée par le complexe cytoplasmique DICER/TRBP. Le duplex de microARN ainsi généré est intégré au complexe Argonaute, et forme le miRISC à partir d'un seul brin du duplex. Les micros-ARNs sont impliqués dans la répression de l'expression de messagers cibles par dégradation de l'ARN ou par inhibition de sa traduction. Ces étapes peuvent être cytoplasmiques, ou se dérouler dans les Processing-bodies (P-Bodie).

D'après Lin & Gregory, 2015

L'interaction entre le miRNA et sa cible se fait par le biais d'une séquence « seed », très conservée, retrouvée au niveau des nucléotides 2 à 8 du micro-ARN. Cette séquence « seed » est complémentaire ou partiellement complémentaire de séquences contenues majoritairement dans le 3' non-traduit des ARNm cibles [153]. Le mécanisme de répression du messager est dépendant de l'intensité de l'interaction entre le miRNA et l'ARNm cible. Une complémentarité parfaite provoque une dégradation du messager via des capacités endonucléolytiques de certaines protéines du complexe Argonaute [154,155]. Cependant, ce mécanisme n'est absolument pas majoritaire chez les mammifères, et a été reporté pour seulement une vingtaine de transcrits [156,157]. Dans le cas des métazoaires, l'appariement entre le miRNA et sa cible n'est pas parfait et n'induit pas de dégradation du messager, mais une inhibition de sa traduction. En effet, la liaison du complexe miRISC en 3' non-traduit d'un messager cible recrute la protéine adaptatrice TNRC6. TNRC6 provoque une inhibition de l'initiation de la traduction *via* son interaction avec le complexe DDX6--4E-T, qui se lie au facteur d'initiation de la traduction eif4E et inhibe la traduction. L'interaction DDX6-4E-T est également impliqué dans la formation de P-bodies et participe au

confinement des messagers cibles dans ces structures subcellulaires [158]. TNRC6 est également impliqué dans la régulation de la stabilité du messager ; elle permet le recrutement de complexes de dé-adénylation comme CCR4-NOT et PABPC. La réduction de la taille de la queue poly(A) induit un enlèvement de la coiffe et une dégradation de la cible (Figure 9).

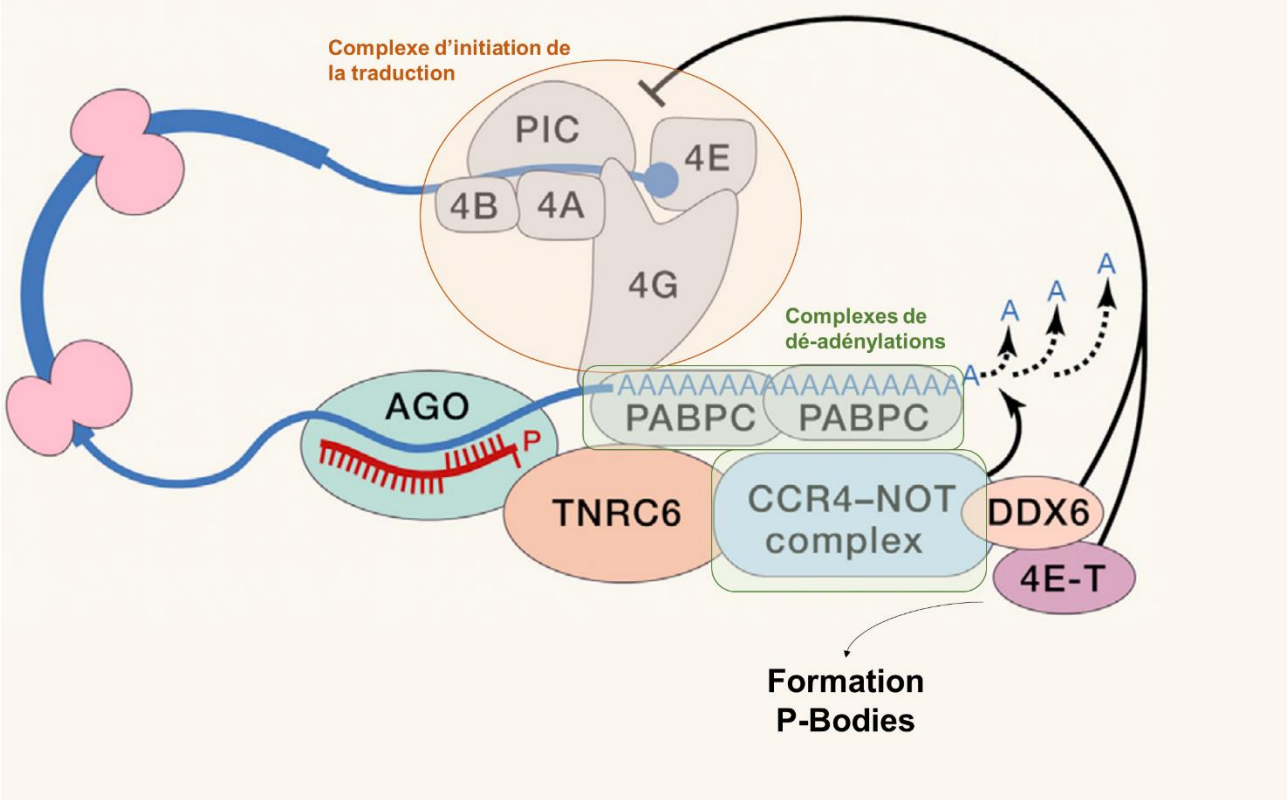

Figure 9 : Mécanismes d'action de miRNA chez les métazoaires

La fixation du complexe miRISC sur un ARNm cible provoque la répression de son expression par 2 mécanismes :

- *Le recrutement des facteurs de dé-adénylation PABPC, PABPC et CCR4-NOT par TNRC6 provoque une réduction de la queue poly(A) du messager, ce qui favorise l'enlèvement de la coiffe et la dégradation du messager.*
- *TNRC6 recrute également un complexe protéique composé de CCR4-NOT--DDX6--4E-T, qui inhibe l'inhitiation de la traduction et qui participe à la mise en place de P-bodies et à la séquestration des ARNs cibles.*

D'après Bartel, 2018

b) Dérégulation des micro-ARNs dans les LAM

La versatilité des micro-ARNs a permis de les relier à de nombreux processus cellulaire. Il n'est pas étonnant de les voir dérégulés dans les cancers, et la LAM ne fait pas exception.

De nombreuses études ont mis en évidences l'importance des micro-ARNs dans le processus de leucémogénèse. L'ensemble des études menées sur le sujet dénombre une vingtaine de micro-ARNs dérégulés dans les LAM [159]. Par exemple, miR-155, un des micro-ARN les plus étudiés dans la LAM, est retrouvé surexprimé un grand nombre de sous-type

de LAM [159]. MiR-155 est induit par c-MYB et inhibe l'expression du FT PU.1, indispensable à la différenciation myéloïde. L'inhibition de l'oncogène c-Myb ou de l'oncomiR miR-155 rétablit l'expression de PU.1 et la différenciation des blastes leucémiques [160,161]. A l'inverse, miR-29b n'est plus exprimé dans certaines LAM, dû à la perte de CEBPα ou par une répression transcriptionnelle par différents oncogène comme c-Myc. La perte de miR-29b favorise la prolifération des blastes par différents processus comme l'inhibition de l'apoptose, ou la surexpression du facteur pro-survie KIT [162,163,164].

Plus généralement, l'analyse fonctionnelle des micro-ARNs dans la LAM a révélé une expression dépendante du sous-type de LAM c'est-à-dire dépendant de l'environnement génétique et mutationnel. Ainsi, l'expression de miR-182 est spécifiquement perdue dans les LAM mutée CEBPα [165] ; à l'inverse, miR-9 est une cible transcriptionnelle de MLL (Mixed-Lineage Leukemia) et se retrouve augmenté dans les LAM exprimant une protéine de fusion impliquant MLL [166].

c) Utilisation des micro-ARNs comme outil diagnostic/pronostic

Malgré ces profils d'expression type, l'analyse des blastes de 122 patients nouvellement diagnostiqués a permis d'établir une signature pronostique de 5 micro-ARNs (miR-20a, miR-25, miR-191, miR-199a, et miR-199b), associée à une diminution de la survie globale des patients et ce, indépendamment du sous-type de LAM considéré [167].

De plus, les différentes propriétés des micro-ARNs en font des candidats tout indiqués pour de potentiels biomarqueurs. En effet, ce sont des ARNs très conservés parmi les espèces, ils sont retrouvés dans les fluides corporels comme le sérum, la salive ou les urines et enfin, ils sont détectables dans ces mêmes fluides. Ainsi, l'analyse du sérum de 140 patients non traités par rapport à 135 donneurs sains a révélé une augmentation des taux circulants de 6 miRNAs, miR-10a-5p, miR-93-5p, miR-129-5p, miR-155-5p, miR-181b-5p et miR-320d chez les patients et pourrait représenter un outil diagnostic non invasif [168]. Plus particulièrement, miR-181b-5p circulant a été corrélé à une meilleure survie globale [168]. Cependant, à l'heure actuelle, aucune de ces signatures n'est utilisée en clinique.

d) Les micro-ARNs comme cible thérapeutique

A l'image de l'utilisation de miRNA comme biomarqueurs, l'utilisation des micro-ARNs comme cible thérapeutique dans la LAM n'en est encore qu'au stade des études précliniques.

La principale stratégie thérapeutique investiguée consiste en l'utilisation d'oligonucléotides doubles brins porteur de la séquence d'intérêt, les miRNA mimics, qui

permettent la surexpression de micro-ARNs suppresseurs de tumeurs. L'utilisation de nanoparticules porteuses d'un miR-29b améliore la survie de souris dans un modèle de xénogreffe, due à une inhibition de la croissance des cellules leucémiques [169]. L'injection de ces nanoparticules ne s'accompagne d'aucune toxicité rénale ou hépatique. Une autre étude démontre que la surexpression de miR-22-3p, également par un système de nanoparticule, inhibe la progression de la maladie *in vivo* [170].

Une deuxième stratégie consiste en l'inhibition des oncomiRs par l'utilisation d'oligonucléotides antisens et complémentaires du miRNA ciblé (antagomiR). Par exemple, l'inhibition des miR-21 et 196b par l'administration d'antagomiR dans un modèle de LAM MLL-AF9 a montré des propriété curatives sur les animaux xénogreffés [171].

La caractérisation fonctionnelle des micro-ARNs, associée au développement de nouvelles technologies d'administration, permettent d'envisager leur utilisation en clinique, non seulement en tant qu'outil diagnostique/pronostique mais également comme cibles thérapeutiques.

III. Le stress du Réticulum Endoplasmique

A. Le Réticulum Endoplasmique (RE)

1. Généralités

Le Réticulum Endoplasmique est un organite intracellulaire observé pour la première fois par Charles Garnier en 1897, dans des cellules de pancréas exocrines. L'analyse par microscopie optique de cet « ergastoplasme » lui permet déjà de conclure que cette structure est nécessaire au processus de sécrétion de ces cellules (Garnier Ch. Les filaments basaux des cellules glandulaires. Bibliographie anatomique, 1897, V, 278-289.). L'arrivée de systèmes plus résolutifs comme la microscopie électronique ont permis dès les années 40 de décrire plus précisément la structure de cet organite pouvant représenter à lui seul plus de 50% des membranes d'une cellule eucaryote [172,173] : la structure générale du réticulum endoplasmique est un réseau de membranes appelées citernes (cisternae). Une membrane phospholipidique renferme l'espace cisternal (ou lumière de réticulum), qui est continu avec l'espace périnucléaire. Le RE est une structure régionalisée, qui témoigne d'une versatilité fonctionnelle. Trois types de régions se distinguent :

- Le RE rugueux : la surface du réticulum endoplasmique rugueux (ou RER) est associée à de nombreux ribosomes lui donnant un aspect "rugueux" en microscopie. Il est le siège d'une forte activité traductionnelle, et permet de synthétiser les protéines sécrétées, membranaires, et résidentes du RE mais aussi les protéines lysosomales. Pour ce faire, la lumière du RER possède un microenvironnement particulier. On y retrouve notamment des protéines chaperonnes, spécialisées dans l'acquisition de la structure tridimensionnelle des protéines néosynthétisées, mais également un contexte redox particulier, avec une forte concentration en calcium cationique Ca^{2+}, nécessaire à l'activité de ces enzymes [174,175].

- Le RE lisse (ou REL) est marqué par l'absence de ribosome. Il permet la synthèse des lipides membranaires comme les phospholipides, et le cholestérol [176,177]. C'est également la zone de stockage du calcium, qui permet non seulement le bon fonctionnement des protéines chaperonnes du RER, mais permet également l'exocytose des vésicules du flux sécrétoire lorsque qu'il est libéré dans le cytosol [178]. Enfin, le REL est impliqué dans des processus métaboliques comme la néoglucogénèse avec la présence de la glucose-6-phosphatase, ou encore la détoxification via l'action du cytochrome P450.

- Les éléments de transition : c'est une zone de fort trafic vésiculaire, qui permet l'envoi des protéines néosynthétisées et des lipides vers l'appareil de Golgi, mais aussi la réception de vésicules en provenance de cet organite [179,180].

Outre ses fonctions propres, le RE forme également des contacts physiques avec d'autres structures cellulaires. Ainsi, il partage des zones d'interactions avec la mitochondrie, au niveau des MAMs (Mitochondrial-Associated Membrane), avec la membrane plasmique, les endosomes et même l'appareil de Golgi [181,182,183] (Figure 10). Ces multiples communications permettent de faire du RE un « détecteur » des fluctuations énergétiques, et de coordonner l'ensemble des réponses nécessaire à la régulation du métabolisme et du devenir cellulaire (Figure 10) [184].

Le RE est donc impliqué dans des processus aussi divers que fondamentaux, et permet un maintien global de l'homéostasie cellulaire. Par conséquent, de nombreuses altérations, comme le développement de maladies ou des variations dans le microenvironnement cellulaire (carences, hypoxie…) peuvent affecter ces multiples fonctions et conduire à un stress du RE.

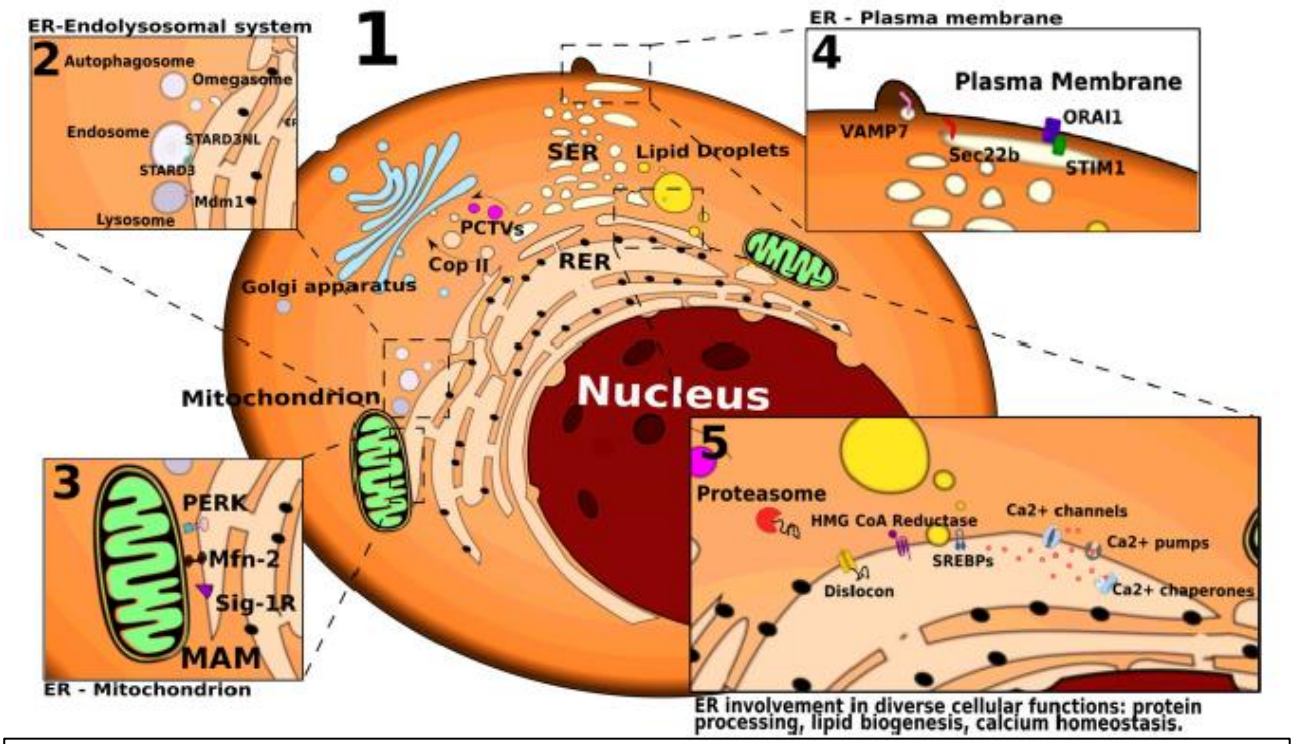

Figure 10 : Implications pléiotropiques du Réticulum Endoplasmique

Le Réticulum endoplasmique est à la base du flux sécrétoire et permet la production des protéines sécrétées et membranaires (panel 1 et 4). De plus, il est impliqué dans le stockage du calcium cationique ainsi que de la synthèse lipidique (panel 5). Cependant, il existe des zones contacts et d'échange avec d'autres organites comme les endosomes et les lysosomes, via STARD3, STARD3NL et Mdm1 (panel 2), ou avec la mitochondrie au niveau des MAMs, via Mfn-2, Sig-1R et PERK (panel 3).

D'après Almanza et al., 2019

2. Troubles de l'homéostasie du RE

a) Perturbations de l'homéostasie lipidique

Différentes études ont permis de lier étroitement le RE au métabolisme des lipides. Il permet non seulement la production d'une partie des lipides membranaire mais il est également impliqué dans leur stockage : dans les adipocytes par exemple, la formation des gouttelettes lipidiques est dépendante de l'activité d'une enzyme du RE, DGAT1, qui catalyse l'estérification des acides gras libres en triglycérides [185]. Il n'est donc pas surprenant qu'une dérégulation dans le métabolisme lipidique entraîne un stress du RE. Ainsi, une trop forte concentration en acides gras libres est à elle seule responsable de l'activation d'un stress du RE dans différents types cellulaires comme les cellules pancréatiques, hépatiques, musculaires ou encore cardiaques [186,187,188,189]. Dans ce cas, l'activation du stress du RE permet de réduire la lipotoxicité en induisant le stockage des acides gras sous forme de gouttelettes lipidiques [190,191]. Le stress du RE est également retrouvé activé dans le cas de certaines maladies impliquant des troubles du métabolisme lipidique, telles que la stéatose hépatique (excès de graisse dans les hépatocytes), la

dyslipidémie (concentration trop élevée/basse dans le sang) ou encore l'insulinorésistance dans le diabète de type 2 [192,193,194].

Néanmoins, le lien entre lipides et induction du stress du RE n'a pas encore été clairement élucidé. Les modifications de la composition de la membrane du RE restent l'hypothèse majeure à ce jour. En effet, une diminution des phospholipides insaturés au niveau de la membrane du RE conduit à l'activation du stress du RE [195,196]. En outre, des protéines virales enchâssées dans la membrane du RE peuvent induire un stress du RE : c'est le cas des protéines du virus de l'hépatite C (HCV), NS4B, ou du Flavivirus ; elles provoquent des variations de la composition de la membrane afin de produire des particules virales qui bourgeonnent à partir du RE. Le cycle viral et les modifications de membrane qu'il engendre a pour conséquence d'activer un stress du RE chronique au niveau de la cellule hôte [197,198].

b) Perturbations de l'homéostasie calcique

Le calcium cationique Ca^{2+} est un ion très polyvalent, impliqué dans de nombreux processus comme la sécrétion, l'activité neuronale ou la contraction musculaire. Cependant, il est très délétère pour les cellules et constitue un message apoptotique lorsque sa concentration cytosolique est trop élevée. Le calcium est donc majoritairement stocké dans la lumière du réticulum endoplasmique, à une concentration pouvant atteindre 5mM quand celle du cytosol est activement maintenue à 1µM [199]. Le calcium est par ailleurs un cofacteur crucial pour les chaperonnes résidentes du RE : il maintient un environnement oxydant optimal pour leur activité et une diminution trop importante de la concentration de calcium intraluminale conduit à une accumulation de peptides mal-conformés, cause systématique d'un stress du RE [200,201] (Figure 9).

c) Perturbations de l'homéostasie protéique

De façon générale, tout évènement qui altère les capacités de production des protéines est susceptible d'induire un stress du RE.

Comme évoqué ci-dessus, les acides gras libres peuvent être directement médiateurs d'un stress du RE, mais ils peuvent également moduler la synthèse des peptides intraluminaux. En effet, la palmitoylation est une modification post-traductionnelle réversible qui engendre l'ajout d'un résidu palmitate, un lipide, sur une cystéine. Cette modification impacte la stabilité de la protéine, ses capacités d'agrégation ou encore sa localisation [202,203]. Une trop forte concentration en palmitate libre conduit alors à une palmitoylation aberrante des protéines néosynthétisées, conduisant à un stress du RE [204].

De plus, l'acquisition de la forme tridimensionnelle des protéines nécessite un certain nombre de modifications co-traductionnelles, comme la formation de ponts disulfure. La formation de cette liaison covalente est catalysée par la PDI (Protein Disulfide Isomérase), mais implique un environnement oxydant [205]. En cas d'hypoxie, la diminution de concentration en O_2 perturbe l'action de la PDI, mais également l'efflux des protéines du RE vers le Golgi, conduisant à un stress du RE [206,207,208]. A l'inverse, les traitements chimiothérapeutiques, les rayons ou encore une inflammation peuvent induire un stress oxydatif, par la production excessive de ROS (Reactive Oxigen Species). Or, une oxydation excessive des protéines conduit à une induction du stress du RE [209].

La formation de protéines fonctionnelles passe également par des modifications de certains de leurs résidus : la N-glycosylation consiste à lier un complexe osidique au niveau d'un résidu asparagine. Cette modification post-traductionnelle est réversible, et permet notamment d'augmenter la solubilité et la stabilité des protéines [210]. Cependant, une carence en glucose affecte directement ce processus, conduisant là encore à un stress du RE (Figure 11).

Le stress du réticulum endoplasmique est donc le point de convergence de nombreux stress, endogènes comme exogènes, délétères voir fatals pour la cellule. Afin de restaurer l'homéostasie du RE, une voie physiologique de réponse au stress est activée : l'Unfolded Protein Response ou UPR.

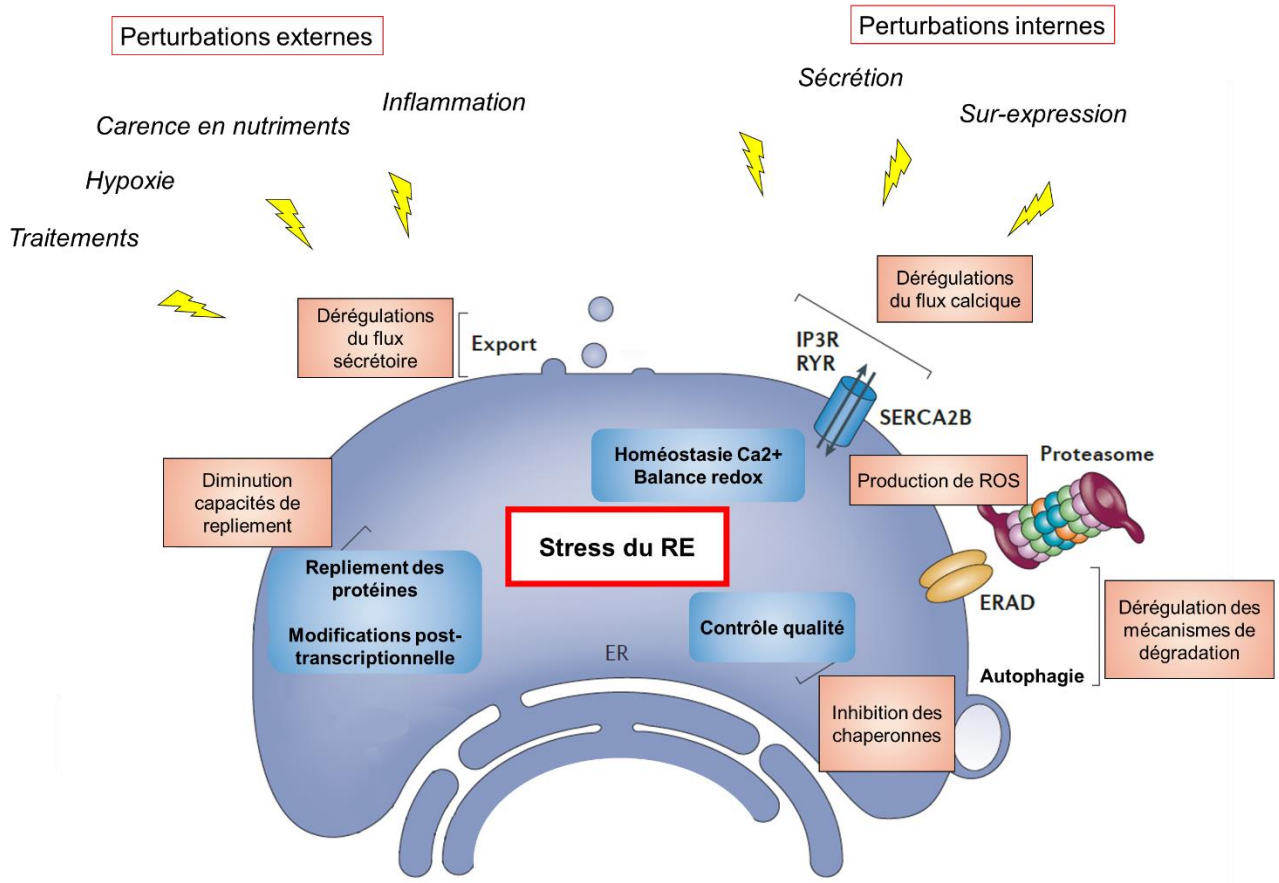

Figure 11 : Activation du stress du RE

De nombreuses perturbations, externes ou internes à la cellule, sont susceptibles d'activer un stress du RE. Ces perturbations vont affecter 3 grandes caractéristiques du RE :

- *perturbation de l'homéostasie calcique et de la balance REDOX, par des fluctuations de la concentration calcique intraluminale (l'inhibition des pompes SERCA par exemple), ou encore la production de ROS,*
- *perturbation de la maturation et de l'export des protéines néosynthétisées, en affectant les mécanismes de repliement/modifications post-transcriptionnelles indispensables à l'acquisition de protéines fonctionnelles, ainsi que le trafic vésiculaire,*
- *perturbation du contrôle qualité de ces protéines, via l'inhibition des protéines chaperonnes et des mécanismes de dégradation des peptides mal-conformés.*

D'après Hetz & Chevet & Harding, 2013

B. L'Unfolded Protein Response ou UPR

1. Activation de l'UPR

Les médiateurs de l'UPR sont trois protéines transmembranaires du réticulum : ATF6 (Activating Transcription Factor-6), PERK (PKR-like ER-associated protein Kinase) et IRE1α (Inositol Requiring Enzyme-1). En l'absence de stress, ces trois protéines sont maintenues inactives par le biais d'une interaction avec la protéine chaperonne BiP (Binding Immunoglobulin Protein), au niveau de leur partie luminale. Lorsque des protéines mal

conformées s'accumulent dans la lumière du réticulum endoplasmique, BiP reprend sa fonction de chaperonne, se dissocie de PERK, IRE1α et ATF6, et permet une levée d'inhibition des trois senseurs [211] (Figure 12).

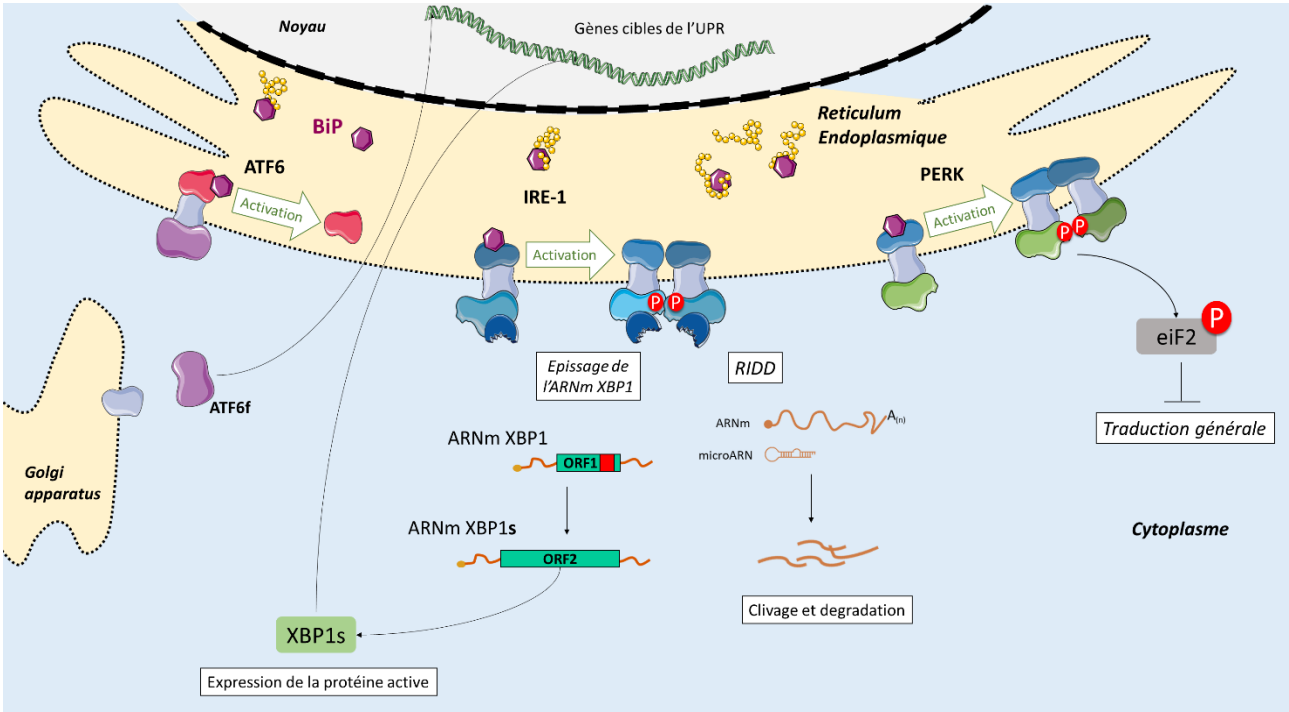

Figure 12 : Les trois voies de l'UPR

L'accumulation de peptides mal conformés dans la lumière du réticulum redirige la protéine chaperonne BiP et libère les 3 senseurs : ATF6 est relocalisé à l'appareil de Golgi et clivé en un facteur de transcription AFT fragment (ATF6f) qui active les gènes cible de l'UPR. PERK se dimérise, phosphoryle le facteur eiF2 et inhibe la traduction coiffe-dépendante. Enfin, IRE1α se dimérise et active une sous-unité RNAse, qui induit la dégradation de certains ARN messager et micro-ARN. De plus, la prise en charge de l'ARNm de XBP1 par IRE1α conduit à un épissage non conventionnel et à l'expression d'un facteur de transcription XBP1s.

Etant donné la multitude de facteurs aboutissant à l'induction du stress du RE, cette voie « canonique » n'est évidemment pas la seule à moduler l'activation des trois senseurs de l'UPR. Des études d'interactions protéine-protéines ont menées à la conception de l'UPRosome, idée suivant laquelle des partenaires protéiques de ATF6, PERK et IRE1α régulent l'amplitude et la durée de leur activation. Cependant, les données restent très parcellaires et axées sur l'étude de IRE1α, le seul des trois senseurs à être commun à l'ensemble des eucaryotes. Ainsi, la chaperonne résidente DNAJB9/ERdj4, par exemple, réprime spécifiquement l'activation de IRE1α : elle forme un complexe protéique avec BiP, inhibe la dimérisation de IRE1α, et de fait son activation [212]. A l'inverse, la chaperonne Hsp47 réduit l'affinité de BiP pour IRE1α ; Hsp47 promeut la dimérisation et l'activation de IRE1α en se fixant sur son domaine intraluminal [213]. Les chaperonnes peuvent également influencer la stabilité de ces senseurs : la Protein Disulfide Isomerase A6 (PDIA6)

déstabilise la dimérisation de IRE1α et PERK par la réduction de leurs ponts disulfure, menant à l'inactivation de leurs signaux [214].

L'intégration des signaux de stress ainsi que les réponses qu'ils engendrent au niveau du réticulum sont donc des mécanismes finement régulés, et qui ne restent pas totalement compris à l'heure actuelle. Une certitude néanmoins est que l'activation des trois senseurs ATF6, PERK et IRE1α influence le devenir cellulaire par la restructuration globale du transcriptome et du protéome.

2. La voie ATF6

ATF6 est un facteur de transcription de la famille des « leucine zipper ». Il existe deux isoformes de ATF6, ATF6α et ATF6β, codés par deux gènes indépendants, respectivement ATF6A et ATF6B. Les deux isoformes présentent de fortes homologies et seul le KO des deux gènes conduit à une létalité embryonnaire à E8,5, suggérant une redondance fonctionnelle entre ces deux protéines. Cependant, malgré leur grande homologie, il semblerait que l'activation des gènes relatifs à l'UPR soit principalement dépendante de ATF6α, ATF6β ayant montré une très faible activité transcriptionnelle [215]. Plus généralement, le rôle de ATF6β n'est pas encore totalement compris : de par sa faible activité transcriptionnelle et sa demi-vie plus longue, il a été proposé en tant qu'inhibiteur de ATF6α, par la formation d'un hétérodimère [216]. Cependant, un KO ATF6β n'influence pas l'activation transcriptionnelle médiée par ATF6α, laissant la question du rôle de ATF6β sans réelles autres hypothèses à ce jour [215].

Suite à l'induction d'un stress du RE, la dissociation de BiP et de ATF6 révèle une séquence d'adressage à l'appareil de Golgi [217]. Cette relocalisation entraîne un clivage par deux protéases résidentes du Golgi, la S1P et la S2P, qui libère un fragment de 50KDa environ, correspondant au domaine cytoplasmique de ATF6, nommé ATF6f (pour ATF6 fragment) [218]. Ce facteur de transcription biologiquement actif est composé d'un domaine de liaison à l'ADN, d'un TAD (Transcriptional Activation Domain), d'un domaine bZIP (basic leucine ZIPper) et enfin d'une séquence NLS (Nuclear Localization Sequence), qui lui permettent l'induction de gènes cibles de l'UPR. La voie ATF6 est plutôt associée à une voie adaptative ; il promeut l'expression de chaperonnes, dont BiP ou encore la calréticuline, et des composant du système ERAD (ER Associated Degradation), qui permettent la rétro-translocation des protéines du RE vers le cytoplasme afin de les dégrader [219,220]. Il favorise ainsi la résolution du stress en augmentant les capacités de prise en charge du RE, tout en augmentant ses capacités de dégradation.

Plus que son action directe sur les gènes cibles de l'UPR, ATF6 a été montré comme un « modulateur » de l'effet d'autres FT ; des expériences d'immunoprécipitations ont montré que ATF6α et XBP1s, une des cibles de IRE1, forment un hétérodimère et conduisent à l'expression d'un réseau de gènes différents de leur simple expression : en outre, l'hétérodimère ATF6α- XBP1s peut augmenter jusqu'à huit fois l'affinité de XBP1s pour ses cibles [221,222]. Des expériences de ChIP (Chromatin Immunoprecipitation) montrent également que ATF6 interagit physiquement avec C/EBPβ au niveau du promoteur de Plk4, un sérine-thréonine kinase impliquée dans la prolifération et la survie. L'activation transcriptionnelle de Plk4 par C/EBPβ est alors réprimée dans le cas d'un stress du RE [223].

Cependant, l'étendue de la régulation dépendante de ATF6 est très probablement sous-estimée, dû au manque d'informations concernant l'ensemble de ses cibles, ainsi que l'ensemble de ses partenaires protéiques. Il a longtemps été considéré comme un doublon de XBP1s, puisque partageant de nombreuses cibles avec ce dernier et sa demi-vie courte rend son étude relativement compliquée.

3. La voie PERK

PERK (pour PKR-like ER-associated protein Kinase) est une sérine-thréonine kinase transmembranaire de type I, codée par le gène EIF2AK3 (pour Eucaryotic Initiation Factor 2 Activating Kinase 3). Elle est nécessaire au développement du pancréas et le KO de PERK, bien que non létal à l'état embryonnaire, entraîne la dégénérescence des cellules β suivi d'un diabète de type 1, ainsi que la mort des individus rapidement après la naissance [224].

En réponse à un stress du RE, la libération du domaine intraluminal de PERK révèle une séquence de dimérisation. L'oligomérisation de PERK engendre sa trans-autophosphorylation, nécessaire à son activation. Le substrat principal de PERK est la sous-unité α du facteur d'initiation de la traduction eiF2 ; sa phosphorylation entraîne une diminution transitoire de la traduction coiffe-dépendante [225].

De nombreuses études ont démontré le rôle ambigu de PERK, qui permet de transmettre aussi bien des signaux adaptatifs que des signaux apoptotiques. Dans un premier temps, l'inhibition de la traduction permet de réduire l'afflux de protéines néosynthétisées, et ouvre une « fenêtre d'opportunité » pour permettre l'adaptation au stress. PERK est d'ailleurs impliqué plus particulièrement dans la résolution des stress oxydatifs ; en effet, une des cibles directes de PERK est le FT Nrf2 [226,227], qui une fois

phosphorylé, est relocalisé dans le noyau où il active des gènes tels que le gène codant pour la NAD(P)H quinone oxidoréductase ou en encore la gluthatione S-transférase, qui sont des antioxydants essentiels à la détoxification cellulaire [228,229].

Toutefois, l'inhibition de la traduction coiffe-dépendante entraîne également la traduction sélective de certains ARNm. C'est le cas de ATF4 (pour Activating Transcription Factor 4), un des FT majeur de la voie PERK et qui appartient à la famille de ATF6 ; en effet, l'activation de PERK entraîne la traduction « préférentielle » de l'ARNm de ATF4 grâce à de nombreuses uORFs (upstream Open Reading Frames) au niveau de son 5'UTR [225]. Des expériences de ChIP-seq ont révélé que ATF4 induisait la transcription de gènes impliquée dans la synthèse et la dégradation des protéines [230].. Comme les autres membres de la famille, la régulation transcriptionnelle relayée par ATF4 est largement dépendante de la formation d'hétérodimère avec d'autres FT, comme les membres de la famille ATF, FOS/JUN, et C/EBP (CCAAT enhancer binding protein) [231,232,233]. Le mécanisme d'action d'ATF4 le mieux décrit est son association avec le FT CHOP (C/EBP Homologous Protein). ATF4 promeut la transcription de CHOP, et leur hétérodimérisation permet, entre autres, la restauration de la traduction canonique par l'activation transcriptionnelle de GADD34, une phosphatase qui déphosphoryle eiF2α [234]. CHOP est également décrit pour augmenter la transcription de messagers pro-apoptotiques tels que Bim et Puma [235,236]. De plus, l'expression de ATF4 est liée à la dégradation par le protéasome de la protéine anti-apoptotique XIAP qui est ubiquitinylée et dégradée plus rapidement lorsque ATF4 est exprimé [237].

4. La voie IRE1

IRE1 est une serine/thréonine kinase couplée à une endoribonucléase (RNase). C'est la branche de l'UPR la plus conservée ; en effet, elle est la seule voie retrouvée chez tous les eucaryotes, des levures jusqu'à l'Homme [238]. Il existe deux isoformes de IRE1, IRE1α et IRE1β, codés respectivement par les gènes ERN1 et ERN2 [239]. Les deux paralogues sont extrêmement proches, à la seule différence que IRE1α est exprimé de façon ubiquitaire, quand IRE1β est seulement exprimé par les cellules épithéliales de l'intestin et les cellules mucosales de l'épithélium aérien [240,241]. De plus, seul le KO de IRE1α est létal au stade embryonnaire, dû à un défaut de développement du foie [242]. Le KO de IRE1β-/- a cependant révélé une activation accrue de l'UPR dans les cellules intestinales des souris, suggérant une action répressive de IRE1β sur l'UPR, et plus particulièrement sur IRE1α [243]. Enfin, la surexpression de IRE1β dans des HEK293 a montré une induction

plus modérée de l'UPR suite à un traitement à la tunicamycine. Ce frein passerait par une répression de l'activité de IRE1α, bien que les mécanismes exacts ne soient pas encore connus [244].

Pour la suite de l'exposé, IRE1 désignera l'isoforme majoritaire IRE1α, sauf mention du contraire.

A l'image de l'activation de PERK, la libération du domaine intraluminal de IRE1 révèle une séquence de dimérisation ; l'homodimérisation de la protéine est suivie d'une trans-autophosphorylation, ce qui entraîne l'activation du domaine RNase [239].

a) La voie du RIDD (Regulated IRE1-Dependant Decay)

IRE1 induit la dégradation de certains ARN par clivage endonucléolytique. Ce processus de RIDD (pour Regulated IRE1-Dependent Decay) permet de diminuer l'afflux de protéines au niveau du RE en jouant directement sur la stabilité des messagers [245]. Outre ses effets bénéfiques sur le « désengorgement » du RE, le RIDD est également impliqué dans l'apoptose. Upton *et al.* ont mis en évidence la dégradation de certains micro-ARNs par le RIDD, les miR-17, miR-34a, miR-96, and miR-125b, chacun impliqués dans la répression traductionnelle de l'ARNm de la caspase 2 [246]. Or, suite à l'activation de IRE1, ces micro-ARN sont dégradés, entrainant une augmentation de l'expression de la caspase 2, et de fait l'induction de l'apoptose. Cependant, le RIDD serait moins un processus spécifique de certains ARN qu'un mécanisme dépendant de l'adressage des ARN au réticulum. En effet, il n'existe pas de sites de clivages consensuels, et dans certains cas, plusieurs sites de clivage ont été décrit pour un même ARN [247]. De plus, Hollien *et al.* démontrent en 2013 que n'importe quel ARN adressé au réticulum est susceptible d'être dégradé par IRE1 [248]. Enfin, les effets du stress du RE et de IRE1 sur l'expression de la caspase 2 restent également controversés [249].

b) La signalisation XBP1s

La cible la mieux caractérisée du domaine RNase de IRE1 reste donc l'ARNm de XBP1 (X box-binding protein 1). La différence avec le RIDD vient de la prise en charge de l'ARN clivé par la tRNA ligase RtcB [250], qui lie les deux fragments ARN entre eux, suite au double clivage par IRE1. En résulte un « épissage non-conventionnel », puisque cytoplasmique, marqué par l'excision de 26 nucléotides au sein de la séquence codante de l'ARNm de XBP1 [251]. Le décalage de phase généré permet la traduction d'un facteur de transcription biologiquement actif, nommé XBP1s (« s » pour spliced), par opposition à la protéine XBP1u (« u » pour unspliced) obtenue à partir de l'ARNm non-épissé. Comme pour

IRE1, le KO de XBP1 est létal au stade embryonnaire, dû à un défaut de développement du foie, et une anémie sévère [252].

La majorité des cibles transcriptionnelles de XBP1s sont liées à la résolution du stress du RE : en effet, des expériences de ChIP-sequencing révèlent qu'environ le tiers de ses cibles sont impliquées dans la synthèse des protéines (15%), le repliement et le transport des protéines (29%), mais également la dégradation via le processus d'ERAD (9,6%) [253]. Il partage d'ailleurs de nombreuses cibles avec le FT ATF6, avec lequel il interagit physiquement par la formation d'hétérodimère (cf Partie III ; B ; 2). Le facteur de transcription XBP1s fera l'objet d'une attention toute particulière et sera développé ultérieurement, en partie IV.

c) Signalisations « non canoniques » de IRE1

Enfin, au-delà de ses activités kinase/RNase, le domaine cytoplasmique de IRE1 est la cible de différentes molécules adaptatrices, qui activent de nouvelles signalisations « non-canoniques » de IRE1. En effet, l'homodimérization de IRE1 engendre la fixation de la protéine adaptatrice TRAF2 (TNFR-associated factor 2), classiquement impliquée dans la réponse au TNFα. Ce complexe protéique est reconnu par :

- ASK1 (Apoptosis Signal-Regulating Kinase 1) [254], une kinase qui une fois activée, phosphoryle JNK (cJun-N-terminal Kinase) : l'activation de la voie JNK en aval de IRE1 constitue alors un message pro-apoptotique par la répression de l'expression de Bcl-2 et l'activation de l'expression de Bim, Bid ou encore Bax [255,256].
- IKK (Inhibitor κB Kinase), une autre kinase en amont de la voie NF-κB. L'activation de cette kinase permet l'activation de NF-κB, et une induction de l'apoptose [257].

Par le biais de son domaine cytosolique, IRE1 promeut donc des voies pro-apoptotiques par l'activation de protéines kinases, à l'image du fonctionnement d'un récepteur membranaire après fixation de son ligand. De ce point de vu, IRE1 apparaît comme un équivalent intracellulaire du récepteur au TNFα, l'activation des voies JNK et NF-κB étant représentatives de la fixation du TNFα sur son récepteur TNFR1.

C. Rôle de l'UPR

Comme évoqué précédemment, l'UPR possède une dualité, et peut aussi bien restaurer les capacités du RE et conduire à l'adaptation de la cellule, que provoquer la mort

par apoptose ou autophagie. L'UPR représente donc un « point de contrôle », qui permet l'intégration des signaux de stress et détermine le devenir cellulaire.

1. Rôle pro-survie

L'activation de l'UPR implique une large reprogrammation transcriptionnelle et traductionnelle, qui permettent de renforcer des mécanismes adaptatifs préexistants et d'affronter les dérégulations de la protéosynthèse. Ces mécanismes d'adaptations jouent sur plusieurs facteurs :

Dans un premier temps, l'activation de PERK et de IRE1 réduit l'afflux de peptides au niveau du RE, respectivement par la phosphorylation de la sous-unité eiF2α, et par la dégradation d'ARN par le RIDD. Cette « pause » traductionnelle est accompagnée d'une augmentation des capacités générales du RE : les FT ATF6, ATF4 et XBP1s activent la transcription de gènes codant pour des protéines impliquées dans la synthèse et dans le repliement des protéines du RE. Plus généralement, ils activent la transcription de gènes codants pour des protéines impliquées dans la biogénèse du RE (synthèse des lipides, trafic vésiculaire…). Ainsi, ces trois acteurs majeurs en augmentent les capacités d'accueil, tant en termes de prise en charge des peptides qu'en termes de volume du RE.

Enfin, le désengorgement du RE est aussi dépendant de la dégradation des peptides mal-conformés : en effet, la machinerie de dégradation des protéines, le protéasome, est cytoplasmique. La dégradation des protéines du RE passent donc par un système de rétro-translocation nommé ERAD (ER-Associated Degradation), qui relocalise les peptides anormaux dans le cytoplasme. Ce processus fait intervenir un grand nombre de partenaires protéiques :

- des chaperonnes, comme BiP ou Edem1, qui recrutent les substrats,
- des protéines adaptatrices comme la famille des Derlin, qui guident les substrats à travers le dislocon c'est-à-dire le canal de rétrotranslocation,
- le dislocon lui-même, un canal qui permet le passage des substrats de la lumière du RE vers le cytoplasme, tout en assurant leur adressage au protéasome par l'action d'ubiquitine-ligases [258].

L'ensemble de ces protéines sont également produites en réponse à ATF6, ATF4 et XBP1s, qui provoquent ici l'augmentation du contrôle qualité des protéines ainsi que des capacités de dégradation du RE.

Les capacités de prise en charge du RE varient énormément en fonction des types cellulaires, et le rôle adaptatif de l'UPR joue un rôle crucial dans les tissus à forte protéosynthèse. C'est le cas des cellules sécrétrices, qui activent l'UPR de façon chronique, afin d'assurer leur production protéique. L'exemple le mieux documenté reste celui des cellules β pancréatiques : en effet, chaque cellule β est capable de produire et de sécréter un million de molécules d'insuline par minute lors d'une prise alimentaire, afin de générer un pic insulinémique. De même, les plasmocytes, des lymphocytes B spécialisés dans la sécrétion des anticorps, peuvent produire leur poids en anticorps par jour, une fois activés. Dans ces 2 cas, ces cellules doivent momentanément s'adapter à une production exacerbée de protéines et, de fait, activent l'UPR à l'état basal [259,260].

2. De l'UPR adaptatif à l'UPR terminal

a) L'UPRosome

Dans le cas où l'homéostasie protéique ne parvient pas à être restauré, l'UPR opère un « switch » vers un programme pro-apoptotique appelé UPR terminal. Le devenir cellulaire est largement influencé par l'intensité et la durée d'exposition à un stress du RE. En effet, une longue exposition ou un stress trop intense conduisent à l'activation de l'UPR terminal. Cependant, les mécanismes d'intégration des signaux de durée ou d'intensité, qui déterminent la transition vers l'UPR terminal, ne sont pas encore totalement connus.

De plus en plus d'études pointent du doigt l'implication de l'UPRosome, c'est-à-dire l'ensemble des facteurs qui modulent l'activité des trois senseurs, et plus particulièrement de IRE1. De nombreux co-facteurs reconnaissent le domaine cytoplasmique de IRE1 dimérisée, et régulent de façon dynamique sa signalisation. IRE1 n'apparaît alors plus comme un senseur de l'état du RE mais plutôt comme un senseur de l'état général de la cellule ; il permet de centraliser un ensemble de signaux et de faire la balance entre signaux anti et pro-apoptotiques : ainsi, HSP72 inhibe l'apoptose médiée par IRE1 en activant la sous-unité RNase, ce qui augmentent la production de XBP1s [261] et à l'inverse, les protéines BAX et BAK se fixent sur le domaine kinase de IRE1 et activent l'apoptose *via* la voie JNK (Figure 13) [262]. Les variations de ces facteurs au cours d'un stress du RE sont donc autant de stimuli qui influencent le devenir cellulaire et qui sont intégrés au niveau de IRE1.

Cependant, il semblerait que IRE1 n'agisse pas seulement comme « la charpente » d'un édifice protéique, mais que les modifications post-traductionnelles qui lui sont appliquées orientent également le devenir cellulaire. C'est notamment le cas de son degré

de phosphorylation : ainsi, une hyperphoshorylation de IRE1 est associée à une hyperactivité endonucléolytique [263]. De plus, parmi les nombreux co-facteurs qui reconnaissent son domaine cytoplasmique, peu ont, en définitive, un effet sur l'activité endonucléolytique de IRE1 mais semblent plutôt agir sur son degré de phosphorylation. Par exemple, la kinase cytosolique ABL se fixe par son domaine kinase et provoque une hyperactivité de IRE1, associée à la mort des cellules β pancréatiques [264]. Le double KO des protéines BAX et BAK provoque une perte de la phosphorylation de IRE1, entraînant une perte du signal de IRE1 et une inhibition de l'apoptose [262]. En définitive, l'état de phosphorylation de IRE1 agirait comme une « horloge interne » et l'accumulation de marques de phosphorylation, comme le signe d'une activation prolongée de IRE1, suggérant l'incapacité de la cellule à restaurer l'homéostasie protéique et activant la mort cellulaire. IRE1 est également ubiquitinée lors d'un stress du RE : CHIP (carboxyl terminus of HSC70-interacting protein) est une E3 ubiquitine-ligase associée au RE, et classiquement impliquée dans le processus ERAD [265]. L'ubiquitination de IRE1 par CHIP stabilise la liaison avec TRAF2 et induit la mort cellulaire *via* l'activation de la voie JNK (Figure 13) [266].

A l'image de IRE1, l'activité soutenue de PERK est associée à l'induction de l'UPR terminal : la protéine NDRG2 (N-myc downstream-regulated gene 2) reconnaît le domaine cytoplasmique de PERK et promeut sa phosphorylation, ce qui est associé à l'induction de la mort de la cellule. A l'inverse, la fixation de la chaperonne $p58^{IPK}$ inhibe le domaine kinase de PERK, et est associée à une meilleure survie des cellules. La voie ATF4-CHOP, en aval de PERK, est également liée à la transition vers l'UPR terminal : classiquement, CHOP a été associé à la transcription de gènes liés à l'apoptose. Mais des analyses de ChIP-seq ont révélé une majorité de cibles en lien avec la synthèse et la dégradation des protéines, donc une implication plutôt adaptative. Cependant, dans le cas d'une activation prolongée de la voie ATF4-CHOP, la synthèse protéique soutenue provoque une déplétion en ATP ainsi qu'une augmentation du stress oxydatif, menant à la mort de la cellule [230].

Les mécanismes de transition de l'UPR adaptatif vers l'UPR terminal commencent donc à être compris, notamment par l'étude de l'UPRosome. L'activité des senseurs IRE1 et PERK est modulée par un ensemble de stimuli, qui mènent à l'atténuation de leur signal

ou à son maintien. Dans ce dernier cas, l'activité prolongée de ces senseurs constitue un signal de mort.

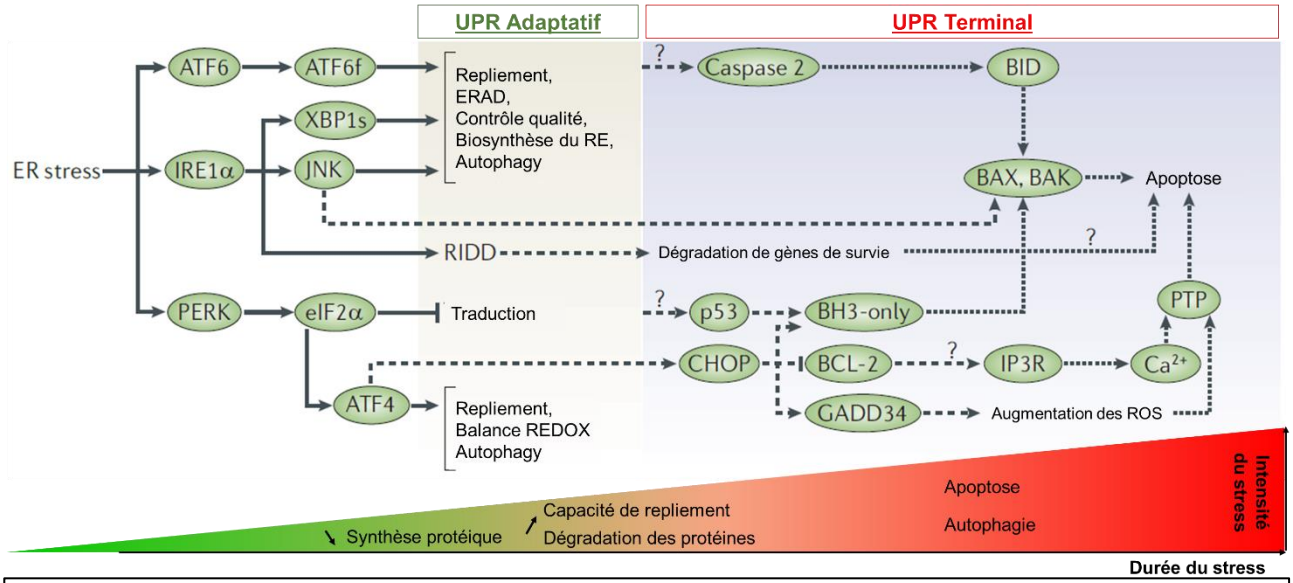

Figure 13 : Orientation du devenir cellulaire par l'UPR

L'UPR oriente le devenir cellulaire en fonction des signaux d'intensité et de durée du stress. Durant la phase adaptative, les trois voies augmentent les capacités générales du RE et les capacités de dégradation des protéines, en promouvant l'expression de protéines chaperonnes, des composants de l'ERAD ou de l'autophagie. La voie PERK permet également de diminuer l'afflux de peptides au réticulum par l'inhibition de la traduction. Cependant, si la phase adaptative ne permet pas un retour à l'homéostasie, UPR induit l'apoptose. L'activation prolongée de la voie PERK/ATF4 induit l'expression de du facteur pro-apoptotique CHOP (C/EBP-Homologous Protein). CHOP est impliqué dans la transcription de facteurs pro-apoptotique de la famille BH3 (BCL-2 homology 3), la repression de facteur anti apoptotique Bcl-2 et la production de ROS via l'expression de GADD34. IRE1α participe également à l'UPR terminal par l'activation de la voie JNK, mais également via le RIDD.

Les flèches pleines illustrent les mécanismes adaptatifs liés à l'UPR, et les flèches en pointillée s les mécanismes liés à l'UPR terminal. Les point d'interrogation indique une méconnaissance des mécanismes impliqués.

D'après Hetz, 2012

3. Modulation par les micro-ARNs

L'UPR est également modulée par des micro-ARNs, qui interviennent en amont, par la modulation de l'expression de BiP, des 3 senseurs ou d'effecteurs de la voie, mais également en aval, puisqu'ils sont la cible des effecteurs de l'UPR (Figure 14).

BiP est la cible de nombreux micro-ARNs, qui diminuent son expression et conduisent à l'activation des trois voies. C'est le cas de miR-30d, -181a et -199a-5p (Figure 14) qui inhibe l'expression de BiP et qui sont sous-exprimés dans différents cancers. La réexpression de ces micro-ARNs conduit à la diminution de l'expression de BiP et a une sensibilisation des cellules cancéreuses aux traitements tels que la trichostatine [267].

L'ARNm des trois senseurs est également régulé par différents micro-ARNs [268], à l'exception de PERK, pour lequel seul le miR-204 est actuellement décrit [269] (Figure 14). Certains micro-ARNs ont été décrits pour intervenir dans la régulation de plusieurs acteurs de la réponse UPR. En effet, l'expression de miR-199a-5p permet à la fois de cibler IRE1 et ATF6. De même, XBP1s et ATF4 sont réprimés par miR-214. Il est envisageable que ces régulations permettent d'orienter l'activation d'une voie préférentiellement aux deux autres.

L'étude des micro-ARNs dans le cadre de l'UPR permet également de mettre en évidence des co-régulations au sein des différentes voies ; ainsi l'expression de miR-30c-2-3p induite par l'activation de la voie PERK, et plus particulièrement via la voie NF-κB, induit une répression de l'expression de XBP1 [270]. De même, le cluster miR-424(322) -503, en aval de la voie PERK, diminue l'expression d'IRE1 et d'ATF6 [271].

Classiquement, l'étude des cibles des effecteurs de l'UPR s'est concentrée sur les cibles codantes. Cependant, les nombreux facteurs de transcription induits par l'UPR activent également la transcription de micro-ARNs. XBP1s active transcriptionnellement quatre micro-ARNs, les miR-148a [272], -153[273], -346 [274] et -1274B [275] (Figure 14). L'expression de miR-148a par activation transcriptionnelle directe permet l'inhibition de Wnt10b, un inhibiteur de la différenciation adipocytaire [272]. De façon intéressante, miR-148a a été relié à d'autres processus de différenciation, particulièrement dans les macrophages, où il permet la maturation finale par activation de la voie Notch [276], mais également dans les plasmocytes, via l'inhibition de Mitf et BACH2 [277,278,279]. Cependant, la régulation par XBP1s n'a pas été montrée pour ces exemples-là. La surexpression de miR-346 par XBP1s a été observé dans différentes lignées cellulaires *in vitro*. MiR-346 a été décrit pour inhiber l'expression de TAP1, ce qui affecte les capacités de présentation de l'antigène par le CMH de type I (Complexe Majeur d'Histocompatibilité de classe I). Cette caractéristique est cruciale dans les cellules dendritiques notamment, où XBP1s a été montré comme nécessaire à ce processus (cf partie IV.E.2). Enfin, miR-153 et miR-1274B ont été reliés au processus d'angiogenèse. En condition d'hypoxie, XBP1s promeut l'expression de miR-153, qui réprime l'expression de HIF1α et inhibe l'angiogenèse dans un modèle de cancer du sein. Ces exemples permettent de mettre en évidence la part non-négligeable des ARN non codants dans la mise en place des phénotypes médiés par les effecteurs de l'UPR.

Enfin, les microARN effecteurs de la réponse UPR interviennent également dans l'orientation du destin cellulaire, en favorisant la survie ou l'apoptose lors d'un stress du RE. Comme évoqué précédemment, la sous-unité RNase de IRE1 clive et entraine la dégradation de micro-ARNs, tels que miR-17, -34a, -96, et -125b. Ces micro-ARNs sont impliqués dans le contrôle de l'expression de la caspase 2, et leur dégradation est lié à

l'induction de l'apoptose [246]. ATF4 induit l'expression de miR-211, lequel réprime l'expression de CHOP, et inhibe l'apoptose [280]. A l'inverse, ATF4 est également impliqué dans l'expression de miR-29a, qui réprime l'expression de la protéine anti-apoptotique Mcl-1, et active l'apoptose [281]. CHOP est également impliqué dans l'expression du méga-cluster miR-379, qui réprime, entre autres, EDEM3 et participe à l'aggravation du diabète [282].

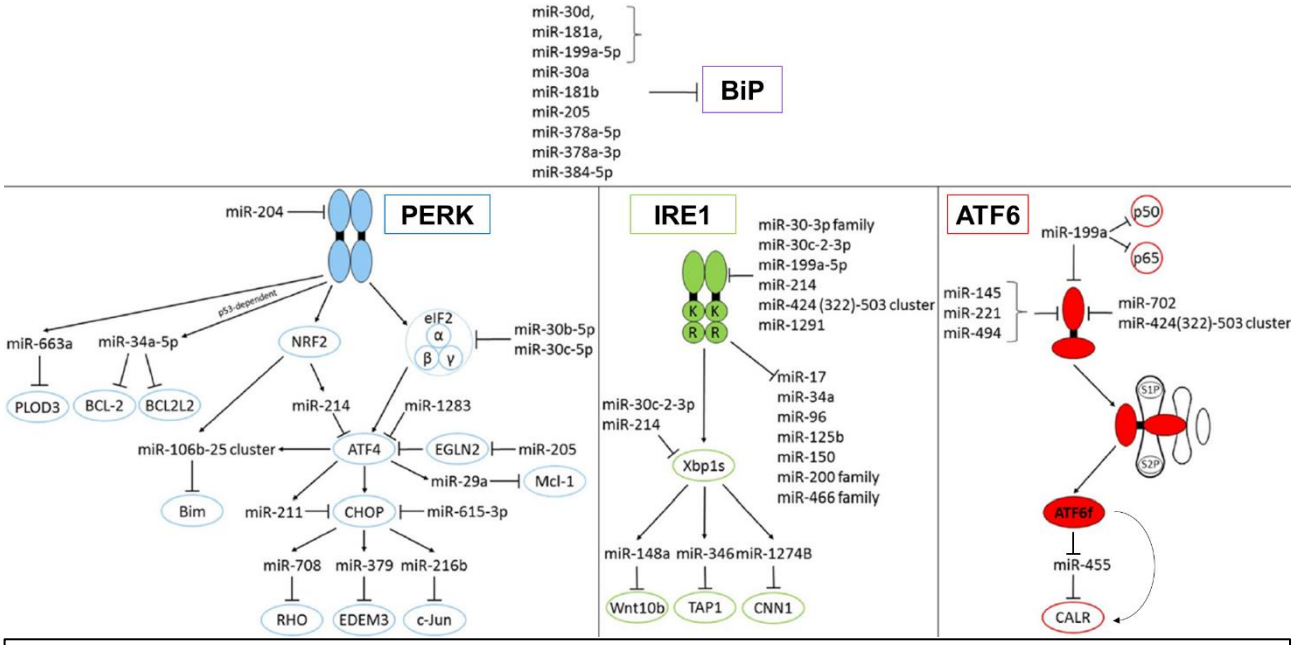

Figure 14 : Implication des micro-ARNs dans l'UPR

D'après McMahon et al., 2017

4. L'UPR dans l'hématopoïèse

Bien que le rôle de l'UPR ne soit plus à démontrer dans les cellules sécrétrices, les phénotypes associés aux KO de différents effecteurs révèlent également une implication dans l'hématopoïèse ; par exemple, le KO de ATF4 engendre une réduction du nombre de CSH dans le foie fœtal des souris au cours du développement embryonnaire [283], celui de XBP1s, une létalité embryonnaire due, entres autres, à une anémie sévère [252]. Ces deux phénotypes suggèrent alors que l'UPR pourrait avoir un effet non seulement sur la mise en place des CSH mais également sur la différenciation hématopoïétique (Figure 15).

	IRE1	XBP1	ATF6	PERK	ATF4
Phénotype					
Létal à l'état embryonnaire	Oui	Oui	Non	Non	Non
Mort post-natale	-	-	Non	Oui	Oui
Effets tissu-spécifique					
Diminution de la sécrétion d'Ig par le cellules B	Oui	Oui	-	Non	-
Altérations du pancréas exocrine	Oui	Oui	-	Oui	-
Altérations du pancréas endocrine et de la sécrétion d'insuline	Oui	Oui	Oui	Oui	Oui
Altérations du stockage des lipides hépatiques	Oui	Oui	Oui	Oui	Oui
Altération de la formation osseuse	-	-	-	Oui	Oui
Cécité	-	-	-	-	Oui
Altération du métabolisme du glucose	Oui	Oui	-	Oui	Oui

Figure 15 : Phénotypes associés au KO général des effecteurs de l'UPR

De façon générale, les effecteurs de la réponse UPR sont des protéines essentielles, et leur perte engendre la mort des individus, à l'exception de ATF6, qui nécessite le KO de ATF6α et ATF6β pour être létal. Les phénotypes liés à ces KO montrent une implication toute particulière de l'UPR dans le pancréas, et plus généralement dans le métabolisme du glucose. Cependant, des études tissus-spécifiques révèlent une implication de l'UPR dans l'hématopoïèse : le KO de IRE1 et XBP1 est accompagné d'une perte de la sécrétion des immunoglobuline (Ig) dans les lymphocytes B, et la perte de XBP1 provoque une anémie létale. La perte de la voie PERK/ATF4, quant à elle, provoque des altérations de la formation osseuse.

a) UPR et CSH

Plusieurs études ont relié l'activation de l'UPR à une expansion des CSH : en effet, les CSH sont des cellules quiescentes, avec un métabolisme et une protéosynthèse très basse, des conditions qui ne provoquent pas d'induction de l'UPR. A l'inverse, l'expansion des CSH est synonyme d'une augmentation de leur activité métabolique, conditions propices à l'induction de l'UPR. Une augmentation de l'expression des protéines chaperonnes est d'ailleurs observée à mesure que les progéniteurs progressent dans la différenciation. Mais plus qu'un marqueur de l'activation des CSH, l'UPR pourrait être un garant de leur intégrité. Un modèle de souris KO pour BiP a d'ailleurs montré qu'une activation prolongée de l'UPR entraîne l'apoptose spécifiquement des LT-HSC, sans affecter les ST-HSC [284]: la sensibilité accrue au stress du RE est perdue une fois les CSH activées, révélant une épuration sélective des CSH quiescentes. Plus particulièrement, en comparaison des progéniteurs, une forte activité de la voie PERK-ATF4 dans les CSH mène rapidement à la mort de ces dernières (Figure 13). L'activation spécifique de la voie PERK jouerait ici le rôle de « garde-fou », en éliminant les CSH soumises à un stress qui pourrait les endommager [285,286,287]. A l'inverse, l'activation de l'axe IRE1α-XBP1s améliore les capacités de reconstitution des CSH après transplantations en série [288,289]. Plusieurs études confirment également que la réduction du stress du RE, par l'expression de protéines chaperonnes telles que DNAJB9, ou par un traitement au TUDCA (chaperonne exogène), ou encore par HIF2-α en cas d'hypoxie, augmente les capacités de reconstitution des CSH ; à nouveau, ces résultats suggèrent l'importance de la « qualité » des CSH, qui sont

maintenues lorsque leur environnement garantit leur intégrité, c'est-à-dire en l'absence de stress du RE (Figure 16) [285,286,290].

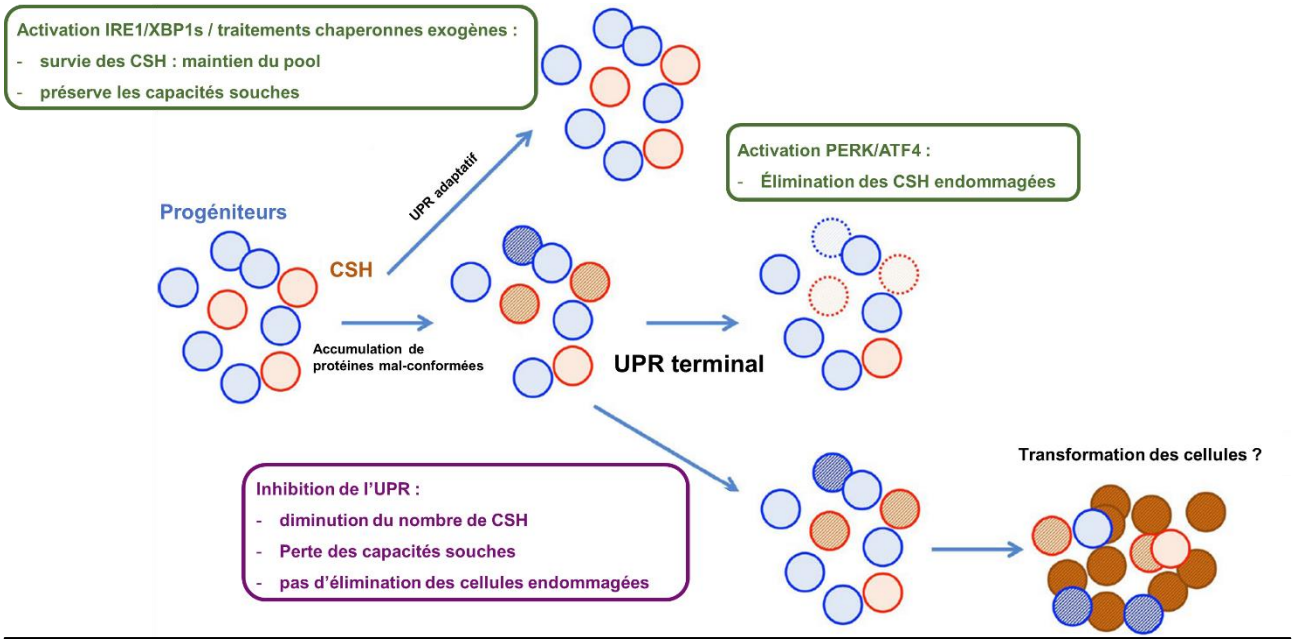

Figure 16 : Implication de l'UPR dans le maintien du compartiment hématopoïétique souche

La population des CSH a montré une sensibilité très forte à l'UPR terminal, grâce à une forte expression basale de ATF4. Cependant, dans le cadre des recherches visant à améliorer les transplantations de CSH, il apparaît qu'une activation de la voie IRE1/XBP1s, ou encore des traitements de chaperonnes exogènes telles que le TUDCA permet de maintenir un « pool » intègre de CSH en favorisant le maintien en quiescence de ces cellules, en augmentant leur survie et par conséquent, en préservant leurs capacités souches. A l'inverse, il semble que l'inhibition de l'UPR dans les CSH soit liée à un épuisement de ces cellules, combiné à un maintien des cellules endommagées.

D'après Sigurdsson & Miharada, 2018

b) UPR et différenciation hématopoïétique

Comme exposé ci-dessus, la perte de BiP dans le compartiment hématopoïétique induit l'UPR de façon chronique et affecte les LT-HSC. Cependant, l'analyse de la composition de la moelle de ces souris révèle une hématopoïèse altérée : une diminution de la lymphopoïèse globale est observée, accompagnée d'une augmentation de la différenciation terminale myéloïde, granulocytaire et monocytaire, illustrant ainsi l'implication de l'UPR dans l'hématopoïèse générale [284].

Déjà en 2001, Reimold *et al*. montraient que XBP1s était nécessaire à la différenciation des plasmocytes, des lymphocytes B spécialisés dans la production et la sécrétion d'anticorps [291]. L'utilisation d'un modèle rapporteur XBP1-GFP a permis de visualiser que l'expression de XBP1s augmente dès le stade pro-B, donc très en amont de l'acquisition de l'activité sécrétoire, faisant de XBP1s un FT clé de l'induction de la différenciation des plasmocytes [292,293] (Figure 17). De façon intéressante, la répression

épigénétique de IRE1α par méthylation de son promoteur, est retrouvé dans des cas de lymphomes B ; dans ces pathologies, le traitement par un agent hypométhylant restore l'expression de IRE1, et induit une régression tumorale, due à la réexpression de XBP1s [294]. De plus, la différenciation plasmocytaire nécessite l'activation spécifique de la voie IRE1α-XBP1s, les voies ATF6 et PERK n'étant pas induites [293,295,296]. Cet exemple révèle également l'indépendance des différentes voies, IRE1α-XBP1s dans ce cas précis, qui peuvent être activées de façon sélective dans les progéniteurs B, sans faire intervenir l'ensemble de l'UPR. Ces mécanismes d'activation spécifique ne sont pas encore connus, néanmoins certaines hypothèses avancent qu'une différence d'expression des trois senseurs pourrait expliquer ces effets.

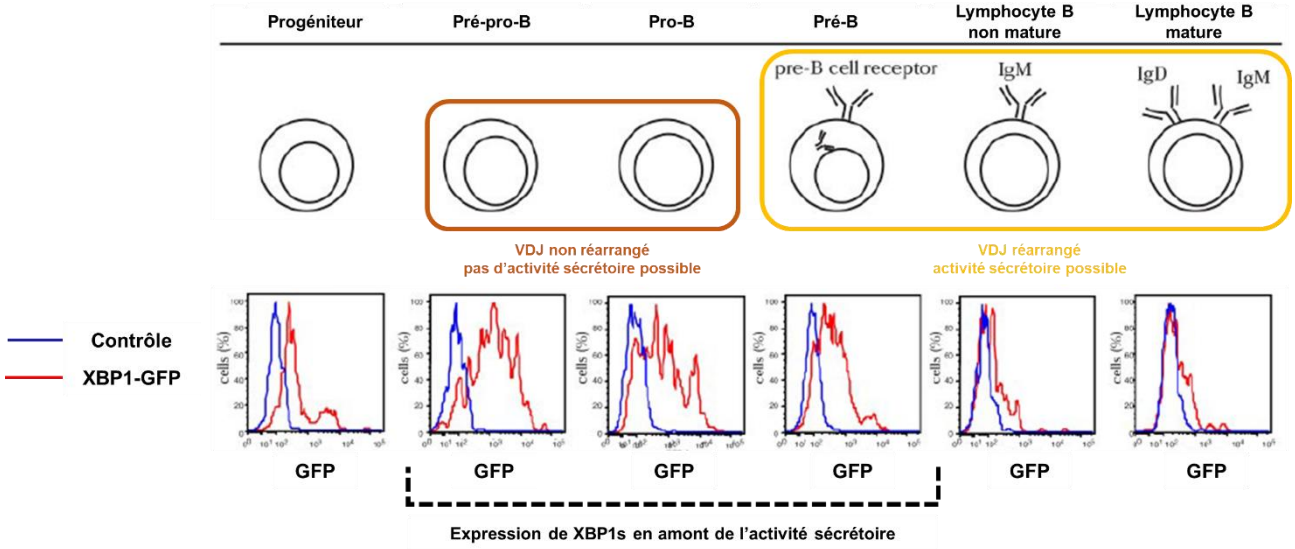

Figure 17 : Activation de la voie IRE1/XBP1s au cours de la différenciation B

Des CSH murines transduites par un système rapporteur XBP1-GFP ou non (contrôle) sont réinjectées dans des souris irradiées. Après la prise de greffes, les cellules issues de l'injection sont triées et l'activation de la GFP est évaluée en fonction du stade de différenciation : cette expérience a permis de visualiser l'activation de la voie IRE1/XBP1s à des stades très précoces de la lymphopoïèse B, avant même l'acquisition d'un phénotype sécrétoire.

D'après Brunsing et al., 2008

La lymphopoïèse T est également tributaire de l'activation de la voie IRE1α-XBP1s, l'utilisation du même modèle rapporteur ayant montré une expression de XBP1s dans des précurseurs de lymphocyte T cytotoxique [292]. Cependant, contrairement aux lymphocytes B, il n'existe aucune preuve de l'absence d'activation des voies ATF6 et PERK au cours de ce processus, qui pourrait tout à fait requérir l'activation de l'UPR dans son ensemble.

Plus récemment, un modèle murin de KO spécifique de XBP1s dans le compartiment hématopoïétique a révélé une implication de l'axe IRE1α-XBP1s dans la différenciation myéloïde ; en effet, la perte de XBP1s provoque une accumulation de progéniteurs myéloïdes au stade GMP (Granulocyte-Monocyte Progenitor), ainsi qu'une perte sélective des éosinophiles matures. Les éosinophiles sont un lignage granulocytaire, spécialisés

dans la production de granules et impliqués dans la lutte contre les parasites et les bactéries. L'activité sécrétoire apparaît donc comme un prérequis crucial dans la maturation de ces cellules, qui nécessite l'activation soutenue de l'axe IRE1α-XBP1s [297]. Enfin, plusieurs études ont déjà montré l'importance de XBP1s dans les cellules dendritiques ; l'utilisation d'un modèle rapporteur murin révèle une activation constitutive de IRE1α dans ces cellules [298,299]. De plus, la perte spécifique de XBP1s dans les cellules dendritiques affecte leur survie mais également leur capacité de présentation d'antigène et d'activation des lymphocytes T [300].

5. Modulation de l'UPR dans les cancers

L'UPR est donc un mécanisme ubiquitaire, impliqué dans de nombreux processus cellulaires aussi fondamentaux qu'éclectiques, comme la sécrétion d'insuline dans les cellules β pancréatiques, ou encore la différenciation des plasmocytes. C'est un point de contrôle, capable d'influencer le devenir cellulaire, et pour toutes ces raisons, il est retrouvé dérégulé dans de nombreuses pathologies, dont les cancers. Sur ce dernier point, l'UPR a été longuement étudié, dans les tumeurs solides notamment, et il est maintenant communément admis que les cellules cancéreuses l'activent de façon chronique ; en effet, en évoluant au sein d'un environnement défavorable, l'activation basale de l'UPR confère des capacités d'adaptation aux conditions d'hypoxie, manque de nutriments, voire aux chimiothérapies, elles-mêmes inductrices d'un stress du RE [301,302]. Deux stratégies de modulation pharmacologique de l'UPR ont alors émergé comme approche thérapeutique : la première propose d'inhiber une ou plusieurs voies impliquées dans l'UPR afin d'affaiblir les mécanismes d'adaptation, la seconde au contraire, propose d'hyperactiver l'UPR afin d'induire l'UPR terminal. L'ensemble des molécules citées ci-dessous, leurs utilisations en oncologie, et plus particulièrement dans le cadre d'hémopathies malignes, sont résumés en Figure 18.

a) Inhibition de la réponse UPR

A l'heure actuelle, la majorité des inhibiteurs de l'UPR en oncologie sont des inhibiteurs spécifiques de l'activité RNase de IRE1α. La découverte de ces inhibiteurs a été rendue possible par l'utilisation de criblages moléculaires à haut-débit, utilisant une protéine IRE1α recombinante humaine, associée à différent substrat, comme un ARN XBP1 fluorescent [303,304] ou encore un ARN XBP1 couplé à la luciférase [305]. L'inhibition spécifique de IRE1α a montré des effets antiprolifératifs et cytotoxiques dans différentes hémopathies

malignes comme le Myélome Multiple, mais aussi les LAM, les LMC (Leucémies Myéloïdes Chroniques), ou encore des LAL-B (Leucémies Aigües Lymphoïdes B) [306–308] . De façon intéressante, les inhibiteurs utilisés ont tous montré une inhibition plutôt spécifique de la maturation de l'ARN XBP1s, sans affecter le RIDD [309]. Concernant PERK, deux inhibiteurs sont utilisés dans les études anticancéreuses, le GSK2606414 et le GSK2656157 [310,311] ; ces molécules inhibent l'activité kinase de PERK de façon hautement sélective et empêche la phosphorylation de eif2α. Ces deux inhibiteurs ont montré des effets anti-tumoraux sur des xénogreffes de pancréas et de Myélome Multiple (GSK2656157) [312] mais également sur modèles *in vitro* de LAP (Leucémies Aigües Promyélocytaires) en association avec un traitement à l'ATRA et L'ATO (Arsenic Trioxide) (GSK2606414) [313]. Cependant, les études in vivo du GSK2656157 ont révélé une toxicité sur les rongeurs, marquée par une insuffisance pancréatique, le développement de diabète, ainsi qu'une importante perte de poids de certains animaux. Bien que de plus en plus explorée dans le cadre du développement de thérapies ciblées, l'utilisation de ces inhibiteurs n'en est encore qu'au stade des études pré-cliniques, avec assez peu d'utilisation dans des modèles animaux [314,315].

b) Activation de la réponse UPR

Dans le cadre de thérapies basées sur la modulation de l'UPR, l'hyper-activation de l'UPR a déjà montré des propriétés anti-cancéreuses. Le rationnel de cette stratégie a été surtout étudié dans le Myélome Multiple (MM), pathologie pionnière dans le développement de thérapies basée sur la modulation de l'UPR ; le MM est une hémopathie maligne B qui touche les plasmocytes, des lymphocytes B activés en différenciation terminale, qui produisent et sécrètent de façon aberrante et continue des immunoglobulines, activant l'UPR de façon basale. En 2012, une AMM est obtenue pour le VELCADE® (bortézomib), un inhibiteur du protéasome, en tant que traitement de première ligne pour le MM. En effet, l'inhibition du protéasome empêche la dégradation des peptides mal conformés, et active l'UPR terminal dans les plasmocytes cancéreux. L'utilisation du bortézomib est actuellement évaluée dans deux autres hémopathies malignes, les LAM (phase I-II d'essai clinique ; références NCT01861314, NCT04173585, NCT01371981) ainsi que les Leucémies Aigües Lymphoblastiques ou LAL (phase III d'essai clinique : NCT02112916), en association avec une chimiothérapie classique. Outre le bortézomib, d'autres inducteurs de l'UPR ont été étudiés, notamment des inhibiteurs de protéines chaperonnes. Ainsi, l'inhibiteur de la chaperonne BiP, BMTP-78, a montré une synergie d'action avec la chimiothérapie dans la LAM *in vitro* ; cependant, les premiers tests *in vivo* sur des rongeurs et des primates ont

révélé une forte toxicité cardiaque [316]. D'autres inhibiteurs de BiP comme l'épigallocatechingallate (EGCG) induit *in vitro* l'apoptose des cellules cancéreuses et les chimiosensibilisent à la vincristine dans un modèle de LAL (Leucémies Aigües Lymphoïdes) [317]. De plus, des études cliniques ont également montré qu'un traitement à l'EGCG est lié à une diminution significative de la taille des ganglions chez des patients présentant une LCL (Leucémie Chronique Lymphoblastique). Cependant, malgré une bonne tolérance, ces essais ont été menés sur des patients en début de maladie, asymptomatiques, et jamais en combinaison avec un agent chimiothérapeutique [318,319].

Enfin, de façon inattendue, certains ligands de IRE1α ont montré un effet activateur ; c'est le cas des inhibiteurs de kinases de type I, des ligands compétitifs qui maintiennent IRE1α dans un état conformationnel qui permet l'activation de sa sous-unité RNase [320]. Des études *in vitro* ont rapporté que de tels ligands permettaient d'induire l'épissage de XBP1, de façon modérée comme le 1NM-PP1, l'APY29 ou le Sunitinib, mais leur manque de sélectivité pour IRE1α les rend difficilement éligibles pour des investigations plus poussées. Plus récemment, un crible a mis en évidence un nouveau composé activateur de IRE1α, hautement spécifique, le G-1749, mais la caractérisation de cet activateur n'en est encore qu'à ses débuts, le mécanisme d'action n'étant pas encore totalement compris [321]. De même, on recense un activateur de ATF6, le composé 147, utilisé seulement dans un modèle murin d'infarctus [322]. Malgré l'absence d'utilisation de ces activateurs dans des modèles de cancers, l'ensemble de ces résultats montrent qu'une activation plus spécifique et plus fine de l'UPR est envisageable, par l'activation spécifique d'une seule des trois voies.

		Type de cancer	Effets	Utilisation en cancérologie
Inhibition de l'UPR	***Inhibiteurs PERK***			
	GSK2656157	Myélome Multiple	Inhibition de la prolifération	Xénogreffes de lignées cellulaires
		pancréas	Inhibition croissance tumorale, diminution angiogénèse	Xénogreffes de lignées cellulaires
	GSK2606414	LAP	Potentialise effet de l'ATO	Lignées cellulaire, cellules primaires in vitro
	Inhibiteurs IRE1			
	STF-083010	LAL	Cytotoxicité	Cellules primaires *in vitro* ; Greffes de cellules primaires murines transduites par BCR-ABL
		Sein	Sensibilise au tamoxifen	Xénogreffes de lignée cellulaire
	MKC-3946	LAM	Cytotoxicité	Lignées cellulaire *in vitro*
		Myélome Multiple	Inhibition de la prolifération ; synergie avec Bortézomib	Xénogreffes de lignées cellulaires
	B-I09	LCL	Cytotoxicité ; Synergie avec ibrutinib	PDX
	MKC-8866	Sein	Inhibition de la croissance tumorale	PDX
		Prostate	Inhibition de la croissance tumorale	Xénogreffes de lignées cellulaires
Activation de l'UPR	***Inhibiteur BiP***			
	Epigallocatechin gallate (EGCG)	LAL	Induction de l'apoptose ; sensibilise à la vincristine	Phase I-II d'essai clinique
	BMTP-78	LAM	Induction de l'apoptose	Lignées cellulaires ; cellules primaires *in vitro* ; forte toxicité in vivo
	Inhibiteur du protéasome			
	Bortezomib	Myélome Multiple	Induction UPR terminal	Traitement 1ère intention
		LAM	Induction UPR terminal	Essai clinique, phase I-III
		LAL	Induction UPR terminal	Essai clinique phase III
	Activateur IRE1			
	1NM-PP1	-	-	-
	APY29	-	-	-
	Sunitinib	-	-	-
	G-1749	-	-	-

Figure 18 : Modulation de l'UPR dans les cancers et perspectives thérapeutiques

IV. X-box Binding Protein 1 (XBP1)

A. Découverte

Le facteur de transcription XBP1 a été initialement mis en évidence dans des lignées cellulaires dérivées de lymphocytes B, caractérisées par un défaut d'expression du Complexe Majeur d'Histocompatibilité de classe II (CMH de type II). En 1990, l'équipe de Laurie Glimcher lie ce défaut d'expression à une séquence très conservée au niveau du promoteur, la séquence X-box ; en effet, pour au moins deux de ces lignées, les auteurs identifient un facteur de transcription de type bZIP (basic leucine Zipper) absent, et capable de se lier sur ces éléments régulateurs très conservés pour induire la transcription des gènes du CMH de type II. Par conséquent, ils décident de nommer ce facteur de transcription X-box Binding Protein 1 ou XBP1 [323].

B. Lien avec l'UPR

L'axe IRE1-HAC1 (homologue de XBP1) est la seule branche de l'UPR à être conservée parmi tous les eucaryotes. La caractérisation de cet axe chez la levure *Saccaromyces Cerevisiae* a donc permis de comprendre les mécanismes d'expressions et les co-dépendances pouvant exister entre ces différents facteurs. En 1996, l'équipe de Peter Walter identifie chez la levure un facteur de transcription de type bZIP, Hac1p, uniquement exprimé lors d'un stress du RE. Cette étude montre également que Hac1p subit un épissage qui permet l'expression d'une protéine plus stable et active, Hac1p^{i}, qui promeut la transcription de gènes codant pour des protéines résidentes du RE *via* des séquences UPRE dans leur promoteur [324]. Ils démontrent l'année suivante que IRE1α est une enzyme bi-fonctionnelle, responsable de l'épissage non-conventionnel de l'ARNm de HAC1 [325]. Ces caractéristiques de l'UPR sont généralisées en 2001, quand Yoshida *et al.* décrit l'homologue de HAC1 chez les métazoaires : XBP1 [326]. Ici encore, l'ARNm de XBP1 est pris en charge par IRE1α qui excise 26 nucléotides dans le cadre ouvert de lecture. La protéine produite XBP1s est plus longue, 376 acides aminées contre 261 acides aminés chez

l'humain, car elle possède à la fois un domaine de liaison à l'ADN ainsi qu'un domaine de transactivation (Figure 19).

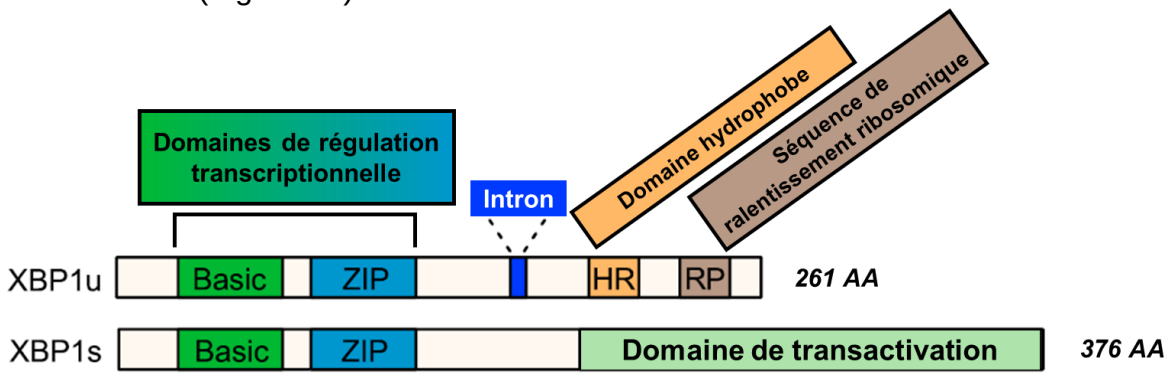

Figure 19 : Comparaison des isoformes protéiques humains XBP1u et XBP1s

Les deux isoformes possèdent la même composition du côté C-terminal, constitué des domaines de liaison à l'ADN. Cependant, ils diffèrent par leur côté N-terminal, plus long pour la protéine XBP1s puisque composé du domaine de transactivation. XBP1u, quant à lui, possède un domaine hydrophobe ainsi qu'une séquence permettant le ralentissement du ribosome, afin de permettre l'épissage en cas d'activation de IRE1. La présence de domaines hydrophobes est également responsable de la demi-vie très courte de cette protéine (environ 10min contre 20min pur XBP1s), qui ancrent XBP1u au réticulum et permettent sa prise en charge très rapide par le protéasome.

D'après Hetz & Papa, 2018

C. Régulations de l'expression de XBP1s

1. Régulations transcriptionnelles

A l'échelle cellulaire, lors d'un stress du RE, un rétrocontrôle positif est mis en place par l'action de ATF6 et XBP1s lui-même sur la transcription de *Xbp1* de façon directe, permettant un maintien du signal et une régulation de XBP1s par l'activation de IRE1α [326]. Cependant, bien que des analyses de séquençage révèlent une expression relativement ubiquitaire de *Xbp1* (Figure 20), les écarts d'expression dans les tissus et l'implication de XBP1s dans des processus de différenciation, indépendants d'un stress du RE, suggèrent également une régulation au niveau de la transcription de son ARN (Figure 20).

Dans les lymphocytes B, la transcription de *Xbp1* est réprimée par le facteur BSAP/ PAX5 (B cell lineage-specific activator protein/PAired boX gene 5) par liaison directe à son promoteur [327]. Lors de la maturation plasmocytaire, le facteur de transcription BLIMP1 (B lymphocyte-induced maturation protein) réprime BSAP/ PAX5, et provoque une levée d'inhibition sur la transcription de *Xbp1* [328]. De plus, des études montrent que le promoteur proximal de *Xbp1* est la cible de différents facteurs de transcriptions. En effet, un site putatif de liaison pour le facteur C/EBPβ a été décrit au niveau du promoteur de *Xbp1* [329]. C/EBPβ

est un acteur clé de la différenciation adipocytaire, entres autres, et active transcriptionnellement XBP1, nécessaire à la différenciation terminale des adipocytes [330].

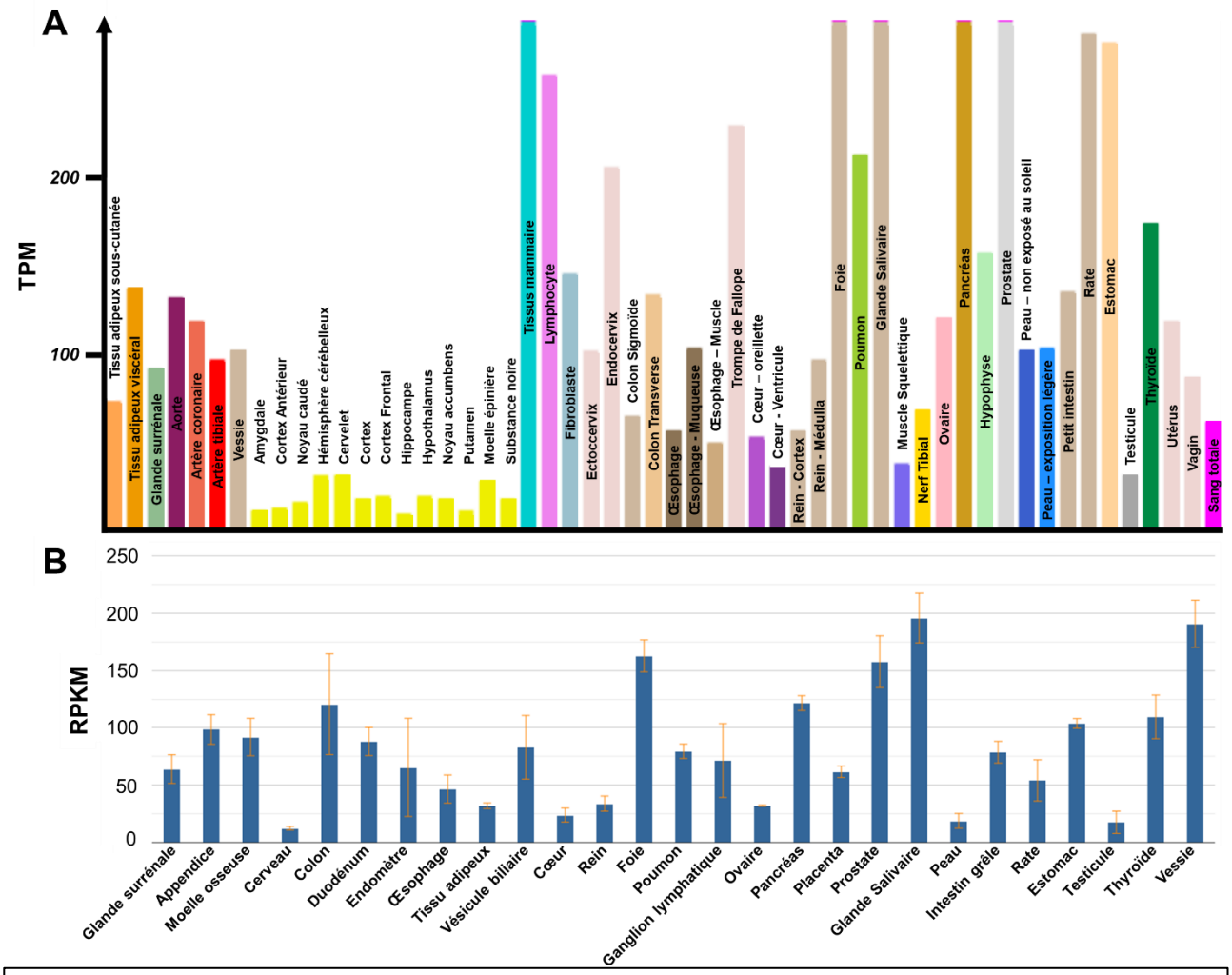

Figure 20 : Expression globale de l'ARNm Xbp1

A) Quantification de l'expression de Xbp1 par séquençage d'ARN dans 54 tissus issus de 948 donneurs sains (Source : https://www.genome.ucsc.edu/index.html)

B) Quantification de l'expression de Xbp1 par séquençage d'ARN dans 27 tissus issus de 95 donneurs sains (Source : https://www.ncbi.nlm.nih.gov/)

2. Régulations post-transcriptionnelles

a) Adressage au Réticulum Endoplasmique

L'adressage de *Xbp1* à IRE1α est un mécanisme co-traductionnel ; le peptide néosynthétisé possède un domaine hydrophobe très conservé qui permet l'ancrage du complexe *Xbp1*-ribosome-peptide à la membrane du RE par la SRP (Signal Recognition Particle) et le translocon Sec61 [331,332]. Ce dernier assure l'adressage spécifique du complexe ribosomique grâce à son interaction directe avec IRE1α [333]. Enfin, une séquence AP (Arrested Peptide) permet d'arrêter momentanément la traduction de l'ARN *Xbp1* afin

de permettre son clivage [331,334]. L'ARN épissé est ensuite prise en charge par la tRNA ligase RtcB, qui raccorde les 2 brins d'ARN, et génère la protéine active [335,336]. XBP1u, quant à elle, est dégradée très rapidement grâce à la génération d'un domaine transmembranaire qui l'ancre à la membrane du RE. Ce domaine est reconnu par des composants de l'ERAD, notamment la SPP (Signal Peptide Peptidase) qui recrute l'ubiquitine-ligase TRC8 afin d'induire la dégradation de XBP1u par le protéasome [337] (Figure 18).

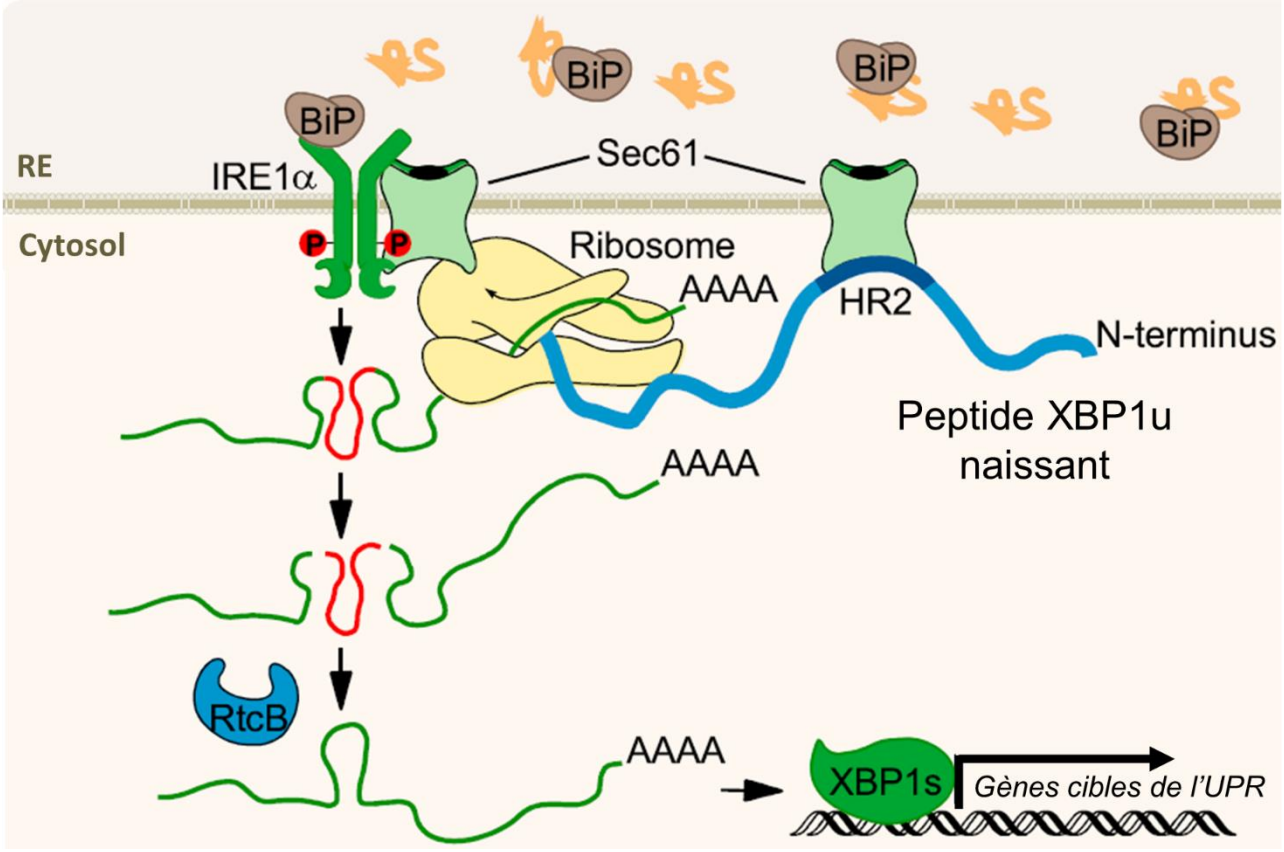

Figure 21 : Mécanisme d'adressage de l'ARNm Xbp1 au RE

Le domaine hydrophobe HR2 de l'ARNm XBP1u interagit avec Sec61, formant lui-même un complexe avec IRE1, et permet un rapprochement de l'ARN en cours de traduction avec l'endoribonucléase. Une séquence induisant le ralentissement du ribosome permet l'épissage en lui-même. La ligation de l'ARN épissé par RtcB permet la fin de la traduction et l'expression de XBP1s.

D'après Hetz & Papa, 2018

b) Régulation de l'épissage

La régulation de l'épissage de l'ARNm de *Xbp1* est foncièrement liée à la régulation de l'activité RNase de IRE1α. Comme exposé en partie III.B.4.c et III.C.2, l'activité de IRE1α est modulée par de nombreux cofacteurs qui reconnaissent son domaine cytoplasmique, lesquels modifient son activité catalytique. Le degré d'oligomérisation de IRE1α affecte également son « orientation catalytique » : la formation de dimère promeut sélectivement le mécanisme de RIDD alors que la formation de cluster est plutôt liée à une augmentation de l'efficacité de l'épissage de XBP1 [309].

c) *Impact des micro-ARN*

L'évolution de l'expression de XBP1s au cours du temps est un processus dynamique, qui requiert de nombreuses régulations intermédiaires. Les micro-ARNs ont donc été proposés comme acteurs à part entière de la modulation de l'expression de XBP1s. En effet, L'ARNm de XBP1 est la cible de différents micro-ARNs comme miR-34c-5p [338], miR-34a-5p [339], miR-30a et miR-241 [340]. Chacun de ces micro-ARN ciblent aussi bien la forme épissée que non-épissé et ont été démontrés comme interagissant directement avec l'ARNm XBP1. La répression de XBP1 par miR-34c-5p ou miR-34a-5p, dans différentes lignées cellulaires, provoque une induction plus rapide de l'UPR terminal après un traitement à la Tunicamycine ou la Thapsigargine, deux drogues classiquement utilisées *in vitro* pour induire un stress du RE [338,339]. Dans un modèle d'insuffisance cardiaque, XBP1 est réprimé par l'action de miR-30a et miR-241 ; la perte de XBP1s provoque la mort des cardiomyocytes, ainsi que la diminution de l'expression du facteur pro-angiogénique VEGF et inhibe la fonction cardioprotectrice de XBP1s [340].

3. Modifications post-traductionnelles

Les modifications post-traductionnelles sont impliquées dans la modulation de l'activité de nombreux facteur de transcription, XBP1 ne faisant pas exception. Ces modifications influencent principalement sa localisation, sa stabilité mais aussi son efficacité transcriptionnelle.

a) *SUMoylation*

Les Lysines Lys276 et Lys297, situées dans le domaine de transactivation C-terminal de XBP1s, sont les cibles de la PIAS (Protein Inhibitor of activated STAT2), une SUMO E3 ligase (Small Ubiquitin-Like Modifier E3 ligase) [341]. La SUMOylation de XBP1s n'influence pas sa localisation ni sa capacité à s'hétérodimériser, mais conduit à une diminution de son activité transcriptionnelle [341]. De plus, la perte des peptides SUMO, par mutation des résidus lysine ou par l'action de la protéase SENP1 (Sentrin/SUMO-specifique), augmente l'activité de XBP1s [341,342].

b) *Phosphorylation*

XBP1s est la cible de différentes kinases. La voie MAPK (Mitogen-Activated Protein Kinase), plus particulièrement p38 MAPK, phosphoryle XBP1s sur ses résidus Thréonine

Thr48 et Sérine Ser61 et permet sa nucléarisation. Ce mécanisme, observé sur des foies de souris, permet la relocalisation nucléaire de XBP1s spécifiquement lors d'une prise alimentaire. L'inhibition de p38 MAPK, a l'inverse, abolit la translocation nucléaire de XBP1s, suggérant non pas une modulation de l'activité de XBP1s, mais plutôt une étape nécessaire à son action [343]. La kinase CDK5 a également été décrite pour phosphoryler XBP1s sur sa Sérine Ser61 dans un modèle *in vitro* de maladie de Parkinson [344]. Dans les deux cas, la phosphorylation de XBP1s favorise sa translocation nucléaire et agit comme un degré supplémentaire de régulation, dans des tissus connus pour activer de façon basal l'UPR.

c) *Acétylation*

Enfin, la stabilité et l'activité transcriptionnelle de XBP1s peuvent être modulées par les mécanismes d'acétylation/déacétylation. L'acétylation par p300 est corrélée à une stabilisation de la protéine ainsi qu'à une augmentation de l'activité transcriptionnelle [345]. A l'inverse, XBP1s est la cible de différentes déacétylases, comme la famille des Sirtuines et notamment SIRT1 et SIRT6. La déacétylation favorise alors la dégradation de XBP1s [345,346]

4. Interactions protéiques

L'action de XBP1s est également modulée par les interactions qu'il forme avec d'autres protéines. Ainsi, comme évoqué plus tôt, XBP1s forme un hétérodimère avec ATF6, et promeut la transcription de gènes liés au stress du RE [326]. Cependant, en condition d'hypoxie, HIF1α peut également former un complexe avec XBP1s et active l'expression de AGT, précurseur de ANGII (Angiotensin II), un peptide sécrété avec des propriétés de vasoconstriction [347]. De même, l'interaction entre XBP1s et les sous-unités p85α et p85β de PI3K augmente la translocation nucléaire de XBP1s [348].

A l'inverse, certaines interactions peuvent être inhibitrices. La formation d'un hétérodimère avec l'Histone Acétyl-Transférase GCN5 provoque une accumulation nucléaire ainsi qu'une stabilisation de XBP1s. Cependant, cette interaction inhibe son activité transcriptionnelle et peut être considérée comme un inhibiteur compétitif, GCN5 titrant XBP1s et l'empêchant de se fixer sur ses gènes cibles [349].

D. Cibles transcriptionnelles de XBP1s

1. Cibles codantes

Dès 2003, des expériences de ChIP dans un modèle de MEF (Mouse Embryonic Fibroblast) surexprimant XBP1s ont permis de déterminer une partie des cibles directes de XBP1s. Ces cibles transcriptionnelles sont des protéines chaperonnes résidente du RE, comme Erdj4, des composants de l'ERAD, comme EDEM (ERAD enhancing mannonidase-like) ou Hrd1 (ubiquitine-ligase), ou encore des protéines impliqué dans le repliements des peptides, comme PDI-P5 [350]. Des méthodes indirectes ont également permis d'étudier les cibles de XBP1s ; en effet, dans un modèle murin de lymphocyte B $Xbp1^{-/-}$, la perte de XBP1s est corrélée à la diminution de VDP (Vesicle Docking Protein) et Tmp21 (Transmembrane Trafficking Protein 21), des protéines impliquées dans le trafic vésiculaire. A l'inverse, la surexpression de XBP1s est corrélée à l'augmentation de ces mêmes protéines [351]. Enfin, un modèle de surexpression de XBP1s dans des fibroblastes NIH-3T3 révèle une synthèse accrue de phospholipides, par à une augmentation de l'activité de la CCTα, (choline cytidylyltransferase) un enzyme impliqué dans la biogénèse des phospholipides. La surexpression de XBP1s dans ces cellules s'accompagne également d'une expansion du RE [352]. Ces résultats seront confirmés par la suite, grâce à une expérience de ChIP sur puce (ChIP-on-chip) réalisée sur plusieurs types cellulaires (cellules musculaires squelettiques, plasmocytes, cellules β du pancréas) [253]. Les auteurs mettent en évidence environ 150 cibles communes aux trois tissus et les regroupent en 19 catégories. Environ un tiers de ces cibles sont liées aux fonctions du réticulum (chaperonne, composants de l'ERAD, synthèse des protéines…). Cependant, les analyses révèlent également des cibles impliquées dans la différenciation cellulaire, la réplication et la réparation de l'ADN, ou encore l'apoptose. Déjà dans la levure, HAC1 régule la réparation des cassures doubles brins : en effet, il interagit avec les histones aux sites de cassures et promeut la réparation via le processus de NHEJ (Non Homologous End Joining) [353]. Chez les métazoaires, XBP1s est lié à l'expression d'un cluster de gènes impliqués dans les mécanismes de réparation de l'ADN (DNA Damage Response ou DDR) comme BRCA1 ou RAD51. De fait, la perte de XBP1 est associée à une augmentation des foci γH2AX et a une diminution de l'activation des mécanismes de réparation, traduisant une incapacité de la cellule à détecter les éventuelles cassures de l'ADN [354].

2. Cibles non-codantes

XBP1s, comme les autres effecteurs de l'UPR a été surtout étudié à travers ses cibles codantes. Néanmoins, quatre micro-ARNs ont été décrit comme des cibles transcriptionnelles directes de XBP1s : miR-148a [272], -153[273], -346 [274] et -1274B [275]. L'action de ces cibles est décrite plus précisément en partie III.C.3.

E. Rôle de XBP1s dans l'hématopoïèse normale et cancéreuse

1. XBP1s et différenciation hématopoïétique

Le rôle de XBP1s dans la différenciation hématopoïétique est décrit précédemment dans la partie III.C.4.

2. XBP1s et fonctionnalité des cellules immunes

Dans les cellules dendritiques, XBP1 n'est pas seulement lié à la maturation, mais également à leur fonctionnalité. En effet, la délétion de XBP1 dans des cellules dendritiques activées aboutit à une hyperactivation du RIDD et une inhibition de la présentation antigénique [299]. De plus, dans le cas de l'échappement immunitaire dans les cancers, une activation constitutive de XBP1s dans des cellules dendritiques associées aux tumeurs induit une accumulation de triglycérides, inhibe la capacité de ces cellules à présenter l'antigène et participe à l'épuisement immunitaire [355] (Figure 22). De façon générale, l'activation des cellules immunitaires *via* les récepteurs TLR ou les récepteurs à cytokine, active la voie IRE1α/XBP1s. Dans les cellules dendritiques, XBP1s est largement impliqué dans leur survie [356]. Dans les macrophages, la voie IRE1/XBP1 stimule la réponse pro-inflammatoire et amplifie le signal du TLR via la production de cytokines telles que l'IL-6, le TNF ou encore l'IFNβ. Ce mécanisme est essentiel dans la mise en place d'une réponse immunitaire liée à une infection par des agents pathogènes [357] (Figure 22). La stimulation du récepteur à l'IL-3 (IL-3R) est également susceptible d'activer la voie IRE1/XBP1. La signalisation en aval de l'IL-3R est cruciale dans la survie des cellules myéloïdes car elle active des mécanismes anti-apoptotiques comme la transcription des facteurs Bcl2 ou BclXL. Dans ce cas, l'activation de XBP1s se fait en 2 temps : d'une part, une activation

transcriptionnelle directe *via* STAT5, et d'autre part, la phosphorylation de IRE1α par la voie PI3k [358].

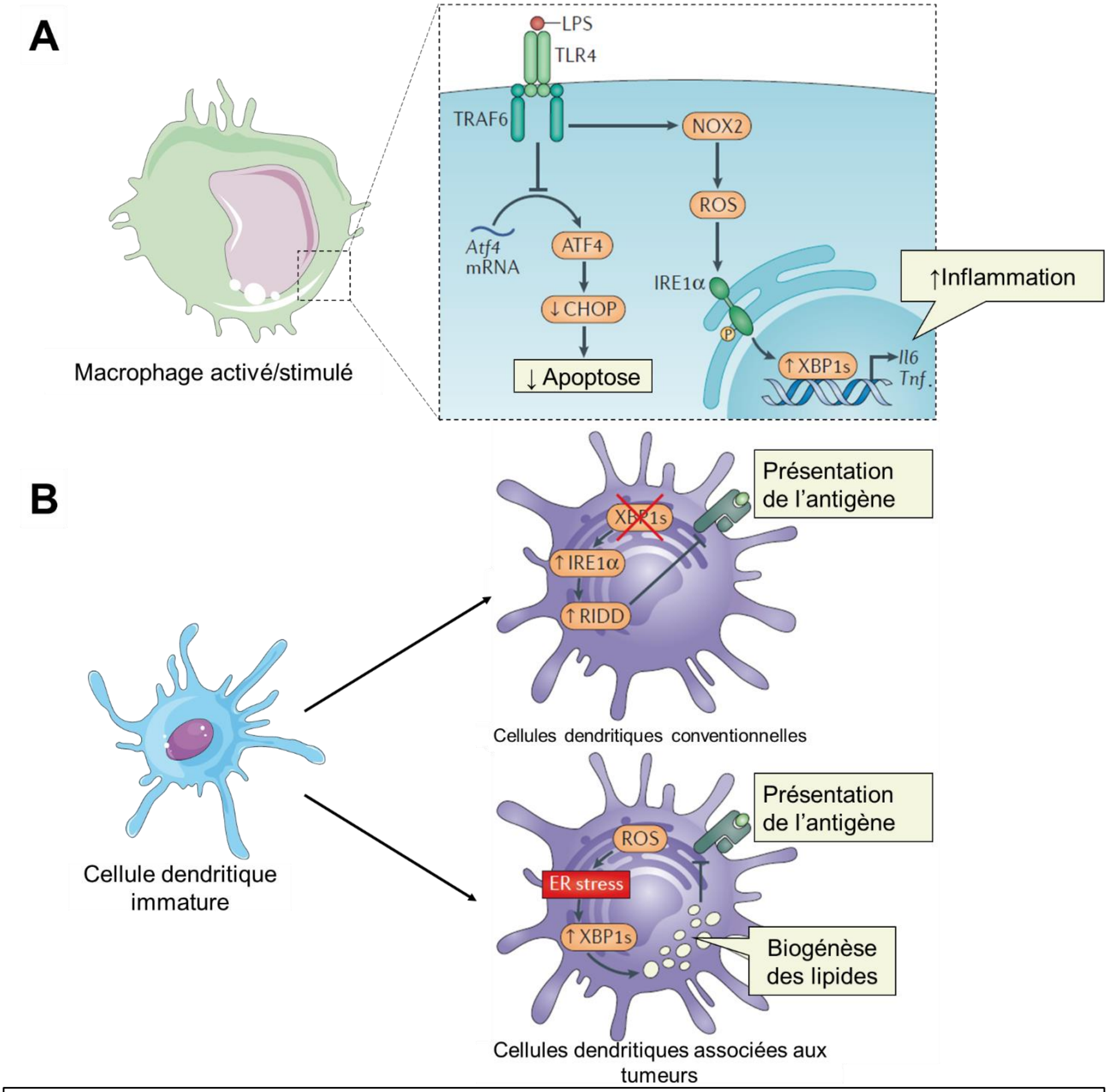

Figure 22 : XBP1s est impliqué dans la médiation de l'inflammation

A) La stimulation du TLR des macrophages promeut l'activation de IRE1 par la production de ROS ; l'activation de XBP1s qui en résulte active transcriptionnellement des médiateurs de l'inflammation tels que l'IL-6 et le TNF. En parallèle, l'apoptose est inhibée par la diminution de la traduction de ATF4.

B) La perte de XBP1s dans les cellules dendritiques conventionnelles matures provoque une suractivation du RIDD. Le RIDD provoque la dégradation des composants du CMH de classe I, nécessaire au processus de présentation de l'antigène. A l'inverse, dans les cellules dendritiques associées aux tumeurs, XBP1s est augmenté et promeut la biosynthèse de lipides, qui perturbent la présentation de l'antigène par le CMH de classe I.

D'après Grootjans et al., 2016

3. Rôle de XBP1s dans les hémopathies malignes

a) Myélome Multiple

Le rôle de XBP1s dans la physiopathologie du Myélome Multiple est décrit précédemment dans la partie III.C.3-4.

b) Les Leucémies BCR-ABL

Les Leucémies Myéloïdes Chroniques (LMC) sont des syndromes myéloprolifératifs, caractérisés par une prolifération aberrante de cellules myéloïdes, sans blocage de différenciation. Les cellules malignes sont caractérisées par une translocation chromosomique t(9 ;22)(q34 ;11), aussi nommée chromosome de Philadelphie. La protéine de fusion BCR-ABL qui en résulte présente une activité kinase constitutive et entraine l'activation de voies de signalisations pro-oncogéniques comme PI3K/Akt, Ras/MAPK et JAK/STAT. Cette translocation est également retrouvée dans 25% des patients atteint de LAL, et sont dites LAL Ph+ (Philadelphie positive).

BCR-ABL active de manière constitutive l'UPR et plus spécifiquement la voie XBP1s [359,360]. Dans les LAL Ph+, une activation transcriptionnelle de *Xbp1* a été mis en évidence par une hypométhylation de son promoteur mais également la répression de régulateurs négatifs, comme BCL6 (B-cell lymphoma 6), par BCR-ABL [306].

Une étude clinique menée sur 55 patients atteints de LAL Ph+ (ECOG E2993) a permis de corréler une forte expression de XBP1 à un pronostic défavorable [306]. L'inhibition de la voie IRE1/XBP1 a donc été proposée comme stratégie thérapeutique potentielle. L'utilisation d'inhibiteurs d'IRE1 (A-106 et STF-083010) a montré une synergie d'action dans des modèles *in vitro* et sur des modèles de PDX (Patients-Derived Xenograft) issus de patients atteints de LAL Ph+ [306].

c) Les Leucémies Aigües Myéloïdes

Dans le cas particulier des LAP, la réponse UPR est une conséquence de l'expression de la protéine de fusion : en effet, l'interaction de PML-RARα et du corépresseur N-CoR (Nuclear hormone receptor CoRepressor) provoque une accumulation de ce dernier dans la lumière du RE [361].

Plus généralement, l'UPR est retrouvé activé dans les cellules issues de patients atteints de leucémies aiguës myéloïdes, indépendamment du sous-type de LAM considéré [362]. Cependant, ce stress n'est pas détectable chez tous les patients. En effet, la détection de XBP1s par RT-PCR permet de ségréger les patients en deux groupes : les patients qui expriment XBP1s (n=17) et les patients qui ne l'expriment pas (n=88) (Figure 23). Il apparaît que l'activation de XBP1 corrèle avec de meilleurs paramètres physiopathologiques comme une meilleure survie sans progression et une meilleure survie globale. Cependant, aucune corrélation entre les paramètres cliniques et l'activation de la voie IRE1/XBP1 n'a encore été décrite à ce jour.

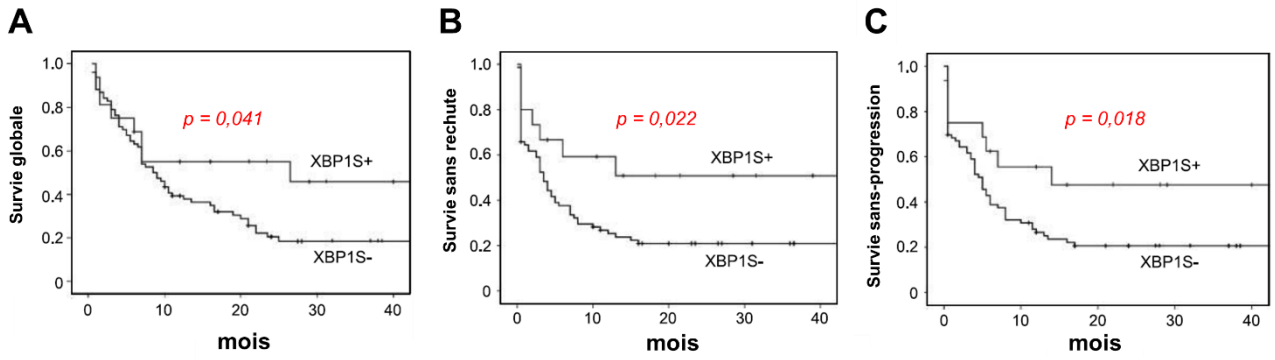

Figure 23 : L'activation de la voie IRE1/XBP1s dans les LAM est corrélée à un bon pronostic

La détection de l'ARNm épissé de XBP1 dans 17 patients sur 105 atteint de LAM en deux groupes distincts : le groupes XBP1S+, qui active XBP1s, et le groupe XBP1S-, qui ne l'active pas. Les auteurs corrèlent l'expression de XBP1s avec de meilleures caractéristiques cliniques : meilleure survie globale (A), meilleure survie sans rechute (B) et meilleure survie dans progression (C).

D'après Schardt et al., 2009

Malgré ces observations, l'utilisation d'un inhibiteur d'IRE1, le A- I06 (2-hydroxy-1-naphthaldehyde, HNA), a montré une activité cytotoxique sur les cellules de leucémie aiguë myéloïde [308]. Mais plus récemment, deux essais cliniques de phase I-II étudient l'impact de l'activation de l'UPR dans le traitement des LAM secondaire et LAM en rechute en incluant le bortézomib dans les régimes de traitement (références essais cliniques : NCT01861314, NCT04173585). Un essai de phase III est également en cours et évalue l'impact du bortézomib sur des LAM *de novo*, porteuses de la mutation FLT3-ITD (référence essai clinique : NCT01371981).

OJECTIF DE LA THESE

Les Leucémies Aigües Myéloïdes représentent 3000 nouveaux cas par an avec un âge médian de diagnostic de 65 ans. La prise en charge des patients repose avant tout sur la chimiothérapie, éventuellement complétée par l'allogreffe de cellules souches hématopoïétiques. Cependant, la toxicité due à cette chimiothérapie empêche parfois l'utilisation de doses optimales. Des thérapeutiques plus spécifiques sont alors utilisées, comme des agents hypométhylants chez les patients les plus âgés et non éligible à la chimiothérapie ou des thérapies ciblées en fonction des anomalies détectées. Cependant, bien que la rémission soit observée dans 80% des cas, les rechutes sont fréquentes et associées à une résistance aux différents traitements. L'amélioration de la tolérance de la chimiothérapie ainsi que la recherche de nouveaux moyens de prises en charge apparaissent donc comme cruciaux dans cette pathologie.

De façon intéressante, l'activation de l'UPR, et plus particulièrement la voie IRE1/XBP1s, est détectée dans 18% des patients atteint de LAM, et corrélée à un bon pronostic, quand la plupart des études réalisées proposent d'inhiber l'UPR [362]. L'activation de la voie IRE1/XBP1s apparaît donc comme une approche intéressante, d'autant plus que l'expression de XBP1s a été reliée au processus de différenciation hématopoïétique. Cependant, la mécanistique sous-jacente et les acteurs impliqués dans ces effets bénéfiques n'ont pas été élucidés.

Au cours de ma thèse, je me suis donc attachée à étudier l'impact de l'expression de XBP1s dans les LAM grâce à un modèle d'expression inductible de l'isoforme épissé. Ce modèle a permis également de réaliser un séquençage des ARNs et des micro-ARNs associés à un ChIP-sequencing spécifiquement dans la LAM. Ce modèle, ainsi que les analyses à grandes échelles qui en ont découlé, ont été le point de départ de mon travail de thèse, qui s'est articulé autour de 3 axes :

- *Déterminer l'impact de la réexpression de XBP1s dans la progression des LAM*

L'utilisation d'un modèle inductible nous a permis de moduler l'expression de XBP1s dans différentes lignées cellulaires de LAM. L'impact de XBP1s a ainsi pu être évaluer seul ou en combinaison avec des traitements, *in vitro* ou dans des modèles *in vivo* de xénogreffes orthotopiques.

- *Déterminer de nouvelles cibles non-codantes de XBP1s impliquées dans ces effets*

Les différentes analyses à grande échelle nous ont permis de déterminer deux micro-ARNs particulièrement relevant, activés transcriptionnellement par XBP1s.

- *Etudier l'implication de ces micro-ARNs cibles dans la progression des LAM*

Nous avons également caractérisé fonctionnellement ces cibles dans la LAM, à travers différents modèles de surexpression et d'inhibition, dans des modèles *in vitro* et *in vivo*. L'utilisation de technique de « pull down » ainsi que différentes bases de données prédictives a permis également d'identifier leurs cibles et de déterminer en partie les cascades moléculaires impliquées dans les LAM XBP1s +.

RESULTATS

Article I

Rôle critique de l’axe XBP1/miR-22/SIRT1 dans la réponse à la chimiothérapie dans les leucémies aiguës myéloïdes

Dans cette présente étude, nous avons cherché à comprendre par quels mécanismes l'expression de XBP1s peut-elle être reliée à un bon pronostic dans les Leucémies Aigües Myéloïdes.

Pour ce faire, nous avons développé un modèle gain-de-fonction, permettant l'expression de l'isoforme épissé de XBP1, sous contrôle d'un promoteur inductible Tet-On, répondant à la doxycycline. La transduction de ce transgène dans 6 lignées différentes de LAM nous a permis d'évaluer l'impact de la surexpression de XBP1s indépendamment des anomalies génétiques présentes dans ces différentes lignées. Ce modèle nous a également permis de moduler l'expression de XBP1s, directement par la dose de doxycycline ajoutée.

Ainsi, nous avons observé qu'une forte induction de XBP1s active l'apoptose dans l'ensemble de nos lignées, en comparaison des cellules parentales TETON, n'exprimant que le transactivateur rtTA. De plus, nous avons réalisé des xénogreffes orthotopiques pour 3 de ces lignées, les OciAML2, les OciAML3 et les HL-60, et pour lesquelles l'induction de XBP1s, dans ce contexte physiologique, permet une amélioration significative de la survie.

Une des hypothèses de Schardt *et al.* à l'issue de l'étude clinique suggérait que XBP1s pouvait être lié à une meilleure sensibilité au traitement. Nous avons alors cherché à étudier l'impact de XBP1s sur la réponse à la chimiothérapie. Afin de se rapprocher des conditions d'activation observées dans la LAM, nous avons induit XBP1s à une dose ne provoquant pas d'apoptose dans les OciAML3, la seule lignée résistante à l'aracytine *in vitro* et *in vivo*. Ce modèle nous a permis de montrer que l'expression de XBP1s potentialise les effets de l'aracytine dans les deux cas.

XBP1s, en tant que facteur de transcription, engendre un remodelage du profil transcriptionnel des cellules qui l'activent. Classiquement, XBP1s a été montré comme activant nombre de gènes codant pour des effecteurs du RE. Cependant, par des analyses de séquençage des ARN couplé à des expériences de séquençage de ChIP, nous avons mis en évidence des cibles non-codantes, dont une potentiellement liée aux phénotypes précédemment décrits.

En effet, nous avons montré que le long ARN non codant MIR22HG est une cible directe de XBP1s, non décrite jusqu'alors. Ce lncRNA est le précurseur du micro-ARN-22, qui apparaît comme le micro-ARN le plus augmenté par XBP1s dans nos analyses de miRnome.

Nous avons montré que la surexpression de miR-22 par transfection transitoire mais également par un modèle inductible, est lié à l'induction de l'apoptose dans les OciAML2 et

les OciAML3. De plus, l'inhibition de miR-22 par transfection d'un antagomiR-22 conduit à une résistance à l'aracytine dans les OciAML3. La purification de complexes ARNm/miR-22-biotine par une expérience de « Biotin pull-down », a permis de visualiser et de confirmer l'interaction entre l'ARNm de la Sirtuine 1 (SIRT1) et miR-22, déjà décrite dans la littérature.

Par ailleurs, nous observons une diminution des niveaux protéiques de SIRT1 à la fois en réponse à XBP1s, mais également après induction de miR-22. A l'inverse, l'inhibition de miR-22 par un antagomiR-22 restitue le niveau d'expression de SIRT1.

Nous avons également relié l'inhibition de SIRT1 aux phénotypes liés à l'activation de l'axe XBP1s/miR-22. L'inhibition de SIRT1 par le biais de 2 siARN transfectés dans 2 lignées cellulaires, OciAML3 et THP1, montre une induction de l'apoptose. De façon intéressante, l'inhibition pharmacologique de SIRT1 ne conduit pas à l'induction de l'apoptose, mais potentialise les effets d'un traitement à l'aracytine.

L'ensemble de ces résultats confirment l'effet anti-leucémique de XBP1s, sous-entendu par l'étude clinique de Schardt *et. al.* Ils suggèrent également l'importance des cibles transcriptionnelles non-codantes de XBP1s, dont le micro-ARN miR-22, qui semblent cruciaux dans les mécanismes anti-cancéreux mis en évidence. Enfin, la description de l'axe XBP1s/miR-22/SIRT1 permet de mettre en évidence de potentiels biomarqueurs prédictifs de la réponse à la chimiothérapie, voire même de nouvelles cibles thérapeutiques.

L'ensemble de ces résultats font actuellement l'objet d'un article en préparation (voir ci-après).

Pivotal role of the Endoplasmic Reticulum stress-related XBP1s/mir-22/Sirtuin 1 axis in apoptosis and response to chemotherapy in Acute Myeloid Leukemia

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ABSTRACT

High proliferation rate of malignant cells must be sustained by a rapid protein synthesis frequently leading to endoplasmic reticulum (ER) overload and accumulation of unfolded or misfolded protein in this cellular compartment. In the ER, protein homeostasis is finely regulated by a mechanism called the unfolded protein response (UPR), involving the activation of three transmembrane proteins of the ER, PERK (Protein kinase RNA-like endoplasmic reticulum kinase), IRE1 (Inositol Requiring Enzyme 1) and ATF6 (Activation Transcription Factor 6). IRE1 activation triggers endoribonuclease activity and cleaves an intron in the open reading frame of the cytosolic mRNA XBP1, a key UPR-specific transcription factor. This unconventional “splicing” induces a translational frameshift and results in the expression of a longer-active-XBP1 protein called XBP1s which upregulates a set of genes involved in the recovery of the cell from the proteotoxic stress.

In solid tumors, ER stress has been widely studied, whereas the role of UPR in malignant hemopathies, and particularly in Leukemia, has been less investigated. While the transcription factor XBP1s was mainly described as a pro-survival protein, UPR activation, especially the IRE1/XBP1 pathway, was associated with a favorable prognosis in Acute Myeloid Leukemia (AML).

In order to precise the role of the IRE1/XBP1 pathway in AML, we have developed a tetracycline-inducible XBP1s model in leukemic cell lines. Our studies clearly demonstrate that the sustained activation of XBP1s expression induce apoptosis both *in vitro* and *in vivo,* whereas a moderate XBP1s expression sensitizes cells to chemotherapeutic treatments. ChIP-sequencing experiments allowed us to identify specific XBP1s target genes including the stress-induced MIR22HG lncRNA, the transcriptional precursor of miRNA-22. Activation of microRNA miR-22 expression by XBP1s or forced expression of miR-22 significantly suppresses viability and sensitizes leukemic cell to chemotherapy, while depleting endogenous miR-22 leads to chemoresistance. Mechanistically, miR-22 was found to target the deacetylase sirtuine 1 (SIRT1), notably described as pro-survival factor. These findings showed that XBP1s acts as a tumour suppressor in AML, and the XBP1/miR22/SIRT1 axis plays a pivotal role in the chemotherapeutic response. We propose MIR22HG/miR-22 to be a promising biomarker of stress level as well as a potential treatment response biomarker in Acute Myeloid Leukemia.

INTRODUCTION

In eukaryotic cells, transmembrane and secreted proteins are transferred into the endoplasmic reticulum (ER) during their translation by ER membrane-bound ribosomes, whereas proteins synthesized by free ribosomes are cytosolic or transported to the nucleus, mitochondria, or peroxisomes [1-3]. The vast majority of cellular stresses (hypoxia, glucose- or amino acid-starvation, oxidative stress, acidosis, etc.) lead to an alteration of endoplasmic reticulum functions by inducing the accumulation of misfolded proteins [4]. To cope with any perturbation caused by misfolded or unfolded proteins accumulation in the ER, cells set up an adaptive and physiological response named unfolded protein response (UPR), which aims to restore normal ER functions [5,6]. The initial intent of the UPR is thus to limit cellular damage and to adapt to the environment changes by reestablishing normal ER function and homeostasis. However, UPR is a dynamic pathway and can also lead to cell death upon intense or too long stressed conditions [7]. UPR-mediated signaling involves the activation of three transmembrane proteins of the Endoplasmic Reticulum (i) the kinase PERK (protein kinase R (PKR)-like ER kinase) also known as EIF2AK3 (eukaryotic translation initiation factor 2-alpha kinase 3) (ii) the bifunctional protein IRE1α (Inositol Requiring Enzyme 1α), and (iii) the transcription factor ATF6 (Activating Transcription Factor 6) [4,8,9]. In normal conditions, the luminal domain of these three proximal sensors binds the protein chaperone BiP (Binding Immunoglobulin Protein), which keeps them in an inactive conformation. When unfolded or misfolded proteins accumulate in the ER, they bind to BIP and disrupt its interaction with the three sensors leading to their activation [10-12].

Upon activation, each sensor elicits downstream signaling. Concisely, the activated cytosolic domain of the PERK protein induces the phosphorylation of its mainly-characterized substrate, the eukaryotic translation initiation factor 2α (eIF2α) at serine-51, leading to inhibition cap-depending-translation, decreasing influx of new proteins at the ER [13,14]. This PERK- mediated-global-translational repression provides an immediate response to ER stress which, in contrast to the other two signaling pathways, does not depend on transcriptional induction. Apart from this general translational downregulation, some mRNA containing specific features in their 5' untranslated region, including uORF (upstream open reading frames) or IRES (Internal Ribosome Entry Sites), and coding for factors essential for cell recovery can be efficiently translated [15-19]. During its activation process, the second sensor, ATF6, is transported and proteolytically processed in the Golgi apparatus in an active transcription factor, ATF6p50 or ATF6f (fragment), which targets UPR response genes including chaperones or proteins involved in the Endoplasmic Reticulum Associated

Protein Degradation (ERAD) mechanism [12,20,21]. The third sensor IRE1α, the most conserved arm of UPR, is a bifunctional protein composed of two cytoplasmic catalytic domains (i) a protein kinase domain and (ii) an endoribonuclease domain [4,22,23]. BIP release induces dimerization of IRE1α which triggers trans-autophosphorylation of its kinase domains and finally activation of the RNAse subunit. The endoribonuclease domain targets cytoplasmic mRNA and microRNAs, leading to their decay by RIDD process (Regulated Ire1-Dependent Decay) [24,25]. Moreover, once activated, IRE1α endoribonuclease removes a 26 nucleotides sequence from XBP1 (X-box Binding Protein 1) mRNA coding sequence inducing a translational frameshift after subsequent re-ligation by the tRNA ligase RtcB [26-28]. This "spliced" mRNA is translated into a 376 amino acids-long isoform XBP1s (spliced), coding for a potent transcription factor, which target stress response genes, essential to restore ER homeostasis [20,21,29]. *Xbp1* mRNA is ubiquitously expressed, but invalidation experiments demonstrated a huge involvement in development and support of secretory tissues such as Paneth cells and β pancreatic cells, but also for proper eosinophil and plasma cells differentiation [30,31]. The IRE1α-XBP1 pathway was also involved in multiple pathophysiological processes such as immunity, obesity and type 2 diabetes, circadian rhythm regulation, neurodegeneration, aging and of course cancer [32,33].

Considering UPR versatile implications, many studies reported its activation in a variety of cancers. In tumors XBP1s expression is frequently associated with a poor prognosis because it drives cell adaptation under stress conditions. Consistently, in triple-negative breast cancer, under hypoxic conditions, XBP1 can cooperate with HIF1a to promote angiogenesis, glucose uptake and thus cancer cells progression and resistance [34]. In some tumors like in Multiple Myeloma, the relevance of XPB1s expression on clinical outcome is more discussed. Indeed, it has been reported that overexpression of XBP1s alone induces a Multiple Myeloma-like syndrome in mice [35]. This suggests a possible oncogenic role for XBP1 in the development of this disease. Moreover, under thalidomide-based treatment, high expression of XBP1s in malignant cells correlates with a lower response to chemotherapy [36]. On the contrary, high *Xbp1* gene expression has been found to correlate with a better response to Bortezomib treatment [37]. Furthermore, two inactivating mutations have been characterized in the *Xbp1* gene of Multipe Myeloma patients, triggering resistance to Bortezomib treatment [38,39]. Taken together, these data suggest a dual role of XBP1s in the progression and treatment of Multiple Myeloma.

Therefore, ER stress in general, and XBP1s in particular, can facilitate both anticancer drug efficacy or chemoresistance development, depending on therapeutic strategies, cellular

context and tumor type. The role of UPR, and more precisely the IRE1/XBP1 axis, in lymphoma and leukemia progression is poorly studied and remains unclear [40]. It was demonstrated that impaired XBP1 activation is a hallmark of Germinal Center B cell-like Diffuse Large B cell Lymphoma and contributes to tumor growth [41]. In Acute Myeloid Leukemia (AML), clinical data from patients correlate XBP1s expression with a favorable outcome upon cytarabine and etoposide-based therapy [42,43].

In order to decipher the role of XBP1s in Acute Myeloid Leukemia, we set up a model enabling inducible expression of XBP1s isoform in six AML cell lines, including the aracytine resistant cell line OciAML3. Our results demonstrate that sustained XBP1s expression can activate apoptotic-signaling pathway both *in vivo* and *in vitro.* Interestingly, a lower and non-toxic XBP1s expression level sensitizes chemoresistant-AML-cell line OciAML3 to Aracytin treatment. By cross-analyzing RNA and ChIP-sequencing, we found that the long non-coding RNA MIR22HG, precursor of microRNA miR-22, is upregulated in an XBP1-dependent manner during ER stress. Interestingly, mature miR-22 is down-regulated in AML patients [44] and represses genes of the DNA damage response [45,46]. We demonstrate miR-22 mediate apoptosis and better treatment response through sirtuin-1 (SIRT1) translational inhibition. Taken together these results identify a novel ER stress-induced axis XBP1/miR-22/SIRT1 as an effector of apoptosis and chemosensitivity in Acute Myeloid Leukemia.

MATERIAL AND METHODS

Cell line generation and shRNA-Mediated Gene Knockdown

The XBP1 spliced isoform coding sequence was PCR amplified with the proofreading Taq polymerase Phusion (thermofisher) using the primer pair Xba XBP1 Fw (GGTCTAGATAATACGACTCACTATAGGGATGGTGGTGGTGGCAGCCGCGCCG) and XBP1 Sal Rev (CCGTCGACTGCAGAATTCGAAGCTTGAGCTCGAGTTAGAC-ACTAATCAGCTGGGG). The EMCV IRES GFP cassette was in parallel amplified with the primers pIres2GFP Sal1 Fw (CAGTCGACGGTACCGCGGGCCCGGGATC-CGCCCCTCTCCCTCCCCCCCCCC) and pIres2GFP BGL2 REV (TTAGATCTTAC-TTGTACAGCTCGTCCATGCCGAG) using the pIRES2GFP vector from Clontech as a template. These two PCR products were digested respectively by Xba1/Sal1 and Sal1/Bgl2 and ligated into the pTRIPz-TRE-Tight plasmid (Open Biosystem) digested by Xba1/ Bgl2 leading to the fusion of the EMCV IRES GFP sequence to the XBP1s open reading frame downstream of doxycycline responsive promoter. Constructs were confirmed by sequencing and used to transduce OciAML2, OciAML3, MV4-11, MOLM-14, THP1 and HL-60 cell lines, already transduced with pTetOn vector (Clontech), allowing expression of the rTTA (reverse Tetracycline Transactivator). Selection of inducible cells was based on EGFP expression and performed by flow cytometry after treatment with a low dose of doxycycline during 10H. Control TetOn cells were only transduced once with pTetOn vector. For generation of stable XBP1 knockdown, wild-type cells were transduced with the lentivirus pTRIPZ-XBP1 shRNA (Dharmacon: reference V3THS_387389, Mature Antisense sequence: TCTTCTAAATCTACCACTT). Stably transduced cells expressing the shRNA were further selected by treatment with 1 µg/mL of puromycin. MiR-22 inducible models were generate using wild-type OciAML2 and OciAML3, stably transduced with shMIMIC Inducible Lentiviral microRNA (Horizon; reference VSH6906-224634676; miRNA sequence: AAGCUGCCAGUUGAAGAACUGU). All transductions are performed through retronectin infection, according to the manufacturer's instructions (Clontech).

Cell culture and treatments

All leukemic cell lines were obtained from the Leibniz Institute DSMZ. Cells were cultured in RPMI supplemented with 10% (OciAML2, OciAML3, MOLM14) or 20% (MV4-11) of Fetal Bovine Serum (FBS), or in IMDM supplemented with 20% of FBS (HL-60), 2mM L-glutamine, 100 U/mL penicillin/streptomycin (all from Invitrogen) and incubated in a

humidified atmosphere of 5% CO2 at 37°C. Aracytine treatment (10µM, 24H) was performed following a 24h -XBP1s induction at 4ng/mL or a 72h-EX-527 treatment at 30µM in OciAML3. Endoplasmic reticulum stress was triggered by treating cells with Tunicamycin (10µg/mL) or Thapsigargin (50nM) for 6h. All drugs used in these studies were from Sigma Aldrich.

Reverse transcription and quantitative PCR

For mRNA and Long-non coding RNA, 500ng of total RNA was reverse transcribed using the PrimeScript RT-PCR Kit from Clontech, according to the manufacturer's protocol. Reverse Transcription (RT) reactions were diluted 20-fold prior to qPCR. Amplification was performed in a total volume of 10µL containing 5µL of a SYBR Premix Ex TaqTM (Tli RNaseHplus), TB Green® Premix Ex Taq™ (Tli RNase H Plus) (TakaraBio), 1µL of both forward and reverse primer (final concentration of 100nM each), and 2µL of diluted cDNA. Primer sequences are given in Table 1. qPCR was performed on the StepOnePlus real-time PCR system (Applied Biosystems), and results was analysed with the StepOne software. For mature microRNA quantification, RT was performed using the miRCURY™ LNA™ Universal RT (Qiagen) microRNA PCR. miR22 expression was assessed by qPCR using hsa-miR-22-3p PCR primer set (Qiagen) and hsa-let-7a-5p PCR primer set for normalization.

Table 1 : Primer list

GENE	FORWARD PRIMER	REVERSE PRIMER
XBP1s	5'-CTGAGTCCGCAGCAGGTGCAG-3'	5'-ATCCATGGGGAGATGTTCTGG-3'
MIR22HG	5'-CCTCGTGCAGCAACCCC-3'	5'-GTGAGGGCGTGAGAGGAAC-3'
SIRT1	5'-ACCTCCTCATTGTTATTGGGTCT-3'	5'-GGGCACTTCATGGGGTATGG-3'
DNAJB9	5'-AAAATAAGAGCCCGGATGCT-3'	5'-CGCTTCTTGGATCCAGTGTT-3'
MDC1	5'-ATTAGCTGCTGTGGAGGCAC-3'	5'-CCCGTAGTGGAATGGAGCAA-3'
P21	5'-ACTCTCAGGGTCGAAAACGG-3'	5'-GCGGATTAGGGCTTCCTCTT-3'
ABL	5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3'	5'-GATGTAGTTGCTTGGGACCCA-3'
HPRT	5'-TGCTTTCCTTGGTCAGGCAGT-3'	5'-CTTCGTGGGGTCCTTTTCACC-3'
TBP	5'-GCCTCCCCCACCCCCTTCTTT-3'	5'-GCCACACCCTGCAACTAACATCC-3'
Actin	5'-CACCATTGGCAATGAGCGGTTC-3'	5'-AGGTCTTTGCGGATGTCCACGT-3'
GAPDH	5'-CAACGACCACTTTGTCAAGCT-3'	5'-CTCTCTTCCTCTTGTGCTCTTGC-3'
ChIP PRIMERS		
DNAJB9	5'-GGTTTAAGCACCGCCTTTTCG-3'	5'GTGGAAAACTGTTGTTGCTGCTA-3'
MIR22HG	5'- CCGGCCAATAGACGGACA-3'	5'- CTTTCGCCTGCTCTTTAGGAC-3'
ACTIN	5'-GGGACTATTTGGGGGTGTCT-3'	5'-TCCCATAGGTGAAGGCAAAG-3'

Western Blotting

Western blots were performed as previously described [47]. After lysis and sonication (10s each separated by a 30s incubation on ice using VCX 130 Ultrasonic Processor (Sonics) at 25% of its power) cellular debris were eliminated by centrifugation (10min at 10,000g, 4°C). The total protein concentration of cell lysates was determined using a Bicinchoninic Acid Assay kit purchased from Sigma. Protein lysates were fractionated on SDS-PAGE 10% and transferred to a nitrocellulose membrane with Trans-Blot® Turbo™ Transfer System (Bio-Rad). Western-blotting was performed using XBP1 (Santa Cruz, sc-7160), phosphorylated eIF2α (Cell Signaling, #9721), cleaved PARP (Cell Signaling, #9541), cleaved caspase 3 (Cell Signaling, #9664), total PARP (Cell Signaling, #9532), SIRT1 (Cell Signaling, #9475), GAPDH (Millipore, MAB374), Actin (Sigma-Aldrich, a5441) antibodies. Proteins were visualized using Clarity™ Western ECL from Bio-Rad.

siRNA and microRNA transfections

XBP1 siRNA (smart pool, Dharmacon), siSIRT1 #1 (s23771, Thermofisher) siSIRT1 #2 (s23770, Thermofisher) as well as negative control siRNA were transfected in Oci AML3 at a final concentration of 10nM or 50nM using the Lipofectamine RNAiMAX tranfection reagent (ThermoFisher) according to the manufacturer's instructions. mirVana miRNA mimic hsa-miR-22-3p and mirVana negative control (ThermoFisher) were transfected in Oci AML3 using the same protocol. Cells were harvested 24 or 48 h later for protein and RNA analyses.

Apoptosis measurement by Flow cytometry

Analysis of apoptosis was done using Annexin V (Annexin-Pacific Blue) and Propidium iodure (PI) (Biolegend # 640928) staining according to standard protocols, followed by flow cytometry using a MACSQuant® VYB from Miltenyl Biotec. Results were analyzed using FlowJo software.

Cell viability assay

Cells were treated or transfected are counted and seeded in 96-well plates (10,000 cells/well, in 100µL RPMI 10%FBS). After 48H cell viability was assessed using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega).

Murine xenograft model

All animal procedures were performed following the principal guidelines of INSERM, and our protocol was approved by the Midi-Pyrenees Ethics Committee on Animal Experimentation. NSG and nude mice were produced at the UMS006 in Toulouse (France) or obtained from Charles River. NSG mice were treated by an intraperitoneal injection of busulfan (20 mg/kg) to induce medullar aplasia. 24h after busulfan treatment, mice were intravenously injected with 2 million of OciAML3, OciAML2 or HL-60 XBP1s-inducible or TETON cells. After a 9-days-engraftment, doxycylin was added at 1mg/mL in drinking water during 8 days and refresh every 3 days. Mice daily monitoring was performed to detect symptoms of disease (ruffled coat, hunched back, weakness, and reduced motility). For *in-vivo*-chemosensitivity assay, mice were daily-intraperitoneally injected with Aracytin at 30mg/kg during 5 days, 3 days after doxycylin-treatment onset. For subcutaneous xenografts, a total of $2.5.10^6$ Oci-AML3 XBP1s cells were injected into both flanks of nude mice. Mouse body weight and tumor volumes were measured every day. Doxycycline (2mg/mL or 0.2mg/mL) was added in the drinking water and refreshed every 3 days, once tumor reached an average volume of $250mm^3$. At the end of the experiment, mice were humanely sacrificed and subcutaneous tumors were harvested, and proteins were extracted for further analyses.

Chromatin Immunoprecipitation

XBP1s expression was induced with 10ng/mL of doxycycline for 48H. ChIP was then perfomed using the ChIP-IT® Express kit (Active Motif). Briefly, in order to cross-link proteins to DNA, cells were treated with 1% formaldehyde for 10min at room temperature, then with glycin according to the manufacturer's protocol. Chromatin was sheared by sonication into relatively uniform 300pb fragments and immunoprecipitated with two different XBP1 antibodies (Santa Cruz, sc-7160 and Biolegend, 619502), or not (Input), using magnetic beads.IgG isotype (Biolegend, 910801) was used as immunoprecipitation control.. DNA was eluted, and then de-crosslinking and purified using phenol-chloroforme extraction. This

protocol was used for ChIP-sequencing sample generation and for ChIP-qPCR. For ChIP-qPCR, 1/10 (2µl) of input or immunoprecipitated DNA was analyzed by qPCR with SYBR green (Takara Bio Inc.) on the StepOnePlus real-time PCR system (Applied Biosystems). Results are represented as the mean value of at least three independent experiments of immunoprecipitated chromatin (percentage of input) with the indicated antibodies after normalization using a control ChIP performed with an irrelevant antibody.

AML patient samples

AML and normal bone marrow samples were obtained from patients at the Department of Hematology (Centre Hospitalier Universitaire de Toulouse [CHU], Toulouse, France) after consent in accordance with the Declaration of Helsinki. Samples were stored at the HIMIP collection. RNA was extract as previously described (ref). 500ng of total RNA was reverse transcribed using the PrimeScript RT-PCR Kit from Clontech, according to the manufacturer's protocol. Reverse Transcription (RT) reactions were diluted 10-fold prior to qPCR. Amplification was performed in a total volume of 10µL containing 5µL of a SYBR Premix Ex TaqTM (Tli RNaseHplus), TB Green® Premix Ex Taq™ (Tli RNase H Plus) (TakaraBio), 1µL of both forward and reverse primer (final concentration of 100nM each), and 2µL of diluted cDNA. Primer sequences are given in Table 1. qPCR was performed on the LightCycler (Roche).

Biotin Pull Down Assay

Biotinylated mimic microRNA 22, purchased from Exiqon, was transfected in 5.10^6 Oci AML3 wild-type cells at 10nM (final concentration) for 24H. Cells were then harvested and resuspend in an IP-Buffer (25mM Tris-HCL (pH7.4), 200mM NaCl, 0.2% Triton, 5mM MgAcetate, 1mM DTT) supplemented with RNAseOUT (Thermo Fisher) and protease inhibitor cocktail (Roche). Samples were sonicated (2 times 10s each separated by a 30s incubation on ice using VCX 130 Ultrasonic Processor (Sonics) at 25% of its power) and lysate was used for pull down. Pierce™ Magnetic beads from ThermoFisher were washed with IP-buffer and blocked with yeast tRNA and BSA for 1H at 4°C. Beads were washed twice and incubated with the lysate for at least 1H30 at 4°C on an end-to-end rotator. Beads was washed 5 times with IP buffer and RNA extraction was performed using TRIzol® total RNA isolation reagent (Invitrogen), following the manufacturer's protocol. Then, 11µL of IP

was used for RT with the Superscript III reverse transcription kit (Invitrogen) and RT reactions were diluted 10-fold prior to qPCR (see primers in table, qPCR part).

Statistical analyses

Results are presented as mean values ± standard deviations (SD). Differences between 2 groups were examined using 2-tailed Student's t-test. Survival analyses were performed using log-rank test. For the subcutaneous xenografts, determination of statistical significance was performed using two-way ANOVA followed by the Bonferroni test. All analyses were performed using GraphPad Prism version 5.03. For all tests, p-values less than 0.05 (*), 0.01(**), 0.001(***) were considered statistically significant.

RESULTS

XBP1s inducible expression in AML cell lines

To identify the role of IRE1/XBP1 pathway activation in Acute Myeloid Leukemia, we established six tetracycline-inducible (Tet-on) cell lines: OciAML3, OciAML2, MV4-11, MOLM-14, THP1 and HL60, in which the XBP1s sequence was cloned under the control of a doxycycline-inducible promoter, allowing regulated expression of the XBP1 spliced isoform. After cell transductions with lentivirus-based bicistronic vector, enabling simultaneous expression of the XBP1s transgene and the green fluorescent protein (eGFP) marker, cells were sorted depending on eGFP expression. In order to validate our model, control cells (TETON) or XBP1s expressing cells are treated with increasing amount of doxycycline. As expected, doxycycline treatment leads to increased XBP1s expression both at the mRNA (Figure 1B, D, F, H and J) and protein level (Figure 1C, E, G, I and K) in each cell lines.

Given that XBP1s regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response, we confirmed the functionality of the exogenously expressed XBP1s protein after induction with low doses of doxycycline by analysing the expression of DNAJB9 (ERdj4), which is highly induced at the mRNA level in response to IRE1/XBP1 axis activation (Supplementary Figure 1A). Moreover, we validated doxycycline treatment didn’t induce XBP1s expression in TETON cells (Supplementary Figure 1B). Finally, increased expression of XBP1s protein upon treatment with doxycycline had no effect on the phosphorylation of eIF2α (Supplementary Figure 1C) indicating that the PERK pathway is not activated upon XBP1s overexpression.

Increased XBP1s expression induces apoptosis both in vitro and in vivo and impaired tumoral progression in vivo

We next studied the effects of XBP1s expression on the viability of AML cell lines. Unexpectedly, flow cytometry analysis demonstrated that increased expression of XBP1s increased apoptosis in all tested cell lines, in a doxycycline-dose-dependent way (Figure 2 A-F), while doxycycline had no effect on apoptosis in TETON control cells. These results were further validated with cleaved-PARP-staining, which increase in OciAML2, OciAML3, MOLM14 and THP1 XBP1s-expressing cells, evaluated by western blot (Supplementary Figure 2A to D).

In order to determine whether increased apoptotic cell death could be responsible for the reduced malignant behavior of tumors, we engrafted subcutaneously nude mice with OciAML3 cells expressing XBP1s. Once tumor reached an average volume of 250mm^3, mice received doxycycline at 2 mg/ml or 0,2mg/ml in the drinking water. XBP1s induction at both concentrations caused dramatic tumor regression compared to untreated mice (Supplementary Figure 2E). At day 23, experiment was stopped and residual tumors were harvested for protein content analyses. Western blot analyses of tumor samples from doxycycline-treated mice compared to non-treated mice, revealed that XBP1s expression was, as expected, strongly increased and was accompanied by the efficient cleavage of both PARP and Caspase 3, confirming apoptosis-signaling induction (Supplementary Figure 2F).

To assess whether activation of XBP1s expression also suppressed tumor progression in a more physiological mouse models, OciAML3, OciAML2 or HL-60 cells expressing XBP1s or control cells (TETON) were intravenously injected into NSG mice. After 9 days of engraftment, drinking water was supplemented with 1mg/ml of doxycycline in order to induce XBP1s expression during 8 days, and survival was further analyzed (Figure 2G). Transient XBP1s expression significantly increased the overall survival in mice for all tested AML cell lines, thereby demonstrating XBP1s anti-leukemic activity *in vivo* (Figure 2H-I).

Increased XBP1s expression potentiates chemotherapeutic treatment in OciAML3 resistant cells

Considering that chronic low-level expression of XBP1s in cancer cells is compatible with cell survival, we chose a doxycycline dose (4ng/mL) that does not induce cell death, to monitor XBP1s expression effect on chemotherapeutic treatment. Apoptotic response in XBP1s-inducible OciAML3 cells, determined by Annexin V/propidium iodide staining followed by flow cytometry, demonstrated that XBP1s expression leads to Aracytin sensitization (Figure 3A), but not to Bortezomib, vinblastine and staurosporine (Figure 3B) compared to TETON control cells. We also investigated the effect of global ER stress induction with Tunicamycin (Tu), in combination with Aracytine. Interestingly, a combinatorial treatment comprising ER stress inducer Tunicamycin and chemotherapy offers synergistic effects on apoptosis induction *i.e.*, induces more apoptosis than the sum of the separate effects of each individual treatment (Supplementary Figure 3).

We confirmed OciAML3 chemosensitization upon XBP1s expression in orthotopic xenograft model. OciAML3 expressing XBP1s or not (TETON) were intravenously injected into NSG mice. After 9 days of engraftment, drinking water was supplemented with 1mg/ml of doxycycline in order to induce XBP1s expression during 8 days. 3 days after doxycycline-treatment start, mice were intraperitoneally injected with Aracytin at 30mg/kg/day during 5 days, or not (Figure 3C). We don't observe survival improvement between mice injected with TETON cells and mice injected with TETON cells treated with Aracytin, confirming OciAML3 chemoresistance *in vivo*. Moreover, as expected, XBP1s expression increase mice survival. More interestingly, XBP1s expression associated with Aracytin treatment improve mice survival even more than XBP1s alone, confirming XBP1s-dependant chemosensitization *in vivo* (Figure 3D).

MIR22HG is a direct target of XBP1s and is up-regulated by endogenous XBP1s upon ER stress

XBP1s is widely known as a transcription factor that regulates many stress response genes, but little is known about its non-coding targets. To address the molecular mechanism by which XBP1s induces apoptosis and sensitizes AML cells to chemotherapy, we performed several large-scale analyses comparing OciAML3 XBP1s to OciAML3 TETON induced at 10ng/mL of doxycycline: Chromatin Immunoprecipitation (ChIP) followed by large sequencing, global RNA sequencing and micro-RNA sequencing allows us to study XBP1s-expression effects on AML cells. We cross-analyzed RNA sequencing ang ChIP sequencing to focus on XBP1s-direct targets. We identified the long non-coding RNA MIR22HG as one of the most enriched lncRNA (Figure 4A and Supplementary Figure 4A). Interestingly, MIR22HG is characterized to be micro-RNA-22 precursor [48] and miRnome analysis revealed that miR22 is the most highly upregulated microRNA following XBP1s activation (Supplementary Figure 4B). The initial ChIP-Seq results were verified by ChIP-qPCR experiments, using primers spanning the putative identified XBP1s binding region in the MIR22HG promoter and two additional primer pairs: one targeting the promoter region of DnajB9, a validated XBP1s target gene (positive control) and the non-relevant β-actin promoter (negative control; Figure 4B). We confirmed efficient enrichment of DNAJB9 and MIR22HG but not of β-actin after XBP1s ChIP and, as expected, we did not observe any significant enrichment when using an IgG antibody as a negative control (Figure 4B). We also wanted to confirm our results, obtained in an artificial-upregulation system. Using RT-qPCR experiments, we quantified Xbp1s, MIR22HG and Dnajb9 mRNAs in 58 AML patient

samples. Analysis reveals positive correlation between Xbp1s and Dnajb9 expression (Pearson coefficient: 0.7931), as expected, and also between Xbp1s and MIR22HG expression (Pearson coefficient: 0.6837) (Figure 4C and D). These observations tend to confirm MIR22HG regulation by XBP1s in AML patients. Finally, up-regulation of the mature micro-RNA miR-22 upon XBP1s induction was also validated by RT-qPCR in three AML cell lines (Figure 5E to G). Taken together these data demonstrate that XBP1s can directly activate transcription of MIR22HG and as a consequence induce miR22 overexpression in AML cells.

To address whether XBP1s endogenous expression leads to up regulation of MIR22HG and miR22 expression under ER stress conditions, OciAML3 cells were transduced by a lentivector which allows the doxycycline-inducible expression of an shRNA targeting XBP1, and were treated with two potent ER stress inducers, Tunicamycine (an antibiotic that inhibits the N-glycosylation process) and Thapsigargine (a sesquiterpene lactone which inhibits the sarcoendoplasmic reticulum calcium transport ATPase SERCA). As expected, Tunicamycine and Thapsigargine increased XBP1s expression, and this up-regulation was partially blocked by XBP1-shRNA induction (Supplementary Figure 5A). Moreover, both Tunicamycine and Thapsigargine increased expression of MIR22HG and this effect was partially reversed by XBP1 shRNA induction after doxycycline treatment (Supplementary Figure 5B). We also demonstrated that ER stress induces an upregulation of miR22 and that inhibition of XBP1s expression by shRNA totally abolishes the induction of miR-22 expression (Supplementary Figure 5C). Taken together, these data clearly demonstrate that MIR22HG/miR22 expression is XBP1s-dependent.

miR-22 has anti-leukemic effect in vitro and participates in the XBP1-induced phenotype

We next investigated the effect of miR22 expression *in vitro*. Apoptosis was determined by Annexin V/propidium iodide (IP) double-staining and anti-PARP western blot after miR22 (mimic 22) transfection compared to control miRNA (mimic neg). Data obtained demonstrated that miR-22 transfection decreases the viability of OciAML3 cells compare to a control miRNA, due to induction of apoptosis as demonstrated by both increase in AnnexinV-positive cells and increase of PARP cleavage staining (Figure 5A and B).

To confirm miR22 effect on apoptotic response, we generated OciAML2 and OciAML3 models expressing miR-22-3p under doxycycline-inducible-promoter control. After 48h

hours of induction with doxycycline-increasing concentrations, apoptosis was evaluated by flow cytometry (Annexin V/propidium iodide staining). As expected, addition of increasing dose of doxycycline elicits an apoptotic response in miR22-expressing cells, but has no effect on TETON control cells (Figure 5C and D). Taken together, these results indicate miR-22 pro-apoptotic effect on the cells.

We also evaluated miR-22 contribution to XBP1s-induced chemosensitization in resistant OciAML3 cell line. XBP1s-inducible OciAML3 cells were transfected with either anti-miR-22 inhibitor (anti-mir-22) or a negative control anti-miRNA inhibitor (anti mir-Neg), and treated at 4ng/mL of doxycycline to induce XBP1s expression. 24h hours later, cells were treated with Aracytine at 10µM, during 24h hours and apoptosis was evaluated by flow cytometry. As expected, transfection of both anti-miR-neg anti-miR-22 and XBP1s induction didn't affect basal apoptosis. However, miR-22-3p inhibition upon Aracytine treatment significantly decrease aracytine-induced apoptosis in XBP1s expressing OciAML3 (Figure 5E). Altogether, these experiments demonstrated that miR-22 participates in the cell response to Aracytine treatment.

XBP1s/miR-22 axis repressed SIRT1 expression in AML cell lines.

In agreement with our data, miR-22 has been previously described to suppress DNA repair and improve response to chemotherapy [45]. Among the described miR22 targets, MCD1, p21 and SIRT1 promote cell survival in response to DNA damage by inducing cell cycle arrest (p21) and DNA repair (MDC1 and SIRT1). To identify direct miR-22 targets in OciAML3, we used the unbiased biotin-labelled pull-down procedure. Biotinylated microRNA 22 or a non-relevant microRNA (miR-39) were transfected in OciAML3 cells, and then pull down with streptavidin-coated magnetic beads. RNA interactants were further analyzed by RT-qPCR. We found that SIRT1 mRNA was efficiently enriched after mir-22 pull down compared to the control biotinylated miRNA, whereas P21 and MDC1 mRNA, as well as GAPDH and ABL mRNAs (negative controls), were not significantly accumulated (Figure 6A). Interestingly, it has been reported that the deacetylase Sirtuin-1 (SIRT1) is frequently overexpressed in Acute Myeloid Leukemia and is mostly considered like an oncogene in this pathology [49-51]. Further analyses reveal no significant reduction of SIRT1 mRNA after miR22 transfection when compared to control miRNA (Figure 6B). However, SIRT1 expression was reduced at protein level, indicating that miR-22 affects SIRT1 expression at the translational level (Figure 6C). We confirm SIRT1 regulation through miR-22 expression using our miR-22

inducible model: protein expression level was also reduced in OciAML2 and OciAML3 cells expressing miR-22 (Figure 6D and 6E). We also confirmed SIRT1 downregulation through XBP1s expression in OciAML3, OciAML2, MOLM14, MV4-11 and THP1 XBP1s-inducible cells. Western blots clearly showed a SIRT1 protein level decrease, proportionally to XBP1s increase (Supplementary figure 6A to E). Finally, we analyzed SIRT1 expression after anti-miR-22 transfection in OciAML3 XBP1s induced at 4ng/mL. Here again, we observed a decrease of SIRT1 in OciAML3 XBP1s transfected with anti-miR control (anti-miR-neg). However, miR-22 inhibition with anti-miR-22 induced SIRT1 re-expression at protein level. All these results allowed us to confirm SIRT1 downregulation by XBP1s/miR-22 axis (Supplementary figure 6F).

SIRT1 downregulation exert anti-leukemic effects in AML cell lines.

We downregulated SIRT1 expression in OciAML3 and THP1 cells by transfecting two siRNAs against SIRT1 mRNA. Both siRNA used efficiently repressed SIRT1 expression (Figure 6G and 6I) and increased apoptosis in both cell lines, compared to control conditions (Figure 6F and 6H). We wanted to test SIRT1 pharmacological inhibition on treatment sensitivity, using the highly-specific-SIRT1-inhibitor EX-527. Interestingly, EX-527 treatment alone doesn't affect cell viability while EX-527 enhanced apoptosis upon Aracytin, compared to Aracytin treatment alone (Figure 6J). Altogether, these results demonstrate SIRT1 implication in XBP1s-dependant apoptosis and chemosensitization.

DISCUSSION

In tumor, cancer cells are prone to many exogenous or endogenous stresses, which can disturb protein quality process and lead to Endoplasmic Reticulum stress. Although XBP1 overexpression has been observed in certain cancers, where it could either contribute to the adaptive response to ER stress, promote cell survival and thus, have a tumor-promoting role [34,52], or have in some case antitumoral effects [38,41,53]

Interestingly in Acute Myeloid Leukemia, XBP1 activation has been correlated with a favorable outcome but underlying molecular mechanisms remain unknown [42]. In order to clarify the role of XBP1 in this pathology, we set up a model enabling inducible XBP1 spliced isoform expression in leukemic cells. We showed that sustained XBP1s expression induce apoptosis whereas a moderate XBP1s expression, compatible with cell viability, potentiates

response to Aracytin treatment in AML resistant cells. We characterize direct XBP1 target genes using ChIP-sequencing, RNA sequencing and micro-RNA sequencing analyses. We identified many target genes, but we focus our analysis mainly on XBP1s non-coding targets, still poorly characterized so far. We found 13 annotated long non-coding RNA specific XBP1 target genes including MIR22HG (*C17orf91*), the precursor transcript of miRNA-22 (Supplementary Figure 4). Interestingly, previous studies demonstrate that MIR22HG is activated in response to hypoxic or chemical stress, two stimuli known to induce ER stress [54,55]. Here, we showed that induction of MIR22HG expression upon ER stress is XBP1-dependent.

Mir-22 was found to be down-regulated in many cancers, and was shown to mainly function as a tumor-suppressing microRNA, including AML [56,57]. Several miR-22 targets were already identified, including mRNAs encoding proteins involved in apoptosis and DNA damage response. Therefore, micro-RNA-22 has been described to suppress DNA repair and promote genomic instability. Indeed, miR22 was previously demonstrated to reduce p21 expression after a sustained stress [58], and the lack of p21 induce apoptosis through the accumulation of DNA damage in leukemic stem cells [59]. However, in our study, no significant interaction between miR-22 and p21 mRNA has been found in OciAML3 cells.

MDC1 and SIRT1 are also two validated miR-22 targets involved in DNA damage response and genomic instability [45,46,60]. MDC1 (Mediator of DNA damage checkpoint protein 1) is an early and critical actor of DNA Damage Response (DDR), which recruits protein at the DNA damage site. Inhibition of MDC1 by miR-22 lead to DNA damage accumulation and it has been suggested the resulting genomic instability could promote tumorigenesis [45]. However, at a later stage, this genomic instability could become deleterious in case of genotoxic insults. In OciAML3 expressing XBP1s, we also failed to characterize a significant interaction of miR-22 with MDC1 mRNA using a biotin pull down assay.

Finally, we showed that the deacetylase SIRT1 mRNA, a member of the mammalian sirtuin family, interacts efficiently with miR-22 in OciAML3 cells, and that miR22 inhibits SIRT1 expression mainly at the translational level, as previously described [60]. SIRT1 was reported to be up regulated in Acute Myeloid Leukemia and was identified as an oncogene in this pathology [49,51]. Consistently, SIRT1 repression in OciAML3 and THP1 using siRNA triggers apoptosis and SIRT1 pharmacological inhibition using EX-527 sensitizes OciAML3 to Aracytine treatment. These results clearly demonstrate that this protein acts as a pro-survival factor in AML cells.

Numerous targets of the deacetylase have been characterized (histones, enzyme, transcription factor…). As a consequence, SIRT1 is involved in many cellular processes including DNA repair [61]. SIRT1 intervene during single-strand breaks as well as double-strand breaks and promote DNA repair instead of apoptosis by deacetylating several factors (XPA, WRN, NBS1, KU, P53…). In Acute Myeloid Leukemia model, SIRT1 pharmacological inhibitor potentiates Aracytine treatment upon Tenovin-6 (Tv-6) [49]. However, Tv-6 is also characterized to inhibit SIRT2, and has never been tested on humans. We decide to use EX-527, highly specific to SIRT1, with no other characterized target, and already use *in vivo*. Interestingly, this inhibitor is also used in clinical trial, in patients with Huntington disease, and it is well-tolerated in patients [62].

SIRT1 has been previously related to treatment response. It was demonstrated on one hand that SIRT1 promotes resistance to chemotherapy in hepatocellular carcinoma [63] and, on the other hand, that downregulation of SIRT1 expression *in vitro* or *in vivo* enhances radiation sensitization, as well as radiation-induced apoptosis [64,65]. Moreover, inhibition of SIRT1 expression by miR-22 leads to DNA damage accumulation and improves response to radiotherapy in breast cancer cells [46]. In this study, we demonstrated that miR-22 upregulation mediated by XBP1s efficiently improve Aracytin sensitivity in AML resistant cells or induces apoptosis by inhibiting SIRT1 expression. Interestingly inhibition of endogenous miR-22 in XBP1s-expressing cells by addition of anti-miR-22, restores SIRT1 expression and partially reverses Aracytine induced apoptosis.

Finally, it was already demonstrated that there is a close relationship between SIRT1 and ER stress. Li *et. al.* demonstrated that hepatic overexpression of SIRT1 attenuates ER stress by inhibiting the UPR response [66]. SIRT1 acts as a negative regulator of XBP1s by mediating its deacetylation and, by this way, repressing its transcriptional activity [67]. Thus, SIRT1 could exert deleterious effects via repressing XBP1s signaling. Moreover, overexpression of XBP1s may contribute to lower expression level of SIRT1. Therefore, a negative feedback loop between SIRT1 and XBP1 could contribute to the regulation of tumor resistance or progression and can be an attractive target for novel therapy in AML.

To conclude, activation of the XBP1/miR22/SIRT1 axis in leukemic cells could define a new therapeutic approach, which can be targeted at different level. Our data suggest that the IRE1/XBP1 axis acts as a tumor suppressor and that activating this branch of the UPR represents an attractive therapeutic strategy to overcome primary resistance in AML patients.

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<u>Figure 1</u>: XBP1s inducible model

(A) Schedule of XBP1s-inducible model generation: AML cell lines are first transduced by a lentivirus expressing the rtTA transgene, next used as control cells in following experiments. The obtained "TETON" cells are transduced a second time with a lentivirus expressing the XBP1s transgene, composed by XBP1s sequence, followed by an IRES and eGFP sequence. GFP positive cells are sorted using flow cytometry after 24h doxycycline treatment. **(B-D-F-H-J)** OciAML3, MOLM14, MV4-11, THP1, and OciAML2 cells are treated with increasing amount of doxycycline during 48h. XBP1s expression is evaluated by RT-qPCR. Expression values are normalized to the housekeeping genes HPRT, MLN51 and ABL, and are depicted as a ratio of mRNA expression in doxycycline-treated cells relative to untreated cells. **(C-E-G-I-K)** XBP1s protein level is evaluated by Western Blot; GAPDH or actin were used as loading controls.

<u>Figure 2:</u> XBP1s expression induces apoptosis in several AML cell lines both in vitro and in vivo

(A-F) OciAML3, MOLM-14, MV4-11, OciAML2, THP1 and HL-60 AML cells are treated with increasing amounts of doxycycline during 48h. Apoptosis is measured by flow cytometry using AnnexinV staining. Data represent mean ± SD (n=3). **(G)** Mice-experiment procedure: NSG mice are intravenously injected with 2 million of OciAML3 **(H)**, OciAML2 **(I)** or HL-60 **(J)** XBP1s-expressing (XBP1s) or control (TETON) cells. After a 9-days-engraftment, doxycycline is added at 1mg/mL in drinking water during 8 days. Survival analysis are performed using log-rank test.

<u>Figure 3:</u> XBP1s expression restore Aracytin sensitivity in chemoresistant cell line OciAML3, both in vitro and in vivo

(A) OciAML3 TETON (control) and XBP1s-inducible cells receive a 24h-treatment of doxycyclin at 4ng/mL, followed by a 24h-treatment of Aracytin at 10µM. Percent of apoptotic cells is measured by flow cytometry using AnnexinV staining. **(B)** OciAML3 TETON and XBP1s-inducible cells are treated with doxycycline (4ng/mL) for 24 hours and then with bortezomib (BTZ), vinblastin or staurosporine (Stauro), respectively at 5nM, 0.5µM and 0.2µM for 24H or left untreated (NT). Data represent mean ± SD (n=3). Statistical analyses are performed using unpaired t-tests; *p≤0.05. **(C)** Mice-experiment procedure: NSG mice

are injected (day 0) with 2 million of OciAML3 TETON (control) and XBP1s-inducible cells. After a 9-days-engraftment of the inducible cells, doxycyclin is added at 1mg/mL in drinking water during 8 days. At day 12, mice are daily injected with Aracytin at 30mg/kg during 5 days. **(D)** Survival analysis of the treated mice are performed using log-rank test.

<u>Figure 4:</u> Identification of the MIR22HG lncRNA precursor of miR-22-3p as a direct target of XBP1s

(A) Snapshots of ChIP-Seq signals (peaks) representing XBP1s-bound genomic regions in OciAML3 cells treated with 10 ng/mL of doxycycline for 48H compared to the input, on the MIR22 Host Gene (MIR22HG) promoter. **(B)** qPCR analysis of MIR22HG and DNAJB9 (positive control) promoter regions immunoprecipitated following ChIP assay on OciAML3 XBP1s cells induced with 10ng/mL of Dox and performed using anti-XBP1 or IgG isotype control antibodies. The enrichment of target gene promoter regions is expressed in % of input. The actin B (ACT B) promoter region is used as negative control and DNAJB9 as positive control. **(C; D)** Pearson's correlation analysis showing gene expression levels (quantified by RT-qPCR) of XBP1s and MIR22HG in a 58-AML-patient cohort. Expression values are expressed in ΔCt, calculated using housekeeping genes Actin, MLN51, GAPDH and TBP. **(E-G)** Mature miR-22 expression levels were assessed by RT-qPCR in OciAML3 (D), THP1 (E) MOLM-14 (F) XBP1s-inducible cells treated with doxycycline. Expression values were normalized to the control let-7a microRNA, and are depicted as a ratio of expression in doxycycline treated cells relative to untreated cells. Data represent mean ± SD (n=3).

<u>Figure 5:</u> miR-22 recapitulates XBP1s-dependent phenotypes

(A) 1) OciAML3 wild-type cells are transfected with a miR-22 mimic (22) or a non-relevant miRNA (Neg) for 48H. Apoptosis is assessed by cytometry using Annexin-V staining.

2) Quantification of 3 independent experiments.

(B) Western Blot analyses on cleaved PARP following miR-22 transfection in OciAML3. **(C-D)** OciAML2 and OciAML3 TETON cells are transduced with a lentivector expressing an inducible miR-22. Transduced cells are treated with increasing amounts of doxycycline during 48H. Apoptosis is measured by flow cytometry using AnnexinV staining. Data represent mean ± SD (n=3). **(E)** OciAML3 XBP1s-inducible cells are transfected with

antisense oligonucleotides against miR-22 (anti-22) or non-relevant oligonucleotide (anti-neg) as control. After transfection, cells are treated with 4ng/mL of doxycycline to induce XBP1 expression and with aracytine (AraC), at 10 µM. After 24 hours, apoptosis is quantified. Presented data represent mean ± SD (n=3). Statistical analysis are performed using unpaired t-tests ; *p≤0.05, **p≤0.01, ***p≤0.001, ****p ≤ 0,0001.

Figure 6: XBP1s/miR-22 axis inhibit SIRT1 expression and compromises leukemic cells survival

(A) RT-qPCR analysis of MDC1, P21, SIRT1, GAPDH, ABL (as negative control) mRNA levels following pull-down with a biotinylated miR22 mimic transfected in OciAML3 wild-type cells. Biotinylated miR-29 mimic is used as non-relevant control. Data represent mean ± SD (n=3). **(B)** qRT-PCR analysis of SIRT1 mRNA expression after mir-22 mimic (mimic-22) transfection in OciAML3 cells. Values were normalized to the expression level of housekeeping gene ABL and are shown as relative SIRT1 mRNA expression compared to non-relevant mimic (mimic neg) transfection. **(C-E)** SIRT1 protein levels were determined after mimic-22 or mimic-neg transfection in OciAML3 WT (C) and also after miR-22 induction in OciAML2 miR-22 (D) and OciAML2 miR-22 (E) cells, by western blotting. GAPDH is used as loading control. **(F-I)** OciAML3 and THP1 are transfected by two siRNA against SIRT1 (si-1; si-2) or a non-relevant siRNA (SCR) during 48h. Apoptosis was measured by flow cytometry using AnnexinV staining (F; H). Data represent mean ± SD (n=3). SiRNA efficacity is evaluated by western blot, by measuring SIRT1 protein levels. GAPDH or Actin as loading control (G; I). **(J)** OciAML3 WT are treated for 96h with the SIRT1 inhibitor EX-527 at 30µM, and with Aracytin at 10µM during the last 24h. Apoptosis is measured by flow cytometry using AnnexinV staining. Data represent mean ± SD (n=3). For all the presented experiments statistical analyses are performed using unpaired *t*-tests; *p≤0.05; **p≤0.01, ***p≤0.001.

Figure 1

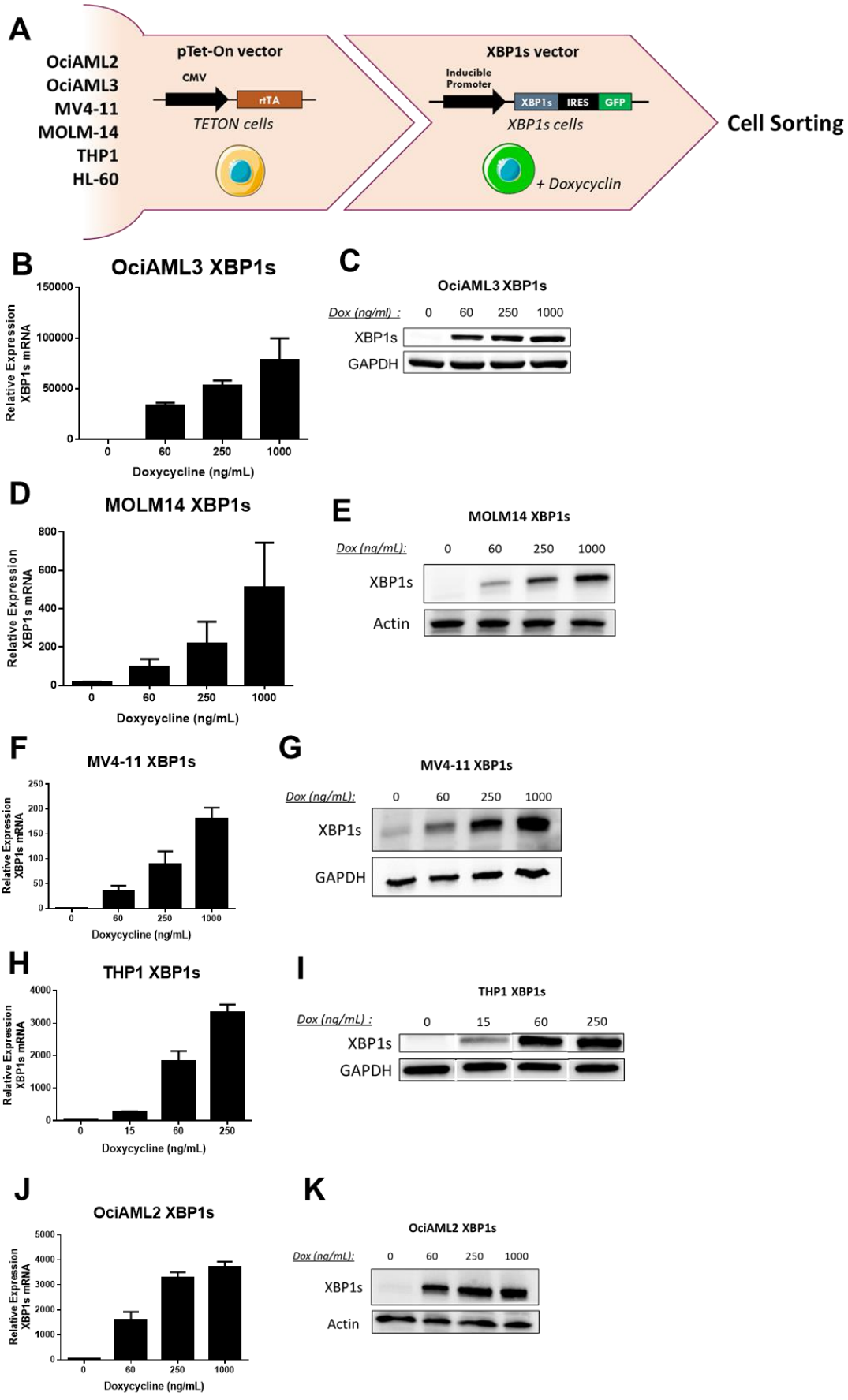

A
OciAML2
OciAML3
MV4-11
MOLM-14
THP1
HL-60
pTet-On vector
CMV
rtTA
TETON cells
XBP1s vector
Inducible Promoter
XBP1s
IRES
GFP
XBP1s cells
+ Doxycyclin
Cell Sorting
B
OciAML3 XBP1s
Relative Expression XBP1s mRNA
Doxycycline (ng/mL)
C
OciAML3 XBP1s
Dox (ng/ml) :
XBP1s
GAPDH
D
MOLM14 XBP1s
E
MOLM14 XBP1s
Dox (ng/mL):
Actin
F
MV4-11 XBP1s
G
MV4-11 XBP1s
H
THP1 XBP1s
I
THP1 XBP1s
J
OciAML2 XBP1s
K
OciAML2 XBP1s

Figure 2

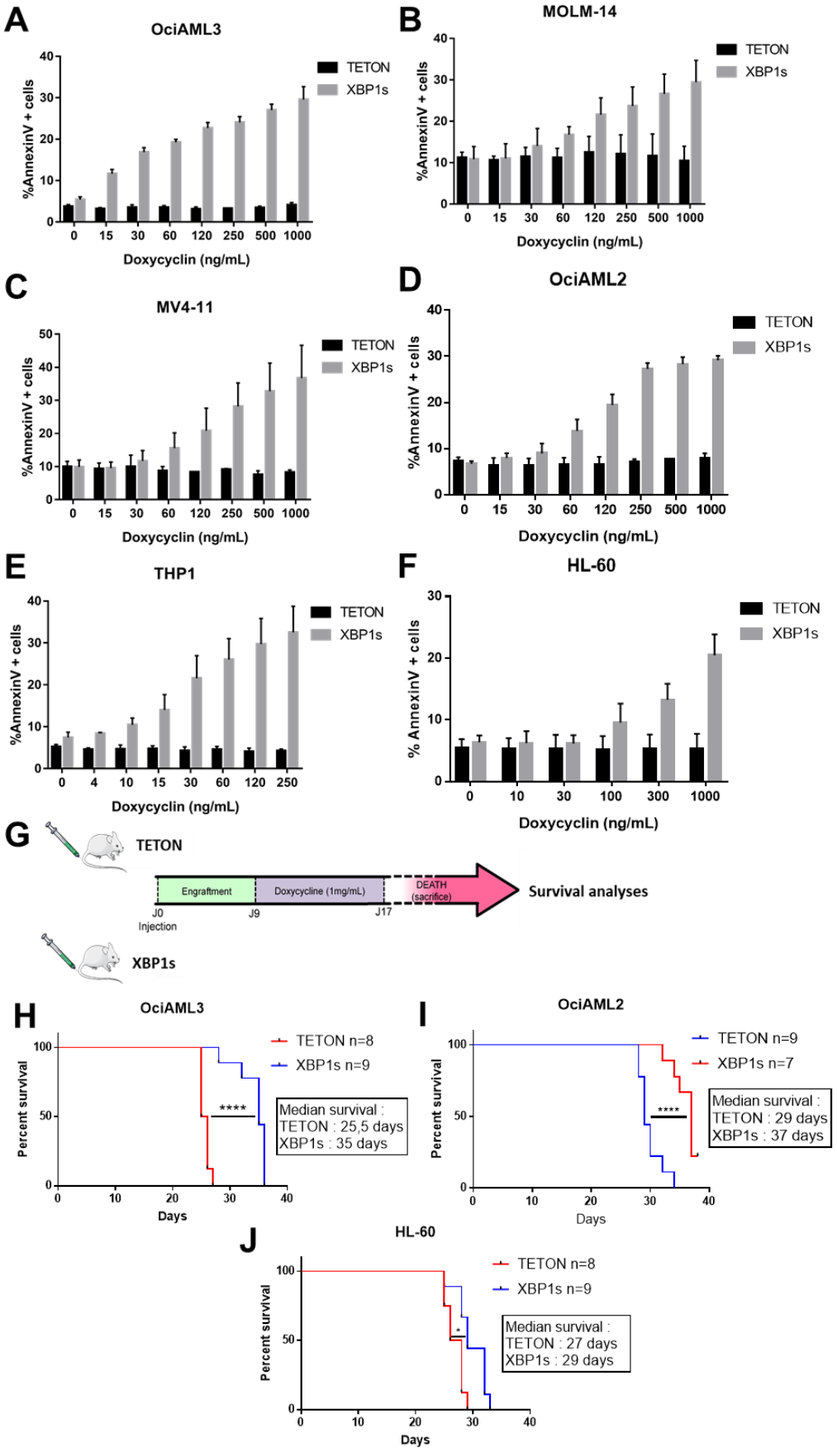

A
OciAML3
%AnnexinV + cells
TETON
XBP1s
Doxycyclin (ng/mL)
B
MOLM-14
C
MV4-11
D
OciAML2
E
THP1
F
HL-60
% AnnexinV + cells
G
TETON
Engraftment
Doxycycline (1mg/mL)
DEATH (sacrifice)
Survival analyses
J0
Injection
J9
J17
XBP1s
H
OciAML3
Percent survival
Days
TETON n=8
XBP1s n=9
****
Median survival :
TETON : 25,5 days
XBP1s : 35 days
I
OciAML2
TETON n=9
XBP1s n=7
Median survival :
TETON : 29 days
XBP1s : 37 days
J
HL-60
TETON n=8
XBP1s n=9
*
Median survival :
TETON : 27 days
XBP1s : 29 days

Figure 3

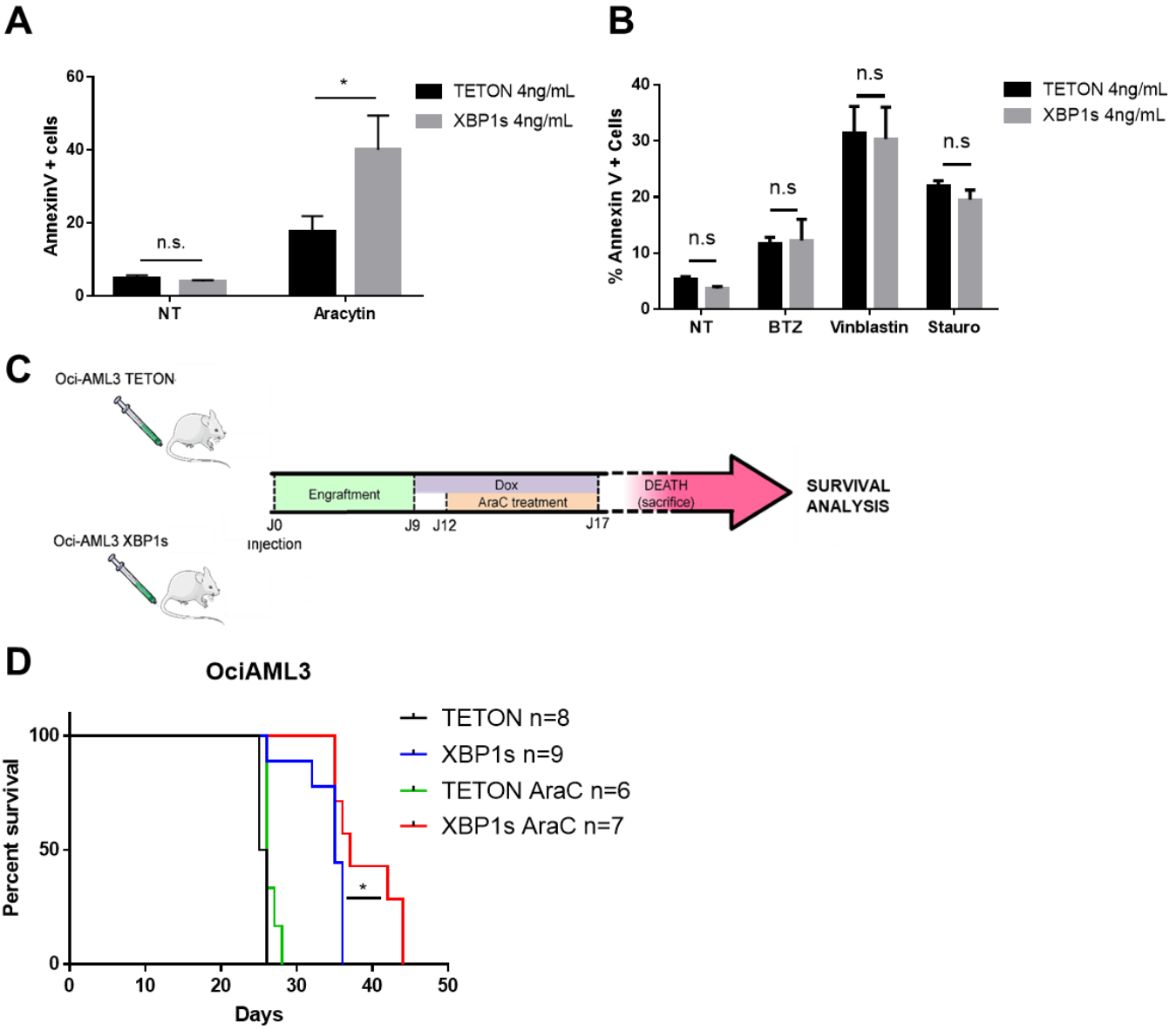

A
AnnexinV + cells
60
40
20
0
n.s.
*
NT
Aracytin
TETON 4ng/mL
XBP1s 4ng/mL
B
% Annexin V + Cells
40
30
20
10
0
n.s
n.s
n.s
n.s
NT
BTZ
Vinblastin
Stauro
TETON 4ng/mL
XBP1s 4ng/mL
C
Oci-AML3 TETON
Oci-AML3 XBP1s
Engraftment
Dox
AraC treatment
DEATH
(sacrifice)
SURVIVAL
ANALYSIS
J0
injection
J9
J12
J17
D
OciAML3
Percent survival
100
50
0
0
10
20
30
40
50
Days
*
TETON n=8
XBP1s n=9
TETON AraC n=6
XBP1s AraC n=7

Figure 4

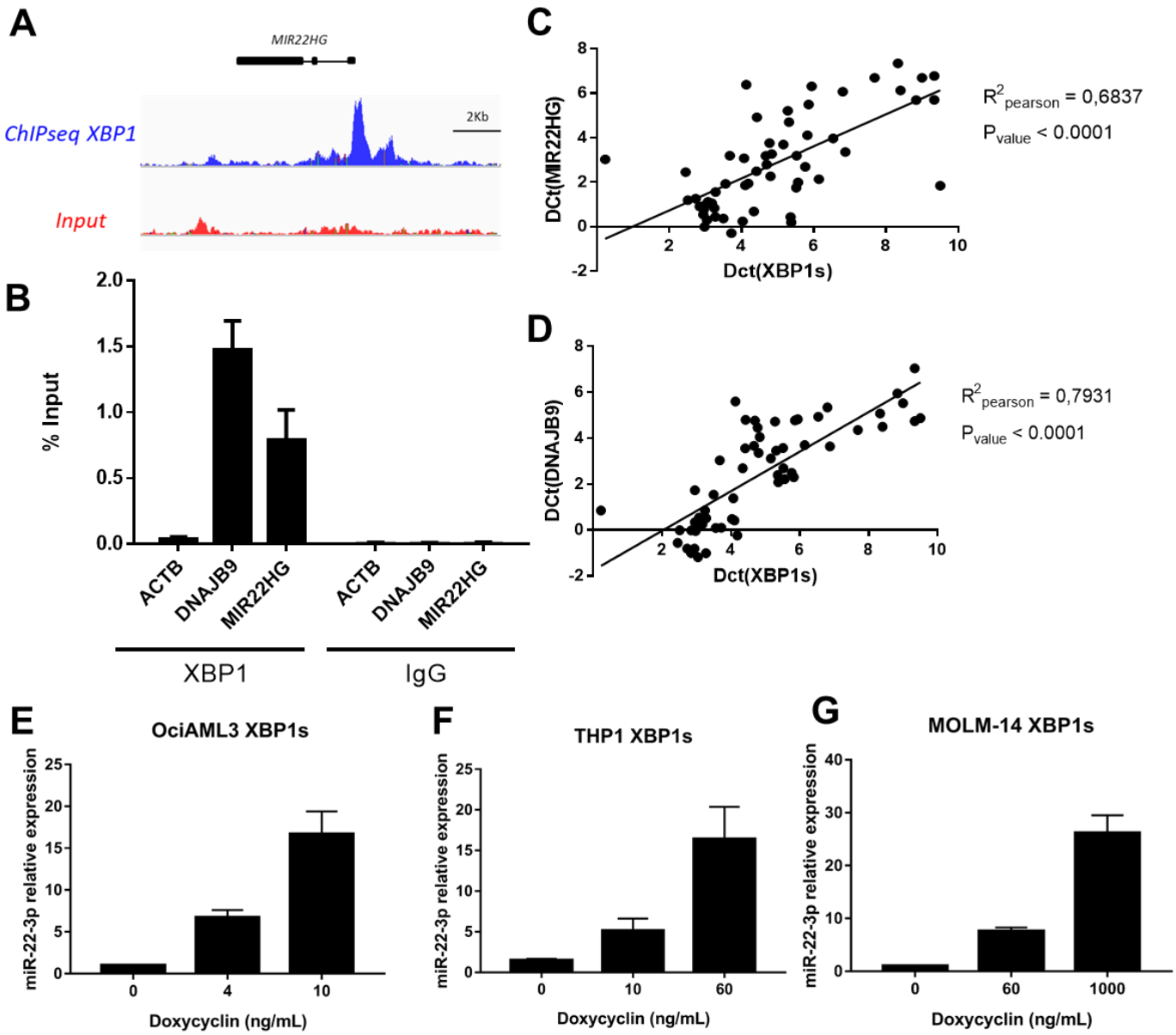

A
MIR22HG
2Kb
ChIPseq XBP1
Input
B
% Input
2.0
1.5
1.0
0.5
0.0
ACTB
DNAJB9
MIR22HG
ACTB
DNAJB9
MIR22HG
XBP1
IgG
C
DCt(MIR22HG)
Dct(XBP1s)
R2pearson = 0,6837
Pvalue < 0.0001
D
DCt(DNAJB9)
Dct(XBP1s)
R2pearson = 0,7931
Pvalue < 0.0001
E
OciAML3 XBP1s
miR-22-3p relative expression
Doxycyclin (ng/mL)
F
THP1 XBP1s
miR-22-3p relative expression
Doxycyclin (ng/mL)
G
MOLM-14 XBP1s
miR-22-3p relative expression
Doxycyclin (ng/mL)

Figure 5

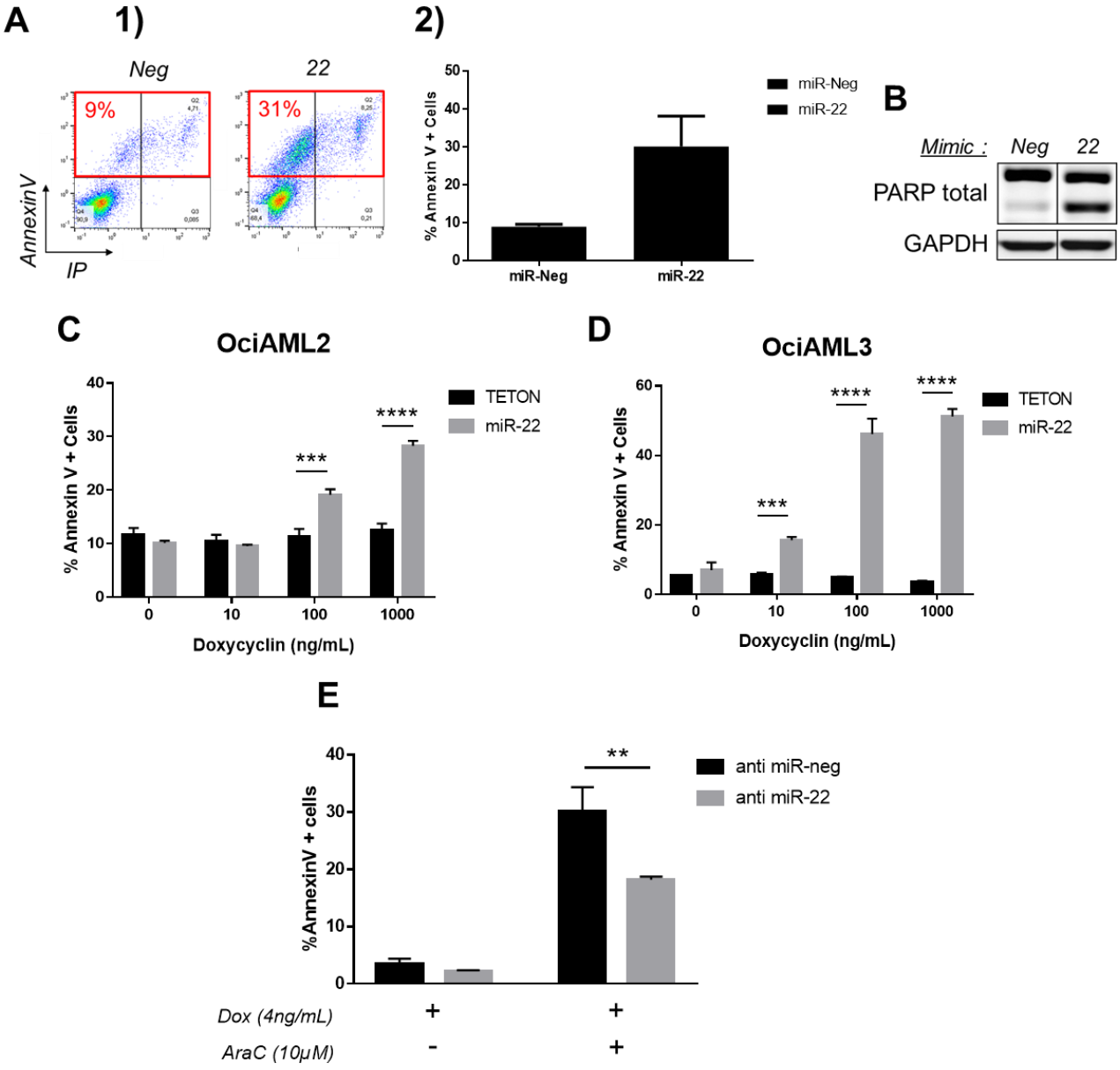
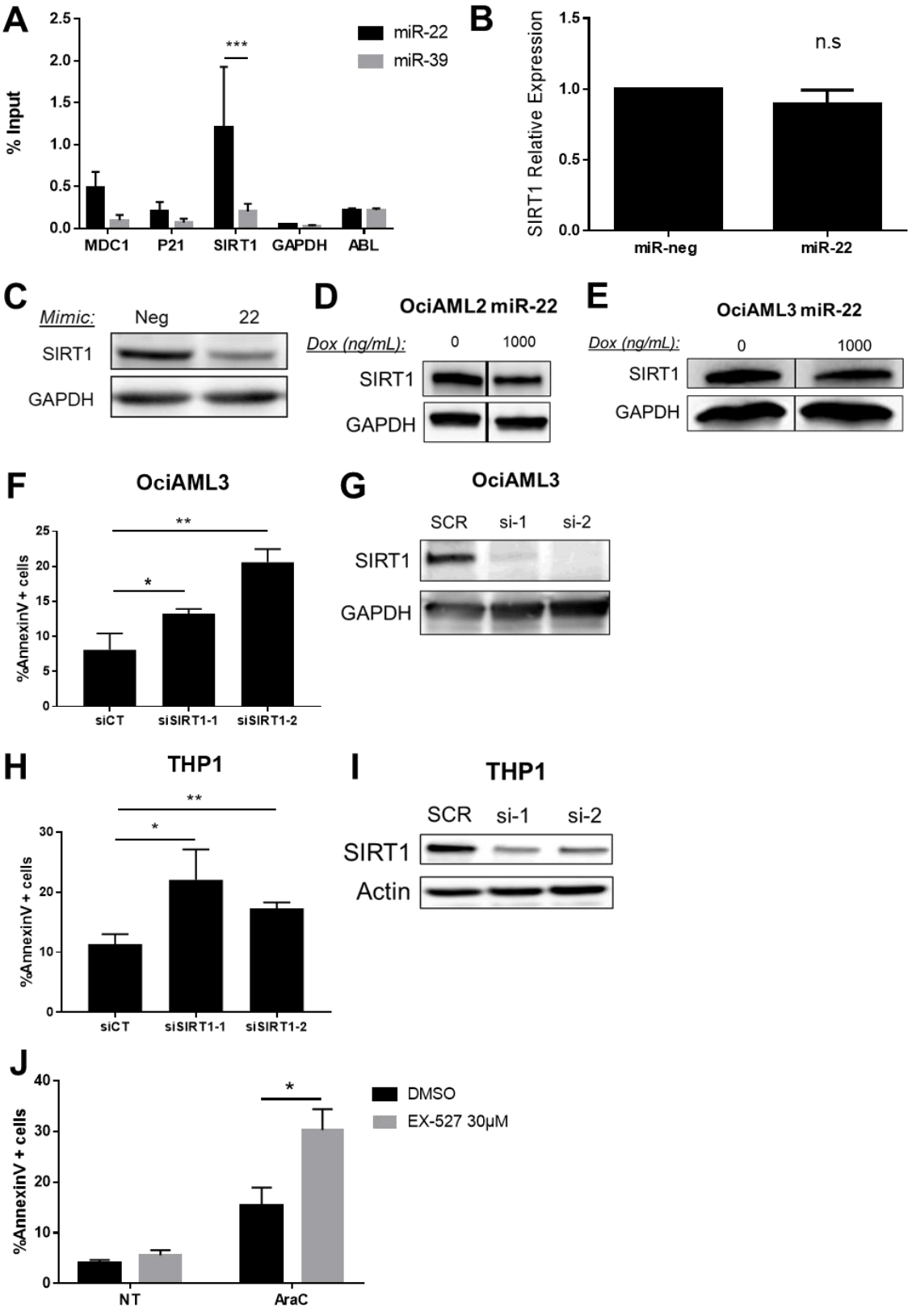
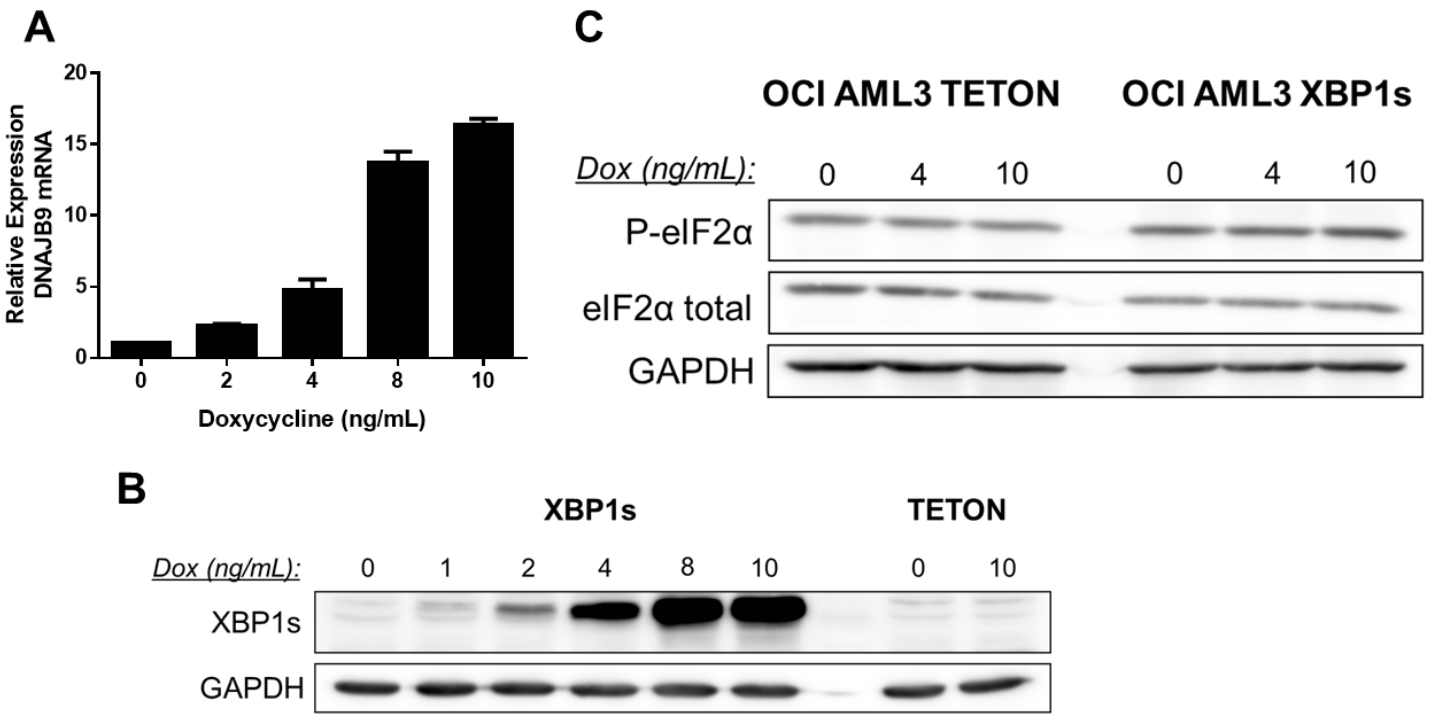

A
1)
Neg
22
9%
31%
AnnexinV
IP
2)
% Annexin V + Cells
miR-Neg
miR-22
B
Mimic :
Neg
22
PARP total
GAPDH
C
OciAML2
% Annexin V + Cells
TETON
miR-22
Doxycyclin (ng/mL)
D
OciAML3
% Annexin V + Cells
TETON
miR-22
Doxycyclin (ng/mL)
E
%AnnexinV + cells
anti miR-neg
anti miR-22
Dox (4ng/mL)
AraC (10µM)

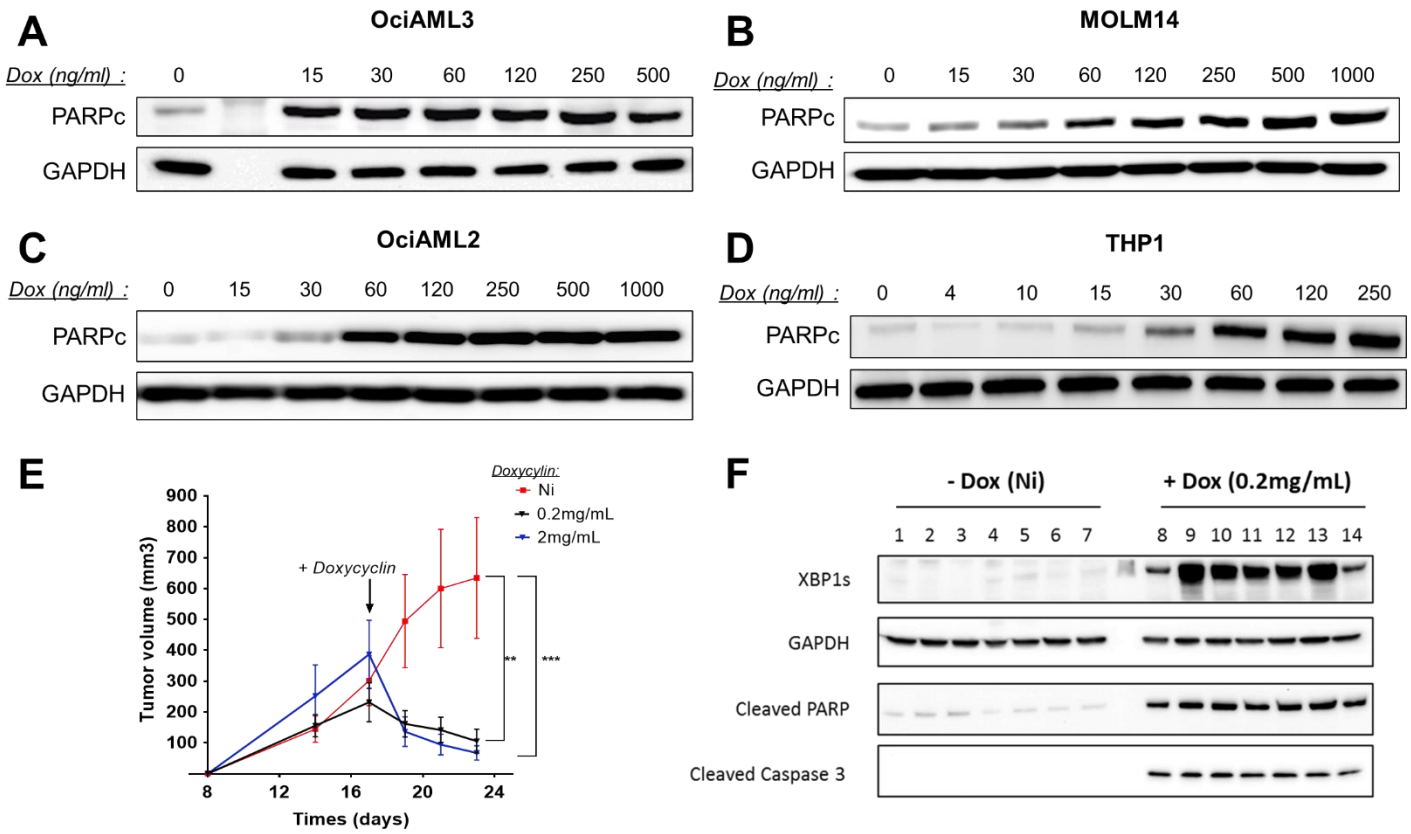
Figure 6


A
% Input
2.5
2.0
1.5
1.0
0.5
0.0
***
miR-22
miR-39
MDC1
P21
SIRT1
GAPDH
ABL
B
SIRT1 Relative Expression
1.5
1.0
0.5
0.0
n.s
miR-neg
miR-22
C
Mimic:
Neg
22
SIRT1
GAPDH
D
OciAML2 miR-22
Dox (ng/mL):
0
1000
SIRT1
GAPDH
E
OciAML3 miR-22
Dox (ng/mL):
0
1000
SIRT1
GAPDH
F
OciAML3
%AnnexinV + cells
25
20
15
10
5
0
**
*
siCT
siSIRT1-1
siSIRT1-2
G
OciAML3
SCR
si-1
si-2
SIRT1
GAPDH
H
THP1
%AnnexinV + cells
30
20
10
0
**
*
siCT
siSIRT1-1
siSIRT1-2
I
THP1
SCR
si-1
si-2
SIRT1
Actin
J
%AnnexinV + cells
40
30
20
10
0
*
DMSO
EX-527 30μM
NT
AraC



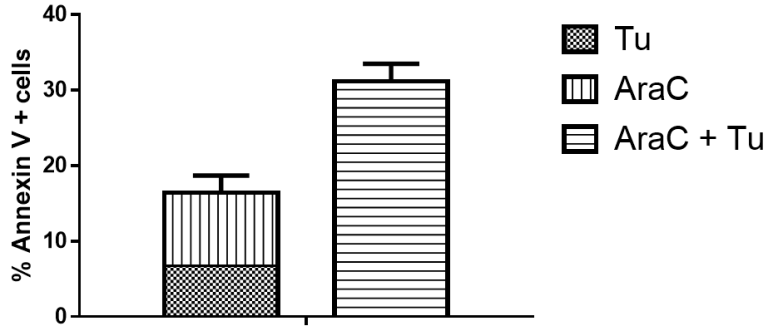
Supplementary Figure 1 **: Validation of the XBP1s inducible expression in OciAML3 cell line**

(A) OciAML3 XBP1s-expressing cells and TETON control cells are treated with increasing doses of doxycycline (Dox; from 0 to 10 ng/mL) for 48H. DNAJB9 mRNA levels are determined by RT-qPCR. Expression values are normalized to the housekeeping gene ABL, and are depicted as a ratio of expression in doxycycline treated cells relative to untreated cells **(B and C)** XBP1s, P-eIF2α and eIF2α total protein expression are evaluated by western blotting. GAPDH is used as loading control.



***Supplementary Figure 2* : XBP1s apoptosis induction compromise tumor development**

(A-D) MOLM14, OciAML3, OciAML2 and THP1 XBP1s-inducible cells are treated with increasing amounts of doxycycline during 48h. Cleaved PARP protein level is evaluated by Western Blot; GAPDH is used as loading control. **(E)** Nude mice are engrafted subcutaneously with OciAML3 XBP1s cells. 17 days post engraftment, mice are exposed or not, to doxycycline dissolved in their drinking water at a concentration of 2mg/ml or 0.2mg/mL (n=8 for each group). The mean tumor volume in mm^3 is calculated every 2 days after doxycycline treatment. Data represent mean ± SD. Statistical analysis are performed by 2-way ANOVA with Bonferroni correction; *p≤0.05; **p≤0.01, ***p≤0.001. **(F)** XBP1 expression, PARP and Caspase 3 cleavage are analyzed by western blotting on protein extracts from tumor samples, with GAPDH as loading control.



Supplementary Figure 4 : **ER stress acts synergistically with aracytin treatment to increase chemosensitivity**

OciAML3 wild-type cells were pretreated (or not) with tunicamycin at 0.05µg/mL for 48H and were then treated (or not) with aracytine (AraC, 10µM) for 24H. Apoptosis was then assessed by Annexin-V/Propidium Iodide (PI) staining. Results of three independent experiments are shown. Data represent mean ± SD (n=3). The left side represents the apoptosis induced by tunicamycin and chemotherapeutic treatment alone and the right side the apoptosis induced by a combinatorial treatment (ER stress inducer Tunicamycin and chemotherapy).

A **RNA sequencing/ChIP sequencing analyses**

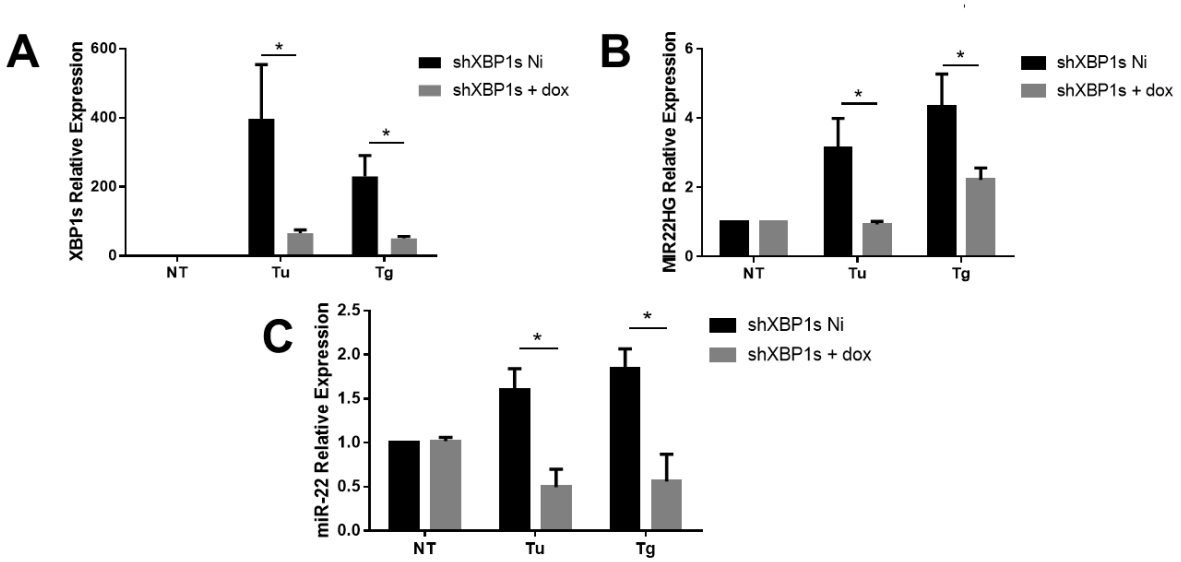
Ensembl Reference	Gene symbol	Fold Change
ENSG00000257605	AC073611.1	7,185064138
ENSG00000272405	AL365181.3	6,702190812
ENSG00000248323	LUCAT1	6,028002143
ENSG00000233452	STXBP5-AS1	5,970801812
ENSG00000236453	AC003092.1	5,910464553
ENSG00000186594	MIR22HG	5,407187766
ENSG00000259343	TMC3-AS1	4,638185842
ENSG00000281649	EBLN3P	1,276765238
ENSG00000227706	AL713998.1	-0,889473826
ENSG00000274536	AL034397.3	-1,225036722
ENSG00000247982	LINC00926	-1,354164141
ENSG00000215246	AC116351.1	-1,616383652
ENSG00000269243	AC008894.2	-2,05944241

B **miRNA sequencing analyses**

miR-RNA	Fold Change
hsa-miR-22-3p	18,7882022
hsa-miR-199a-3p	17,125
hsa-miR-148a-5p	10,60199
hsa-miR-148a-3p	9,91198555
hsa-miR-21-3p	6,56037515
hsa-miR-574-3p	6,0952381
hsa-miR-215-5p	5,5
hsa-miR-29c-3p	3,78571429
hsa-miR-1307-5p	2,95053004
hsa-miR-501-3p	2,875
hsa-miR-21-5p	2,73601533
hsa-miR-877-5p	2,7

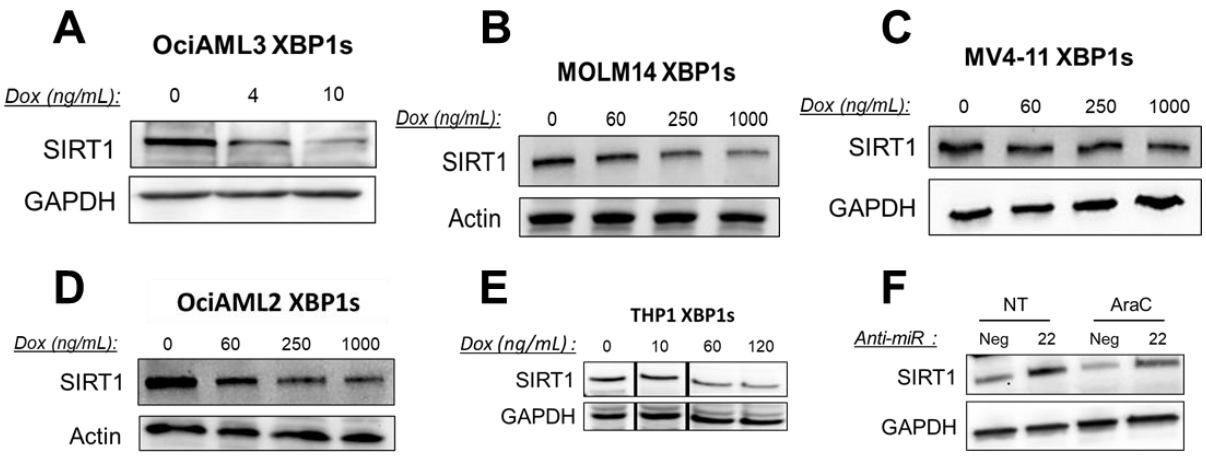
Supplementary Figure 3 : **ChIP-sequencing and micro-RNA sequencing analysis**

(A) List of long non-coding RNA specifically targeted by XBP1s **(B)** List of micro-RNAs upregulated upon XBP1s expression in OciAML3 XBP1s cells compared to TETON control cells after treatment with 10ng/mL of doxycycline for 48H.



***Supplementary Figure 5* : XBP1-dependent expression of MIR22HG and miR-22 during endoplasmic reticulum stress**

(A-C) OciAML3 expressing shRNA against Xbp1s-mRNA are co-treated with doxycycline at 1000ng/mL and tunicamycin (Tu ;10 µg/mL) or thapsigargin (Tg; 50 nM) for 6H, or left without treatment (NT). XBP1s (A), MIR22HG (B) and (C) miR-22 (C) expressions are determined by RT-qPCR. Values are normalized to the expression level of the housekeeping gene ABL (for MIR22HG and XBP1s) or the control let-7a microRNA (for miR-22), and are shown as relative RNA expression compared to non-induced (Ni) and non-treated (NT) condition. Data represent mean ± SD (n=3); *p≤0.05.



***Supplementary Figure 6* : SIRT1 downregulation is miR-22-dependent**

(A-E) SIRT1 protein levels were then analyzed by western blotting in OciAML3, MOLM-14, MV4-11, OciAML2 and THP1 XBP1s-exprerssing cells. **(F)** SIRT1 protein level is assessed by western blotting in OciAML3 XBP1s treated at 4ng/mL and transfected with antisense oligonucleotide against miR-22 (Anti-22) or non-relevant oligonucleotide (Anti-neg). GAPDH is used as loading control.

Article II

XBP1s induit un déblocage de la différenciation myéloïde par l'action combinée de miR-22-3p et miR-148a-3p, dans la Leucémie Aigüe Myéloïde

XBP1s a été largement relié au processus de différenciation dans l'hématopoïèse saine. Il est en effet crucial dans la maturation des plasmocytes, des cellules dendritiques et des éosinophiles. De fait, il a été étudié en détail dans un contexte d'hématopoïèse non-cancéreuse.

Afin de caractériser au mieux les effets anti-leucémiques de XBP1s et de part cette implication dans les phénomènes de différentiation hématopoïétique, nous avons émis l'hypothèse que XBP1s pouvait intervenir sur le blocage de la différenciation des blastes.

L'utilisation des modèles inductibles précédemment décris nous a permis d'exprimer XBP1s de façon chronique *in vitro*, à des doses permettant la survie des cellules, et ce dans trois lignées cellulaires différentes : OciAML3, OciAML2 et HL-60. Nous avons observé que l'activation chronique de XBP1s associée à des traitements inducteurs de la différenciation, tels que la α1,25-dihydroxyvitamin D3 (1,25-D3) ou le DMSO, provoque une augmentation de l'expression du CD11b, un marqueur de différentiation terminal pan-myéloïde. De plus, l'observation de ces cellules révèle des variations morphologiques caractéristiques d'une progression dans la différentiation. Nous avons également confirmé ces résultats *in vivo*, par des xénogreffes orthotopiques dans les 2 lignées OciAML2 et OciAML3.

Les analyses à grande échelle réalisées sur la lignée OciAML3 nous ont permis de mettre en évidence 2 cibles directes de XBP1s. Dans le contexte de la myélopoïèse, miR-22 a été montré comme transcriptionnellement activé par PU.1 et semble, ici encore, une cible intéressante. De plus, un autre micro-ARN intéressant est miR-148a-3p. Ce micro-ARN a déjà été décrit comme cible directe de XBP1s, et il est également relié à des processus de différenciation et de polarisation des macrophages.

Nous avons réalisé à nouveau des modèles inductibles, permettant la surexpression de chacun des deux micro-ARNs.

Dans le cas de miR-148a-3p, la surexpression de ce micro-ARN augmente la réponse à la 1,25-D3 dans les OciAML2 et les OciAML3 *in vitro*. Ces résultats ont également été généralisés dans des modèles de xénogreffes orthotopiques, pour les deux lignées. Des analyses *in silico* ont permis d'identifier le facteur de transcription HEX (Hematopoietically-Expressed Homeobox Protein) comme cible potentiellement directe de miR-148a-3p, bien que cette interaction reste encore à déterminer dans notre modèle. En effet, nous observons une diminution de l'expression de HEX après l'induction de XBP1s mais également après l'induction de miR-148a-3p. Cette diminution de HEX n'est visible qu'au niveau protéique, suggérant une inhibition traductionnelle de ce facteur de transcription. De plus, la répression

de HEX dans le modèle OciAML3, après transfection d'un siARN, potentialise les effets de la 1,25-D3, liant ainsi HEX au blocage de la différentiation dans ces cellules.

Dans le cas de miR-22-3p, la surexpression de ce micro-ARN augmente également la réponse à la 1,25-D3 dans la lignée OciAML2, *in vitro*. Nous avons montré que la surexpression de miR-22 était associé à la répression du facteur de transcription proto-oncogénique c-MYB : en effet, nous observons une diminution de l'expression de c-MYB après l'induction de XBP1s dans les lignées OciAML2 et OciAML3 ainsi qu'en réponse à l'induction de miR-22 dans les OciAML2. De plus, la diminution de ce facteur de transcription par transfection d'un siARN dans les OciAML3 potentialise également les effets de la 1,25-D3.

Bien qu'encore incomplets, ces résultats confirment une fois de plus les effets anti-leucémique de XBP1s. L'axe XBP1s/miR-22 semble particulièrement impliqué dans la médiation de ces effets. Nous avons également confirmer l'activation directe de miR-148a-3p.

XBP1s unlocks myeloid differentiation through miR-22-3p and miR-148a-3p combined action in Acute Myeloid Leukaemia.

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ABSTRACT

Acute Myeloid Leukaemia (AML) is a rare and highly fatal haematological disorder, characterized by progressive invasion of bone marrow, circulating blood and peripheral organs by abnormal myeloid progenitors named blasts. Blasts are defined by differentiation blockade, and keep typical features of myeloid progenitor state, like high proliferation rate and survival increased. AML treatment is based on chemotherapy combining Aracytin with anthracyclin, and mainly target proliferative cells. However, relapses are frequently observed, associated with chemoresistance. Targeting differentiation blockade appears as promising approach to target malignant cells, but AML heterogeneity make them hard to develop. In 2009, clinical study links transcription factor XBP1s expression to favourable outcome in AML. Interestingly, XBP1s has been related to myeloid differentiation, especially in granulocyte-eosinophils terminal maturation. XBP1s is actually a crucial component of an Endoplasmic-reticulum-stress-induced pathway, the Unfolded Protein Response (UPR). UPR is a physiological response, triggers by accumulation of unfolded or misfolded proteins in Endoplasmic Reticulum, and mediates by ER-transmembrane-sensors activation: IRE1 (inositol-requiring enzyme-1), the only sensor conserved among eukaryotes, is a cytoplasmic endoribonuclease which initiates unconventional splicing of Xbp1 mRNA, leading to XBP1s expression, the active form of this transcription factor. Interestingly, XBP1s has been deeply studied in B lymphocytes differentiation, and also related to granulocyte-eosinophils differentiation, but its impact on AML remains unknown. In order to decipher XBP1s effects on myeloid differentiation in AML, we develop inducible-XBP1-spliced-isoform models, using several AML cell lines. We showed that XBP1s expression is linked to myeloid differentiation progression both *in vitro* and *in vivo*. We identify specific-XBP1s-non-coding targets, using ChIP-sequencing data. We focus on two micro-RNAs miR-22 and miR148a-3p, and demonstrated that they are able to promote differentiation, by targeting respectively C-MYB and HEX, two transcription factors related to myeloid differentiation. These results show that XBP1 has anti-leukemic effects by promoting leukemic blasts differentiation in AML, and open up new opportunities in AML differentiation-based therapies.

INTRODUCTION

Acute myeloid leukaemia (AML) is a phenotypically and genetically heterogeneous haematological disease that is one of the most frequently occurring leukaemia in adults (it accounts for roughly 80% of adult leukaemia), with an average age at diagnosis of more than 60 years [1]. AML is characterized by the accumulation of chromosomal translocations and/or multiple mutations resulting in the transformation and clonal expansion of hematopoietic progenitors. These malignant cells named blasts, with impaired differentiation programs, are blocked in a progenitor state with increased survival and high proliferation rate. This leads to the lack of mature myeloid cells and accumulation of immature myeloid blasts in bone marrow and peripheral blood. The general therapeutic strategy for most patients consists in cytarabine-based-induction chemotherapy, associated with an anthracyclin, generally daunorubicin. Despite frequent complete remission after chemotherapy, AML is associated with a poor prognosis since about 80% of patients tend to relapse due to emergence of treatment-resistant clones [2].

However, in one AML subtype, chemotherapy is associated with differentiation-inducing therapy. This kind of therapy restores differentiation programs to induce cell maturation, leading to a decrease in proliferation-rate and to cell death [3]. This therapeutic approach has been developed to cure Acute Promyelocytic Leukemia (APL), a very aggressive AML form, representing 5–10% of cases. This particular AML subtype is characterized by the presence of chromosomal translocation which leads to the fusion of the retinoic acid receptor α (RARα) to the promyelocytic leukemia genes (PML) respectively on chromosome 17 and 15 [4]. In this case, RARα fusion proteins acts as a dominant-negative protein and inhibits RARα signalling, locks differentiation at the promyelocytic stage and promotes survival of myeloid precursor cells [5]. However, all-trans-retinoic acid (ATRA) treatment induce fusion-protein degradation and restore myeloid differentiation. This treatment results in huge improvement of patient survival, with a complete-remission rate up to 80% without relapse [6]. However, even if ATRA has been shown to be ineffective in non-APL subtypes, this therapy provides the first example that terminal-differentiation induction is an efficient way to treat AML.

Except for APL characterized by this very particular translocation, AML is considered as a heterogeneous disease. Mutations and karyotype analyses reveal numerous alterations, with co-occurrence patterns, making differentiation therapy hard to develop. More recently, new oncogenic pathways have been identified, enabling successful development of targeted therapies, partially based on blasts-differentiation recovery: agents that target IDH1/2

mutations or FLT3 mutations became approved for the treatment of AML in 2017, and display anti-leukemic activity by inhibiting these specific mutations, and promoting differentiation in this way [7]. Many other studies have shown that Vitamin D3 analogs, such as α1,25-dihydroxyvitamin D3 (1,25-D3), can potently induced monocytic differentiation *in vitro* and *in vivo* [8,9]. However, while vitamin D3 mechanism and its effect on leukemic cells have been widely studied in a preclinical context, clinical investigation has been quickly stopped. One major limiting factor in the clinical application of vitamin D3 is the dose required, which induces adverse events secondary to systemic hypercalcemia [10,11].

However, several studies highlight new actors involved in myeloid differentiation, notably the transcription factor XBP1s, activated through Unfolded Protein Pathway (UPR).

The Unfolded Protein Response is an evolutionarily conserved pathway activated by accumulation of misfolded protein in Endoplasmic Reticulum. In mammals, UPR is triggered by activation of three ER transmembrane sensors: protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6α (ATF6α), and inositol-requiring enzyme-1α (IRE1α). The luminal part of these proteins integrates signals from the ER lumen, while the cytosolic part is responsible for signaling cascades. In the absence of stress, the ER resident protein chaperone BiP interacts with the three sensors to maintain them in an inactive state. When misfolded protein accumulates in ER luminen, BiP is released from the three sensors, leading to their activation. Once activated, each sensor initiates a downstream signaling [12].

Briefly, the activation of PERK result in the eukaryotic initiation factor eIF2α subunit phosphorylation at serine 51, which induces a decrease in protein synthesis through a rapid and potent inhibition of global translational initiation [13]. However, despite this general translation inhibition, some mRNAs containing either uORFs (upstream open reading frames) or IRESs (Internal Ribosome Entry Sites) in their 5' untranslated region can still be efficiently translated [14-16]. Release of BiP unmasks a Golgi Localisation Signal allowing migration of ATF6 from ER to the Golgi apparatus, where it is processed by two proteases (S1P and S2P) into an active ATF6p50 transcription factor [17,18].

IRE1 initiates the most conserved UPR signaling branch. IRE1 is a type I transmembrane protein containing a serine/threonine kinase domain and an endoribonuclease (RNAse) activity, which becomes functional after IRE1α homodimerization and trans-autophosphorylation under stress conditions [19,20]. IRE1-RNAse domain catalyzes excision of a 26-nt intron within the XBP1 mRNA. This unconventional splicing event, which is then

followed by a ligation step mediated by the tRNA ligase RtcB [21], results in a frame shift in XBP1 open reading frame, which leads to expression of a stable and active transcription factor known as XBP1s (spliced) [12,22]. XBP1s is mainly known to control expression of genes encoding factors that modulate protein folding, secretion, ERAD (ER-Associated Degradation), protein translocation into the ER, and lipid synthesis [23]. Interestingly, XBP1s has been also described to be a key differentiation regulator in several hematopoietic lineages. XBP1s is specifically required for B-lymphocyte maturation. XBP1s knock-out in mouse bone marrow induce accumulation of B-lymphocytes-progenitors and complete ablation of mature B-lymphocytes [24,25]. XBP1s is also essential for the development and survival of dendritic cells [26]. Moreover, it was demonstrated that progenitors of myeloid cells selectively expressed Xbp1s, and that this transcription factor is absolutely required for eosinophil differentiation, suggesting XBP1s may support myeloid differentiation [24,27].

Despite the numerous studies aimed at determining the role of the UPR in hematological malignancies, our knowledge of the role of the IRE1/XBP1 pathway in myeloid leukemia remains unclear [28]. Interestingly, a clinical study highlights XBP1s expression is correlated with favorable outcome in Acute Myeloid Leukemia, regardless of AML subtype [29].

To unravel the role of XBP1s in acute myeloid leukemia, we developed original tool, based on inducible-XBP1s-expression in several AML cell lines, in order to decipher specific implication of this transcription factor in AML cell differentiation. We observed XBP1s expression in several AML cell lines increase CD11b expression and unlock myeloid differentiation both *in vitro* and *in vivo*. Using large-scale analysis, we showed that XBP1s target C-MYB and HEX through 2 micro-RNAs, respectively miR-22-3p and miR-148a-3p. miRNA mediated inhibition of C-MYB and HEX expression and remove differentiation blockade, to allow partial differentiation recovery. Taken together these results identify two novel ER stress-induced axes XBP1/miR-22/C-MYB and XBP1/miR-148a/HEX as actors of differentiation in Acute Myeloid Leukaemia. Activation of the IRE1/XBP1 pathway could be a good therapeutic strategy by increasing differentiation-based therapies effects.

MATERIAL AND METHODS

Inducible-model generation

The XBP1 spliced isoform coding sequence was PCR amplified with the proofreading Taq polymerase Phusion (thermofisher) using the primer pair Xba XBP1 Fw (GGTCTAGATAATACGACTCACTATAGGGATGGTGGTGGTGGCAGCCGCGCCG) and XBP1 Sal Rev (CCGTCGACTGCAGAATTCGAAGCTTGAGCTCGAGTTAGAC-ACTAATCAGCTGGGG). The EMCV IRES GFP cassette was in parallel amplified with the primers pIres2GFP Sal1 Fw (CAGTCGACGGTACCGCGGGCCCGGGATC-CGCCCCTCTCCCTCCCCCCCCCC) and pIres2GFP BGL2 REV (TTAGATCTTAC-TTGTACAGCTCGTCCATGCCGAG) using the pIRES2GFP vector from Clontech as a template. These two PCR products were digested respectively by Xba1/Sal1 and Sal1/Bgl2 and ligated into the pTRIPz-TRE-Tight plasmid (Open Biosystem) digested by Xba1/ Bgl2 leading to the fusion of the EMCV IRES GFP sequence to the XBP1s open reading frame downstream of doxycycline responsive promoter. Constructs were confirmed by sequencing and used to transduce OciAML2, OciAML3, MV4-11, MOLM-14, THP1 and HL-60 cell lines, already transduced with pTetOn vector (Clontech), allowing expression of the rTTA (reverse Tetracycline Transactivator). Selection of inducible cells was based on EGFP expression and performed by flow cytometry after treatment with a low dose of doxycycline during 10H. Control TetOn cells were only transduced once with pTetOn vector. MiR-22 and miR-148a-3p inducible models were generate using wild-type OciAML2 and OciAML3, stably transduced with shMIMIC Inducible Lentiviral microRNA (Horizon Discovery; reference VSH6906-224634676 and VSH6904-224645524; miR-22 sequence: AAGCUGCCAGUUGAAG-AACUGU; miR-148a-3p sequence: UCAGUGCACUACA-GAACUUUGU). All transductions are performed using retronectin infection, according to the manufacturer's instructions (Clontech).

Cell culture and treatments

Leukemic cell lines were obtained from The Leibniz Institute DSMZ. Cells were cultured in RPMI supplemented with 10% (OciAML2, OciAML3) of Fetal Bovine Serum (FBS), or in IMDM supplemented with 20% of FBS and 2mM L-glutamine (from Invitrogen), and incubated in a humidified atmosphere of 5% CO2 at 37°C. OciAML2 and OciAML3 are treated 72h with α1,25-dihydroxyvitamin D3 (1,25-D3) (Sigma-Aldrich) respectively at 50nM

and 10nM, with previous-doxycycline induction respectively at 15ng/mL and 8ng/mL during 72h (XBP1s expressing cells), or respectively at 100ng/mL and 1000ng/mL during 96h (miR-148a-3p-expressing cells). HL-60 XBP1s-expressing cells are treated with DiMéthyl SulfOxyde (DMSO) (Sigma-Aldrich) at 1% of final volume during 72h, with previous doxycycline induction at 60ng/mL during 72h. OciAML2 miR-22-expressing cells are treated 72h with α1,25-dihydroxyvitamin D3 (1,25-D3) (Sigma-Aldrich) at 50nM, with previous doxycycline induction at 30ng/mL. Differentiation was assessed by flow cytometry using a MACSQuant® 10 from Miltenyl Biotec. Results were analyzed using FlowJo software.

Reverse transcription and quantitative PCR

For mRNA and mRNA, 500ng of total RNA was reverse transcribed using the PrimeScript RT-PCR Kit from Clontech, according to the manufacturer's protocol. Reverse Transcription (RT) reactions were diluted 20-fold prior to qPCR. Amplification was performed in a total volume of 10µL containing 5µL of a SYBR Premix Ex TaqTM (Tli RNaseHplus), TB Green® Premix Ex Taq™ (Tli RNase H Plus) (TakaraBio), 1µL of both forward and reverse primer (final concentration of 100nM each), and 2µL of diluted cDNA. Primer sequences are given in Table 1. qPCR was performed on the StepOnePlus real-time PCR system (Applied Biosystems), and results was analysed with the StepOne software. For mature microRNA quantification, RT was performed using the miRCURY™ LNA™ Universal RT (Qiagen) microRNA PCR. miR22 expression was assessed by qPCR using hsa-miR-22-3p PCR primer set (Qiagen); expression normalization is performed using hsa-let-7a-5p PCR primer set and SNORD44 PCR primer set for (Qiagen).

GENE	FORWARD PRIMER	REVERSE PRIMER
XBP1s	5'-CTGAGTCCGCAGCAGGTGCAG-3'	5'-ATCCATGGGGAGATGTTCTGG-3'
c-MYB	5'-GGGAACAGATGGGCAGAAATCG-3'	5'-GCTGGCTTTTGAAGACTCCTGC-3'
HEX	5'-CCAGGTGAGATTCTCCAACGAC-3'	5'-CTCCATTTAGCGCGTCGATTCTG-3'
HPRT	5'-TGCTTTCCTTGGTCAGGCAGT-3'	5'-CTTCGTGGGGTCCTTTTCACC-3'
MLN51	5'TAATCCCAGTTACCCTTATGCTCCA-3'	5'-GTTATAGTAGGTCACTCCTCCATATACCTGT-3'

Murine xenograft model

All animal procedures were performed following the principal guidelines of INSERM, and our protocol was approved by the Midi-Pyrenees Ethics Committee on Animal Experimentation. NSG mice were produced at the UMS006 in Toulouse (France) using breeders obtained from Charles River. NSG mice were treated by an intraperitoneal injection of busulfan (20 mg/kg) to induce medullar aplasia. 24h after busulfan treatment, mice were intravenously injected with 2 million of OciAML3 or OciAML2 XBP1s-inducible or TETON cells. After a 9-days-engraftment, doxycylin was added at 1mg/mL in drinking water during 8 days and then, daily monitoring to detect symptoms of disease (ruffled coat, hunched back, weakness, and reduced motility). Mice were humanely sacrificed to analyse bone marrow engraftment. For miR-148a-3p-expressing-cells xenografts, all mice are sacrified 26 days post-injection. Bone marrow (mixed from tibias and femurs) were crushed into PBS with 1% SVF, washed in PBS, dissociated into single-cell suspension, and staining with human CD45 antibody (Biolegend, BLE368504) and human CD11b antibody (Biolegend, BLE301322). Engraftment was measured by flow cytometry, and represent hCD45 positive fraction. For morphological analyses, 10^5 cells are resuspended in 150µL PBS and centrifugated on glass slides, at 700 rpm for 8 min. MGG staining is performed at room temperature, according to the manufacturer's protocol (Sigma-Aldrich). Cellular morphology is examined with a Cell Observer microscope (Carl Zeiss).

Western Blotting

After lysis and sonication (10s each separated by a 30s incubation on ice using VCX 130 Ultrasonic Processor (Sonics) at 25% of its power) cellular debris were eliminated by centrifugation (10min at 10,000g, 4°C). The total protein concentration of cell lysates was determined using a Bicinchoninic Acid Assay kit purchased from Sigma. Protein lysates were fractionated on SDS-PAGE 10% and transferred to a nitrocellulose membrane with Trans-Blot® Turbo™ Transfer System (Bio-Rad). Western-blotting was performed using XBP1 (Cell Signaling, 83418), c-MYB (Abcam, ab-45150), HEX (Abcam, ab153899), GAPDH (Millipore, MAB374), Actin (Sigma-Aldrich, a5441) antibodies. Proteins were visualized using Clarity™ Western ECL from Bio-Rad.

SiRNA and microRNA transfections

C-MYB siRNA (smart pool, Dharmacon), HEX siRNA (smart pool, Dharmacon) as well as negative control siRNA were transfected in OciAML3 during 72h, at a final concentration of 50nM, using the Lipofectamine RNAiMAX tranfection reagent (ThermoFisher) according to the manufacturer's instructions. mirVana miRNA mimic hsa-miR-148a-3p and mirVana negative control (ThermoFisher) were transfected in OciAML3 using the same protocol.

Apoptosis measurement by Flow cytometry

Analysis of apoptosis was done using Annexin V (Annexin-Pacific Blue) and Propidium iodure (PI) (Biolegend # 640928) staining according to standard protocols, followed by flow cytometry using a MACSQuant® VYB from Miltenyl Biotec. Results were analyzed using FlowJo software.

Cell viability assay

Cells were treated or transfected are counted and seeded in 96-well plates (10,000 cells/well, in 100µL RPMI 10%FBS). Cell viability was assessed using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega), every 24h during 4 days.

Statistical analyses

Results are presented as mean values ± standard deviations (SD). Differences between 2 groups were examined using 2-tailed Student's t-test. Survival analyses were performed using log-rank test. For all tests, p-values less than 0.05 (*), 0.01(**), 0.001(***) were considered statistically significant.

RESULTS

XBP1s expression enhance the effect of differentiating drugs in AML cell lines in vitro and in vivo

Using our previously described XBP1s-inducible-AML cell lines (Oci AML3, Oci AML2, and HL60), validated in Philippe, Jaud et al. (in preparation), we chronically induced XBP1s at doxycycline low doses during 72h, and then added differentiating agents (1,25-D3 for OciAML2 and OciAML3 and DMSO for HL-60) to induce differentiation *in vitro*. We evaluated differentiation rate by flow cytometry, using CD11b-pan-myeloid marker. Without doxycycline addition, the respective doses of 1,25-D3 or DMSO slightly increase CD11b staining of control TETON cells and of cells expressing XBP1s (Figure 1A, C and E). Moreover, XBP1s expression alone, without 1,25-D3 or DMSO treatment, has no significant effect on the number of CD11b positive cells *in vitro*. However, co-treatment with doxycycline and 1,25-D3 or DMSO significantly increase the number of CD11b positive cells in XBP1 expressing cells compared to the control, indicating a higher efficiency of the differentiating treatment under these conditions. May-Grünwald-Giemsa-stained cytospins of OCI-AML3 and OciAML2 TETON control and XBP1s expressing cells, both treated with doxycycline and 1,25-D3 reveals XBP1s expressing cells are associated with specific morphological changes, like bigger cells, with membrane protrusions, and poly-lobed nucleus. In HL-60, morphological changes are less noticeable, and cells keep myeloid-progenitors characteristics like little size, with a big-round nucleus (Figure 1A, C and E).

We also verified effect of 1,25-D3 or DMSO treatment on the expression of XBP1s at the mRNA and protein level. As expected, addition of doxycycline strongly increases the expression level of XBP1s, both at the RNA (Supplementary Figure 1A, C and E) and protein level (Supplementary Figure 1B, D and F) compared to untreated cells in the three cell lines.

We also wanted to confirm these results in more physiological model: to assess whether activation of XBP1s expression enhance differentiation *in vivo*, 2.10^6 OciAML3 or OciAML2 cells, expressing XBP1s or not (TETON) were intravenously injected into NSG mice. After 10 days of engraftment, mice drinking water was supplemented with 1mg/ml of doxycycline in order to induce XBP1s expression during 8 days, and bone marrow was further analysed, after relapse (Figure 1G). Transient XBP1s expression significantly increase the number of hCD45/hCD11b double positive cells in the bone marrow (Figure 1H and G), indicating that XBP1s expression is sufficient to induce differentiation of these cells in vivo. The difference

obtained is not related to a variation in the engraftment level and in the number of CD45-positive cells in the bone marrow (Supplementary Figure 2).

Direct XBP1s targets, miR22-3p and miR-148a-3p, enhance myeloid differentiation in vitro.

It is widely reported that XBP1s regulates many stress response genes, but very few information is available on its noncoding RNA target genes. To investigate the molecular mechanism by which XBP1s stimulate AML cell differentiation and to identify novel XBP1s targets, we carried out microRNA analyses and chromatin immunoprecipitation (ChIP) followed by sequencing after induction of XBP1s expression in OciAML3 cells. Among the miRNAs most up regulated by XBP1s, we identified miR-22-3p and miR148a-3p, which expression level is increased approximately and respectively by 4,2 and 3,3 times (Figure 2A). miR-22 regulation by XBP1s is previously described (Jaud et al.). Mir148a is also directly up-regulate by XBP1s. ChIP-seq results reveal XBP1s fixation at the miR-148a precursor transcription start site, described by Chien et al. (Figure 2A) [30]. Because our sequencing has been realized in OciAML3, we verified that XBP1s effectively activates miR148a-3p expression in OciAML2 and HL-60 (Figure 2C and D). Interestingly, miR-148a-3p has already been related to many differentiation processes, in adipocytes or muscle, and also in macrophages. Moreover, miR-148a-3p has been related to better prognosis in AML. We previously studied miR-22 effect on AML, and demonstrated several anti-leukemic effects related to this micro-RNA expression: apoptosis induction and sensitization to chemotherapy through SIRT1 oncogene repression. However, miR-22 has been related to myeloid differentiation and is a direct target of PU.1 transcription factor. For these reasons, these two micro-RNAs appears as relevant target, potentially involved in XBP1s-related differentiation.

We generated new inducible models, allowing us to induce miR-22-3p or miR-148a-3p overexpression in OciAML2 and OciAML3 cell (Supplementary Figure 3A). While cell viability is not affected by miR-148a expression (Supplementary Figure 3B and C), an up regulation of miR22 expression by treatment with increasing amounts of doxycycline stimulates an apoptotic response in OciAML2 cell line (Supplementary Figure 3D and E). Validation of miR-148a-3p and miR-22-3p induction by doxycycline in each cellular model by RT-qPCR is also performed (Supplementary Figure 3E to G). To study miR-148a-3p

impact on myeloid differentiation, cells were treated with doxycycline for 7 days, to chronically induce miR-148a-3p expression in OciAML2 and OciAML3, and then were treated with 1,25-D3 the last 3 days to induce differentiation. Without doxycycline addition, the vitamin D3 stimulates the differentiation of OciAML2 and OciAML3 control (TETON) or miR148a-3p expressing cells in a very similar range, but miR148a-3p expression after doxycycline addition significantly increase the number of CD11b positive cells compared to the control (Figure 2E and F). These results were confirmed using May–Grünwald–Giemsa staining that shows morphological hallmarks of differentiation when OciAML2 and OciAML3 cells expressing miR148a-3p were treated with 1,25-D3 (Figure 2H and I). Similar results were obtained in OciAML2 expressing miR22-3p (Figure 2G and J): OciAML2 expressing cells or not (TETON) were treated at 30ng/mL of doxycycline, a non-lethal dose, during 5 days. The last 3 days, cells were treated with 1,25-D3 at 50nM. Here again, we observed an CD11b increase when miR-22 is expressed and associated with 1,25-D3 treatment. Interestingly, miR-22 alone show a little but significant increase of CD11b staining. Moreover, MGG reveals morphological changes associated with differentiation progression (Figure 2G and J). Altogether, these results indicate that these two miRNAs are able to stimulate the differentiation of AML cell lines *in vitro*.

miR-148a-3p enhance myeloid differentiation in vivo

In order to confirm the effect of miR-148a-3p expression on differentiation *in vivo*, $2x10^6$ OciAML2 or OciAML3 cells expressing miR148a-3p or control cells (TETON) were intravenously injected in NSG mice. After 10 days of engraftment, mice drinking water was supplemented with 2mg/ml of doxycycline for 8 days in order to transiently induce miR-148a-3p expression (Figure 3A). Sacrifice was performed 26 days after injection and bone marrow was analysed. In both OciAML2 and OciAML3 cells, miR-148a-3p increase the number of CD45/CD11b double positive cells in the bone marrow compared to the TETON control cells, indicating that this miRNA is able to induce differentiation of these cells *in vivo* (Figure 3B and C). Interestingly, engraftment analyses using CD45 staining, reveal a significant decrease of human cells in bone marrow, suggesting miR-148a-3p-related differentiation slow down AML progression (Figure 3D and E).

Repression of HEX by miR-148a-3p induce differentiation in AML cell lines

We next performed in silico analyses to identify potential target of miR-148a-3p. We identified HEX was a good candidate, based on the presence of canonical 8-mer seed site in the 3'UTR (Figure 4A). Moreover, the direct interaction of miR148a-3p and HEX was experimentally verified using AGO2 HITS-CLIP experiments in Pancreatic beta cells [31] and in HeLa cells [32]. HEX is a member of the homeobox family of transcription factors mostly expressed in hematopoietic lineages. HEX is generally overexpressed in acute myeloid leukemia [33,34] and blocks myeloid differentiation at the promyelocyte stage [35]. More recently it was demonstrated that HEX is able to promote myeloid transformation and drive AML development [36,37].

We first verify the effect of XBP1s on HEX expression using the inducible XBP1s expressing cellular models. Addition of doxycycline decreases HEX expression level in XBP1s expressing cells but not in TETON control cells (Figure 4B, C and D). Moreover, HEX expression is also decreased upon miR-148a-3p expression in OciAML2 and OciAML3 (Figure 4E and F). RT-qPCR experiments on XBP1s expressing cell lines reveal no variation of HEX at mRNA level compared to TETON cells, suggesting a translational repression by miR-148a-3p (Supplementary Figure 4A to C).

Finally, to confirm the involvement of HEX in AML cell differentiation, we down-regulate its expression by transfecting siRNA against its mRNA in OciAML3, the only AML cell line easy to transfect. siRNA HEX was transfected during 72h, and 1,25-D3 was added for the last 24h. Interestingly, siRNA slightly decreases HEX expression (Figure 4H), but this inhibition is sufficient to significantly increase the number of CD11b positive OciAML3 cells, more than 3 times compared to control cells, upon 1,25-D3 treatment (Figure 4G). In the absence of differentiating agent treatment, we also observed a slight but significant increase of CD11b positive cells, compared to cells transfected with a non-relevant siRNA (siCT) (Figure 4G).

Repression of c-MYB by miR-22-3p induce differentiation in AML cell lines

It was well documented that MYB is a master regulator of hematopoiesis and contributes to leukemogenesis by promoting proliferation, suppressing apoptosis [38,39] but its ability to block differentiation is clearly critical for its transforming potential [40-43]. Interestingly, we found that c-MYB expression was inhibited by XBP1s in both OciAML2 and OciAML3 cells, compared to TETON control cells (Figure 5A and B). Moreover, unexpectedly in Oci-AML2 cells expressing miR-22-3p we demonstrated that doxycycline addition lowers MYB expression level but not in in TETON control cells (Figure 5C). Here again, RT-qPCR experiments on

XBP1s expressing cell lines reveal no variation of c-MYB at mRNA level, suggesting a translational repression directly mediate by miR-22, or by another process miR-22-dependant (Supplementary Figure 4). Finally, we also confirm that c-MYB is involved in the differentiation of OciAML3 by lowering its expression using siRNA (Figure 5D). As expected, c-MYB expression inhibition alone increases the number of CD11b positive cells, indicating that c-MYB inhibits differentiation. Moreover, c-MYB inhibition strongly enhances, by more than 4-fold, the differentiating effect of low dose of 1,25-D3 (Figure 5E). These data demonstrated that miR-22-3p, through c- MYB repression, is involved in the enhancement of AML cell lines differentiation.

DISCUSSION

In this study, we develop inducible model based on Tet-On strategy to study specifically XBP1s activation, regardless of PERK or ATF6 pathway, or other IRE1α potential targets. We demonstrated that XBP1s induces differentiation in leukemic blasts through 1,25D or DMSO treatment *in vitro* and spontaneously *in vivo*. Our results suggest IRE1α-XBP1 signalling pathway may be a differentiation switch, and modulate cellular fates of AML.

Interestingly, XBP1s is essential for the differentiation in many cell types, including adipocytes, myocytes, and plasma cells [25,44]. In the mammary epithelium, XBP1 deletion results in inhibition of epithelial proliferation and differentiation during lactation [45]. XBP1s is selectively and absolutely required for eosinophil maturation [24], but our results suggest that XBP1s may induce differentiation at earlier stages. Through the stimulation of miR-22-3p expression, XBP1s decrease c-MYB protein level, leading to blast differentiation (Figure 6). C-MYB has been widely associated with different types of cancers and studied in detail in myeloid leukemias. Indeed, it's now commonly accepted c-MYB is expressed by myeloid progenitors to sustain proliferation and survival [46]. Moreover, c-MYB stabilization has been related to Acute Myeloid Leukemia development and progression, especially in MLL-translocation AML subtype, and proposed as therapeutic target [47-49]. c-MYB also induces FLT3 expression [50], a transcription factor frequently mutated and activated in AML, and for which targeted therapeutic agent has been developed [50]. Despite the fact that transcription factors have been considered difficult or impossible to target in a therapeutic context, several c-MYB inhibitors were developed and tested in preclinical leukemia models *in vitro* and *in vivo* [51-53]. In the light of our results, an interesting alternative would be to specifically activate

the IRE1/XBP1 axis to decrease c-MYB expression and sensitize blasts to treatment with differentiating agents.

In addition, XBP1s also target HEX transcription factor through mir-148a-3p transcriptional activation (Figure 6). HEX is broadly elevated in AML, compared to normal hematopoietic cells [37]. Several studies revealed that HEX overexpression blocks myeloid differentiation at the promyelocyte stage, is sufficient to elicit rapid promyelocytic leukemia in mice [35,54] and drives also AML development [36,37]. Moreover, during serial bone marrow transplantation, HEX-deleted HSCs are progressively lost, revealing an intrinsic defect in HSC self-renewal. HEX-deleted mice show markedly impaired hematopoietic recovery following myeloablation, due to a failure of progenitor expansion [55,56]. Thus, given the limited availability of effective therapies for AML, HEX activity inhibition may offer new opportunities for halting the spread of AML. Nevertheless, the regulation of HEX expression in AML remains unknown. Again, this work demonstrates that activation of the IRE1/XBP1 pathway could decrease HEX expression and put the brakes on leukaemia growth.

Actually, many studies have already reported that UPR activation, for example with proteasome inhibitors such as bortezomib, exert anti-leukemic effect. Bortezomib is currently in phase I-II of clinical trial for AML treatment (clinical trials references: NCT01861314, NCT04173585, NCT01371981) [57]. However, our results suggest that specific activation of XBP1s exerts different impact than entire UPR activation, and lead to the differentiation of AML cells. Nevertheless, XBP1 splicing requires IRE1α activation, and until now, selective IRE1 RNase activation has not been extensively explored. Only few drugs inducing this activation have been already described. For example, APY29 - a predicted type I kinase inhibitor act as ligands and bind to IRE1α's kinase ATP-binding site and activates its adjacent RNase domain resulting in a moderate XBP1 splicing [58]. More recently two potent allosteric IRE1 activators, named G-9807 and G-1749, binding the kinase ATP-binding pocket and inducing a conformational remodeling typical of an active kinase were characterized [59]. These compounds are structural analog of AMG-18 that was previously reported to be selective allosteric inhibitor of the IRE1 RNase [60,61], but in this case they induce stimulation of IRE1 RNase activity and promote XBP1 splicing [59]. Novel non-toxic molecules, IXA1, IXA4, and IXA6 selectively activating IRE1 signaling were identified by high throughput screening approach [62]. These compounds act independently of the ATP-binding pocket, and did not globally activate other ER stress related signalling pathways, but only slightly activate XBP1 splicing. Finally, it was also demonstrated that the flavanol Quercetin activates IRE1's RNase activity and promote XBP1 splicing [63,64]. Interestingly,

numerous studies probed the influence of Quercetin in different cancer cell lines, and this compound was proved to possess beneficial effects on hematological cancers [65]. Nevertheless, none of these drugs have been tested in preclinical acute myeloid leukemia models, in which it appears to be particularly relevant to activate the XBP1s pathway for therapeutic purposes.

Classically, XBP1s action has been described through its coding targets, like ER-resident chaperones. ChIP assays in different cell type have revealed 30% of XBP1s targets were related to ER stress response and proteosynthesis [23]. However, significant fraction of XBP1s is non-coding targets. In this work, we shown that miR-148a-3p are directly up-regulated by XBP1s, and we already showed it for miR-22. This non-coding-target fraction appears to be critical in XBP1s-related effects on AML, as we demonstrate that miR-22-3p and miR148a impact myeloid differentiation. Moreover, micro-RNAs also represent interesting druggable targets. For example, miR-22-3p has been study in AML context and show therapeutic potential through miR-22-3p-associated-nanoparticle treatment [66]. Actually, our study focuses on 2 micro-RNAs but large-scale analysis underlines up-regulation of several non-coding RNAs, including lncRNAs, which represent a promising search field in AML, as diagnostic or even therapeutic tools [67-69].

To conclude, our data highlights original XBP1s action on myeloid differentiation in pathological context like AML. Each XBP1s/miR22-3p/C-MYB and XBP1s/miR148a-3p/HEX axes contribute to improve myeloid differentiation and may represent new potential targets for differentiation-based therapies in non-APL AML.

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<u>Figure 1:</u> XBP1s overexpression improve differentiation in several AML cell lines, both in vitro and in vivo

(A-F) OciAML2 (A), OciAML3 (C), and HL-60 (E) control (TETON) or expressing XBP1s are respectively treated at 15ng/mL, 8ng/mL or 60ng/mL of doxycyclin during 6 days to chronically induced XBP1s expression. The last 3 days, VitaminD3 at 50nM (A) or 10nM (B) or DMSO at 1% (C) is added to induce cell differentiation. Differentiation is evaluated by flow cytometry, using CD11b staining. Results represented average from 3 independent experiments +/- Standard Deviation (SD).105 cells are also cytospun and stained with May-Grünwald Giemsa coloration; scale bars represent 20µM (D; E). **(G)** Mice experimental procedure: NSG mice are injected with 2 million of OciAML2 **(H)** or OciAML3 **(I)**, TETON and XBP1s. After a 9-days-engraftment, doxycylin is added at 1mg/mL in drinking water during 8 days. After relapse, percentage of CD45+/CD11b+-double-positive cells is measured in bone marrow using flow cytometry (BM). Statistical analysis is performed using Mann-Whitney test; *p≤0,05; **p≤0,01; ***p≤0,0001.

<u>Figure 2:</u> miR-148a-3p and miR-22-3p are XBP1s-direct target, and improved myeloid differentiation in vitro

(A) List of up-regulated micro-RNAs between OciAML3 XBP1s cells and TETON control cells treated with 10ng/mL of doxycycline for 48H, presented in fold change(RPKMXBP1s/RPKMTETON). **(B)** Snapshots of ChIP-Seq signals (peaks) representing XBP1s-bound genomic regions in OciAML3 cells treated with 10 ng/mL of doxycycline for 48H compared to the input, at the MIR148 putative TSS. **(C; D)** Mature miR-148a-3p expression, upon XBP1s induction, is validated by RT-qPCR in OciAML2 and HL-60. Expression values are normalized to SNORD44 and let-7a microRNAs, and are depicted as an expression ratio in doxycycline treated cells relative to untreated cells. OciAML2 and OciAML3 expressing inducible miR-148a-3p **(E; F)** are respectively treated at 100ng/mL 1000ng/mL of doxycyclin during 7 days to chronically induced miR-148a-3p. The last 3 days, VitaminD3 at 50nM (E) or 10nM (F) is added to induce cell differentiation. CD11b expression is measured by flow cytometry. **(G)** OciAML2 expressing inducible miR-22 are treated at 30ng/mL doxycycline during 5 days to chronically induced miR-22-3p. The last 3 days, VitaminD3 at 50nM is added to induce cell differentiation. CD11b expression is measured by flow cytometry. Results represented average from 3 independent experiments +/-

Standard Deviation (SD). **(H-J)** Cells are cytospun and stained with May-Grünwald Giemsa coloration.

<u>Figure 3 :</u> miR-148a-3p expression improves differentiation in vivo

(A) *In vivo*-protocol representation : immunodeficient NSG mice are xenografted with OciAML2 (B ; D) or OciAML3 (C ; E), expressing miR-148a-3 (148) or not (TETON). After a 10-days-engraftment-delay, doxycyclin is added in drinking water at 2mg/mL to induce miR-148a-3p expression, during 8 days. Mice are sacrified 26 days after injection. **(B ; C)** Differentiation levels are assessed in bone marrow using CD45/CD11b staining. **(D ; E)** Engraftment is evaluated by flow cytometry using CD45 staining in bone marrow.

<u>Figure 4 :</u> miR-148a-3p induces differentiation through HEX repression

(A) Predictive interaction between miR-148a-3p and HEX using DIANAtools ans miR-map databases. **(B-D)** HEX protein expression, evaluated by western blotting in Oci-AML2 (B), Oci-AML3 (C) and HL-60 (D). Actin is used as loading control. **(E ; F)** HEX protein expression, evaluated by western blotting in Oci-AML2 (G), Oci-AML3 (H) miR-148a-3p expressing cells. Actin or GAPDH is used as loading control. **(G)** Oci-AML3 wild type cells are transfected with siRNA control (siCT) or siRNA against HEX mRNA (siHEX) at 25nM during 72h. The last 24h, cells are treated with 10nM of VitaminD3 (VD) or not (NT) to induce differentiation. Cells are analysed by flow cytometry, using CD11b staining. **(H)** siHEX efficiency is tested by western blot. GAPDH is used as loading control.

<u>Figure 5 :</u> miR-22-3p induces differentiation through C-MYB repression

(A-B) C-MYB protein expression, evaluated by western blotting in OciAML2 (A), OciAML3 (B) after XBP1s induction during 6 days, Actin and GAPDH are used as loading control. **(C)** C-MYB protein expression, evaluated by western blotting in OciAML2 control (TET) or expressing miR-22 (22) induced at 30ng/mL of doxycycline during 5 days.**(D)** OciAML3 wild type cells are transfected with siRNA control (siCT) or siRNA against C-MYB mRNA (siMYB) at 25nM during 72h. The last 24h, cells are treated with 10nM of VitaminD3 (VD) or not (NT) to induce differentiation. Cells are analysed by flow cytometry, using CD11b staining. **(E)** siMYB efficiency is tested by western blot. GAPDH is used as loading control.

<u>Figure 6 :</u> XBP1s-related differentiation in AML cells

A main hallmark in Acute Myeloid Leukemia is differentiation blocked : stabilization of myeloid progenitors factors like C-MYB, as well as quiescence and stemness factors like HEX prevent differentiation progression. AML cells are *de facto* more resistant to apoptosis or chemotherapeutic treatments. XBP1s expression in those cells up-regulates two micro-RNAs, miR-22-3p and miR-148a-3p, which target respectively C-MYB and HEX. Repression of these two factors unlocks blockade in different AML cell type and allows partial differentiation recovery.

Figure 1

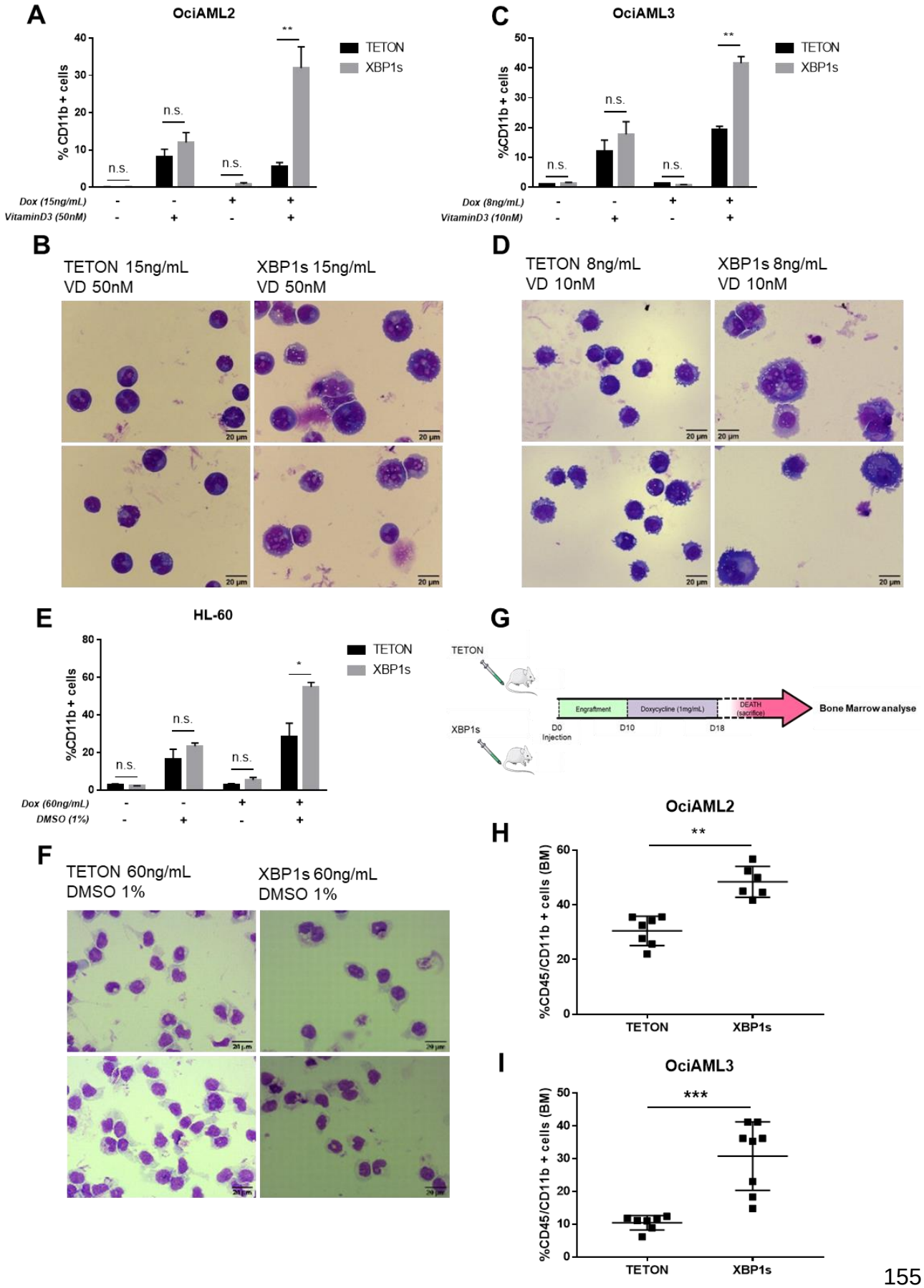
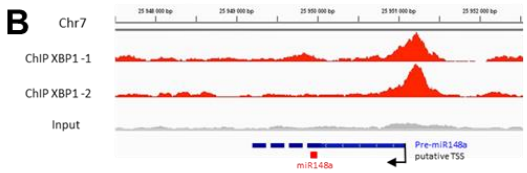
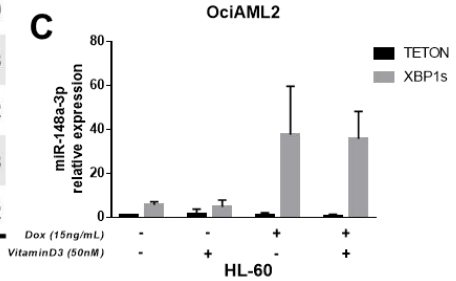

A
OciAML2
% CD11b + cells
TETON
XBP1s
n.s.
**
Dox (15ng/mL)
VitaminD3 (50nM)
C
OciAML3
%CD11b + cells
Dox (8ng/mL)
VitaminD3 (10nM)
B
TETON 15ng/mL
VD 50nM
XBP1s 15ng/mL
VD 50nM
D
TETON 8ng/mL
VD 10nM
XBP1s 8ng/mL
VD 10nM
E
HL-60
%CD11b + cells
*
Dox (60ng/mL)
DMSO (1%)
F
TETON 60ng/mL
DMSO 1%
XBP1s 60ng/mL
DMSO 1%
G
TETON
XBP1s
Engraftment
Doxycycline (1mg/mL)
DEATH (sacrifice)
Bone Marrow analyse
D0
Injection
D10
D18
H
OciAML2
%CD45/CD11b + cells (BM)
I
OciAML3
***

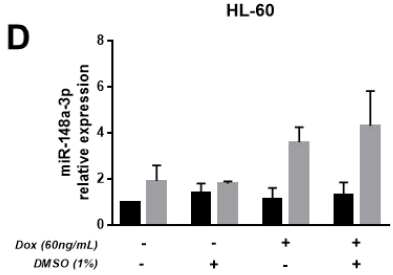
Figure 2

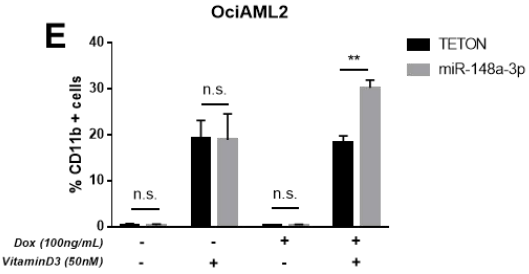
A

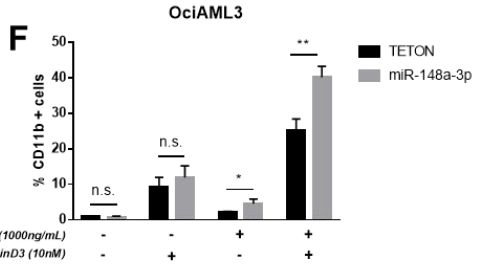
miRNA	$FC_{XBP1/TETON}$
hsa-miR-22-3p	4,23175512
hsa-miR-199a-3p	4,09803208
hsa-miR-148a-5p	3,40626319
hsa-miR-148a-3p	3,30917408
hsa-miR-21-3p	2,71377832
hsa-miR-574-3p	2,60768258
hsa-miR-215-5p	2,45943162

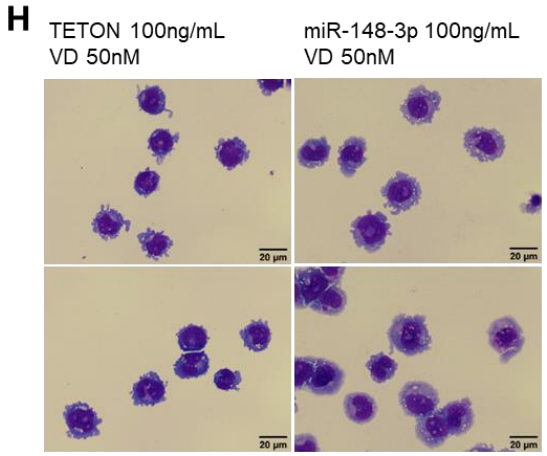


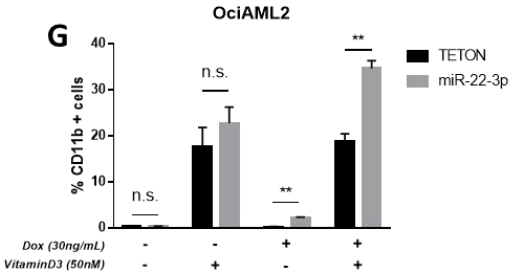


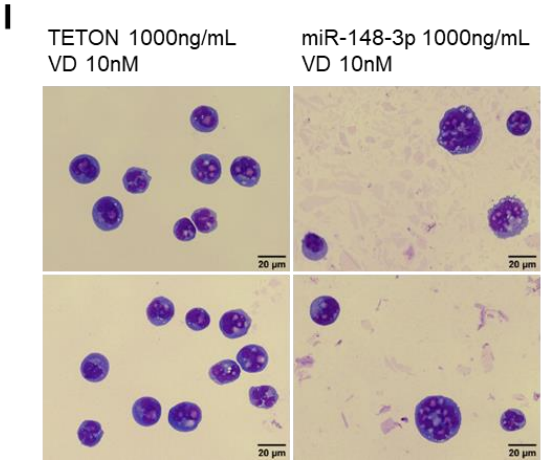












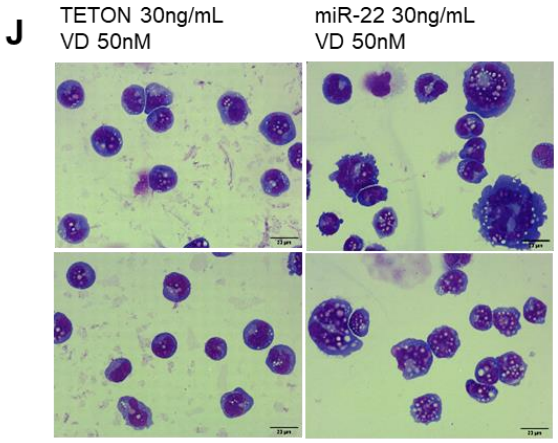


Figure 3

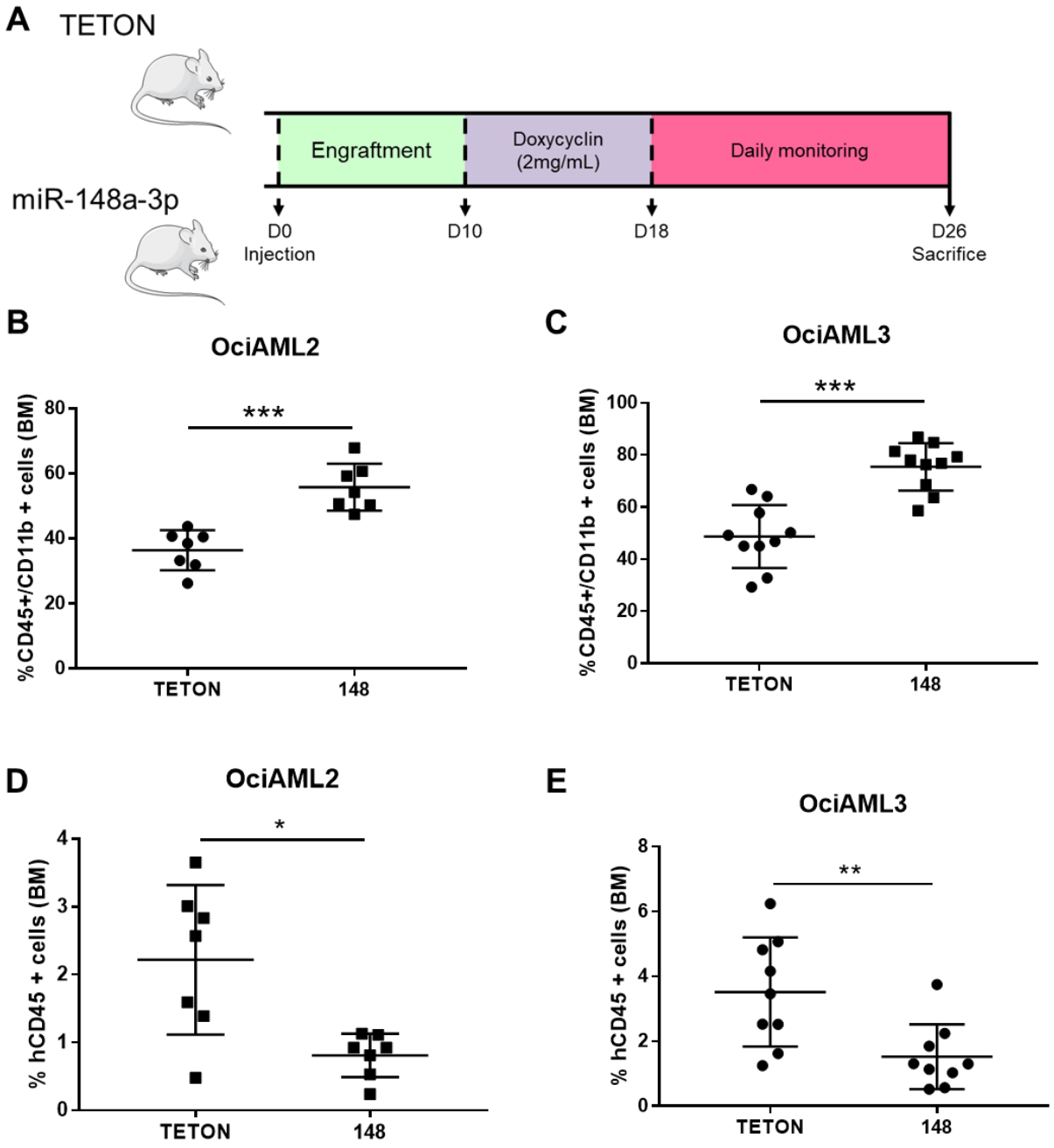

A
TETON
miR-148a-3p
Engraftment
Doxycyclin (2mg/mL)
Daily monitoring
D0 Injection
D10
D18
D26 Sacrifice
B
OciAML2
% CD45+/CD11b + cells (BM)
***
TETON
148
C
OciAML3
% CD45+/CD11b + cells (BM)
***
TETON
148
D
OciAML2
% hCD45 + cells (BM)
*
TETON
148
E
OciAML3
% hCD45 + cells (BM)
**
TETON
148

Figure 4

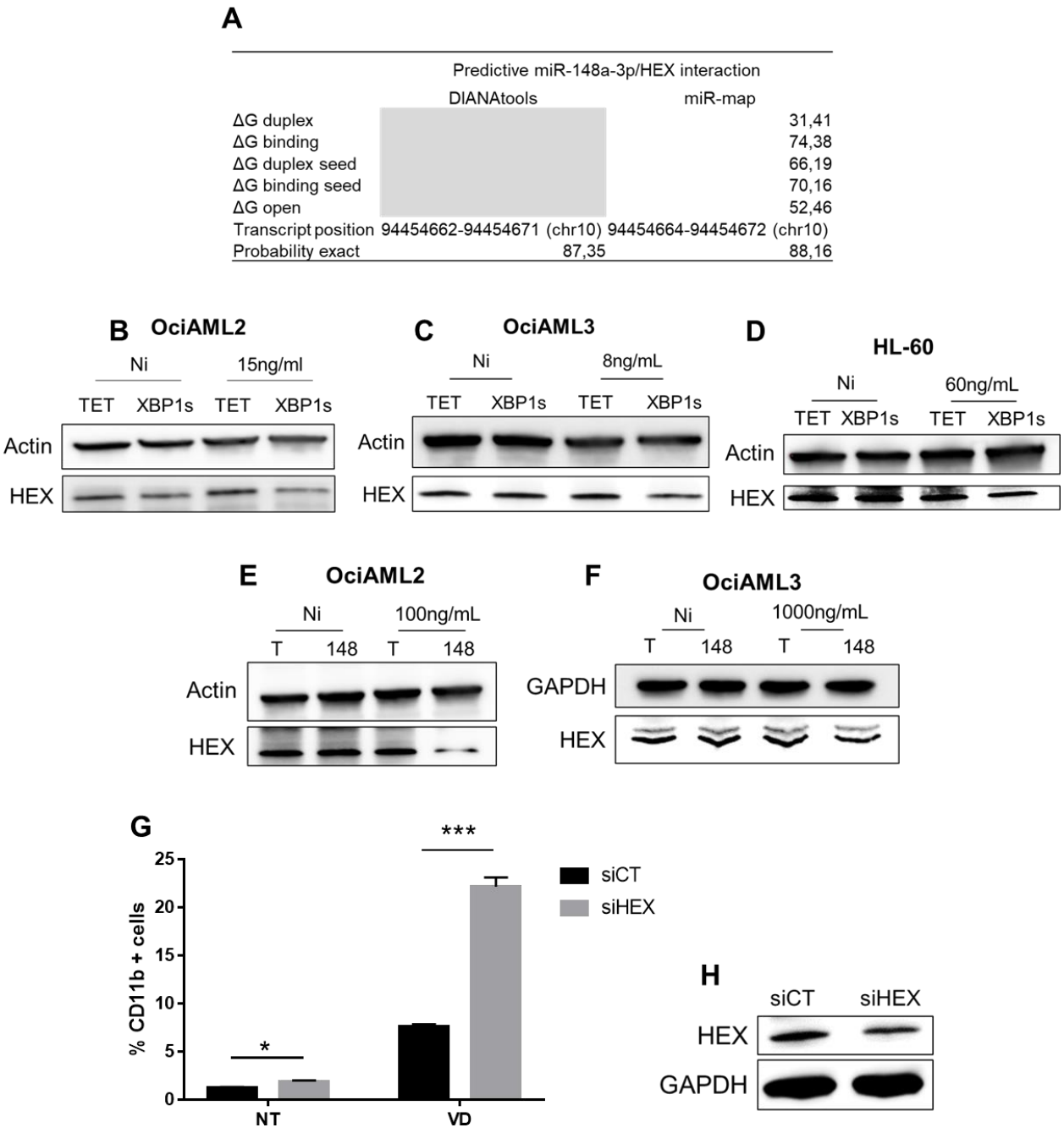

A
Predictive miR-148a-3p/HEX interaction
DIANAtools
miR-map
ΔG duplex 31,41
ΔG binding 74,38
ΔG duplex seed 66,19
ΔG binding seed 70,16
ΔG open 52,46
Transcript position 94454662-94454671 (chr10) 94454664-94454672 (chr10)
Probability exact 87,35 88,16
B OciAML2
Ni
15ng/ml
TET XBP1s TET XBP1s
Actin
HEX
C OciAML3
Ni
8ng/mL
TET XBP1s TET XBP1s
Actin
HEX
D HL-60
Ni
60ng/mL
TET XBP1s TET XBP1s
Actin
HEX
E OciAML2
Ni
100ng/mL
T 148 T 148
Actin
HEX
F OciAML3
Ni
1000ng/mL
T 148 T 148
GAPDH
HEX
G
% CD11b + cells
25
20
15
10
5
0
*
***
NT
VD
siCT
siHEX
H
siCT siHEX
HEX
GAPDH

Figure 5

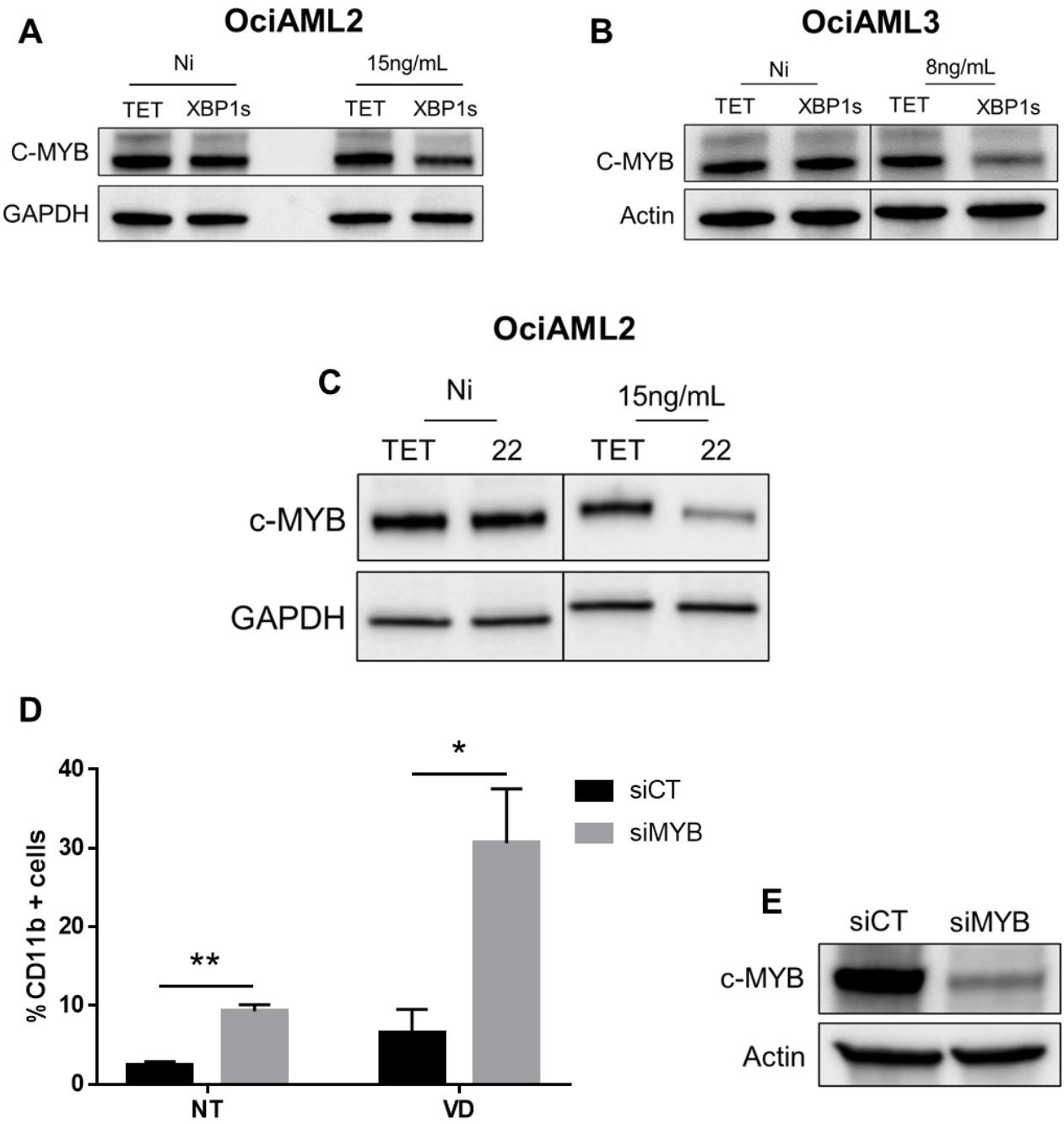
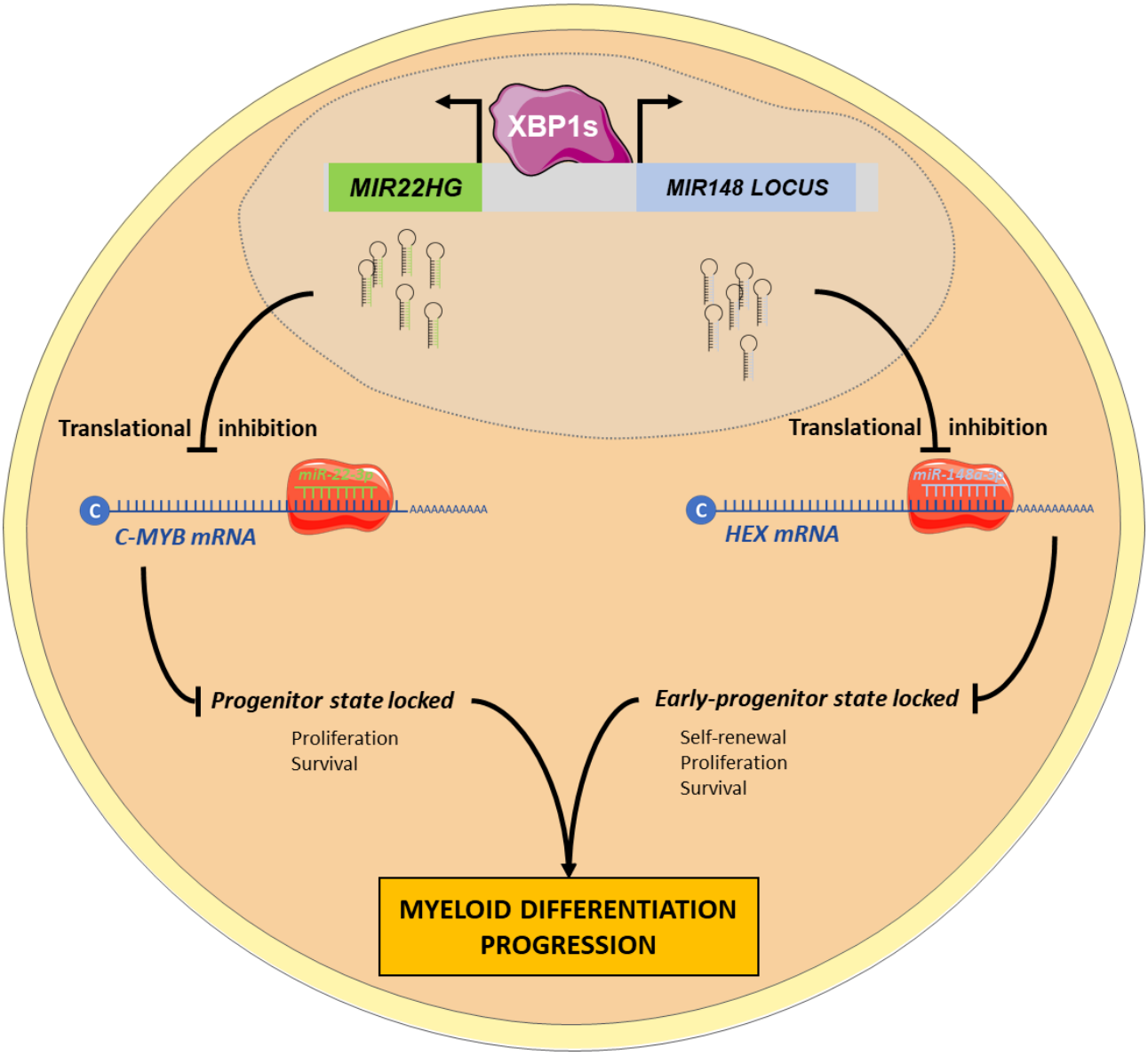
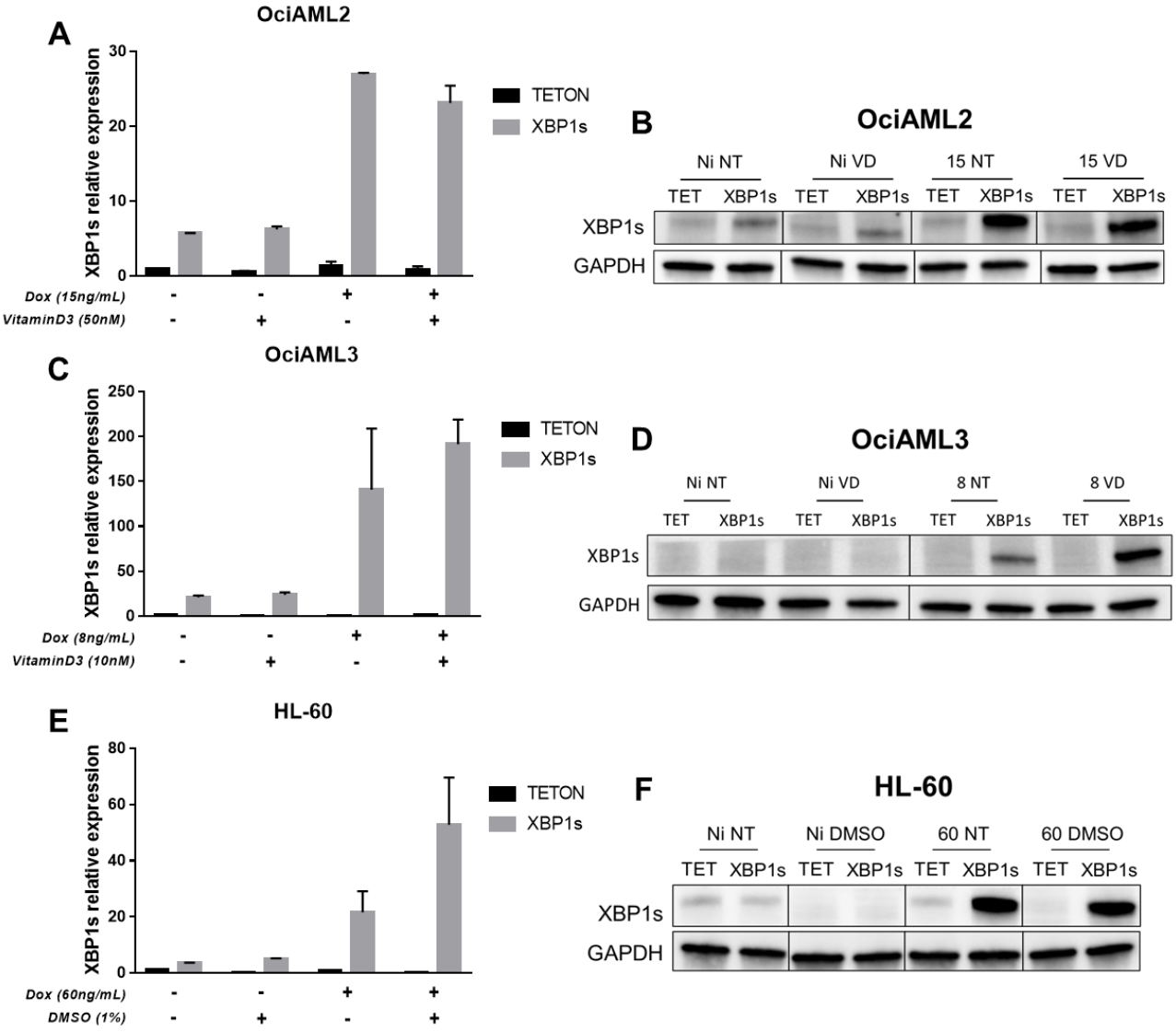

A
OciAML2
Ni
15ng/mL
TET
XBP1s
TET
XBP1s
C-MYB
GAPDH
B
OciAML3
Ni
8ng/mL
TET
XBP1s
TET
XBP1s
C-MYB
Actin
OciAML2
C
Ni
15ng/mL
TET
22
TET
22
c-MYB
GAPDH
D
% CD11b + cells
40
30
20
10
0
*
**
siCT
siMYB
NT
VD
E
siCT
siMYB
c-MYB
Actin

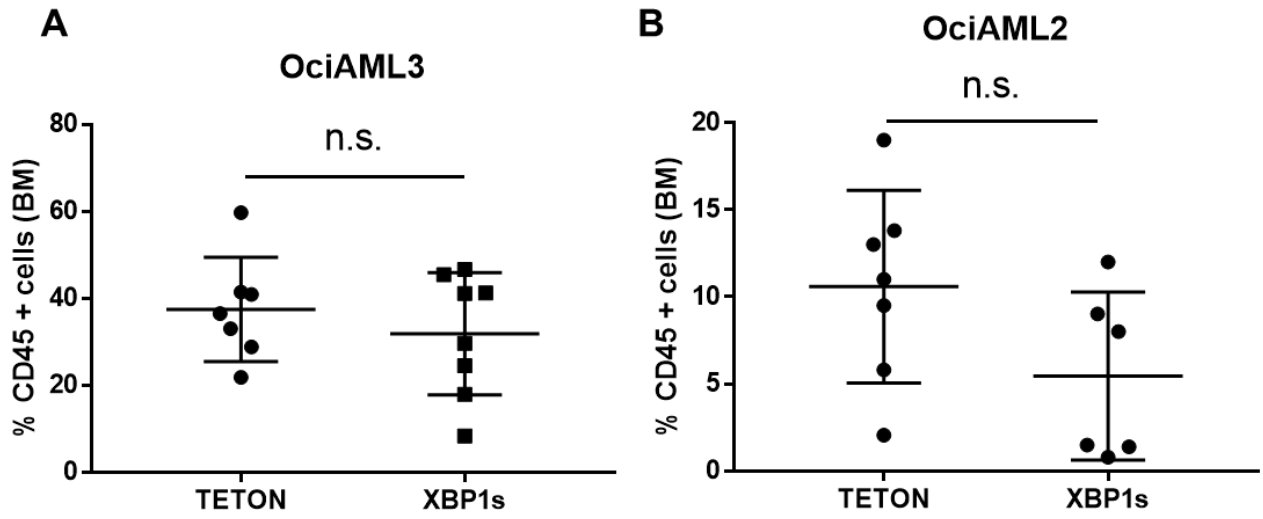
Figure 6


XBP1s
MIR22HG
MIR148 LOCUS
Translational inhibition
Translational inhibition
C-MYB mRNA
HEX mRNA
Progenitor state locked
Proliferation
Survival
Early-progenitor state locked
Self-renewal
Proliferation
Survival
MYELOID DIFFERENTIATION PROGRESSION



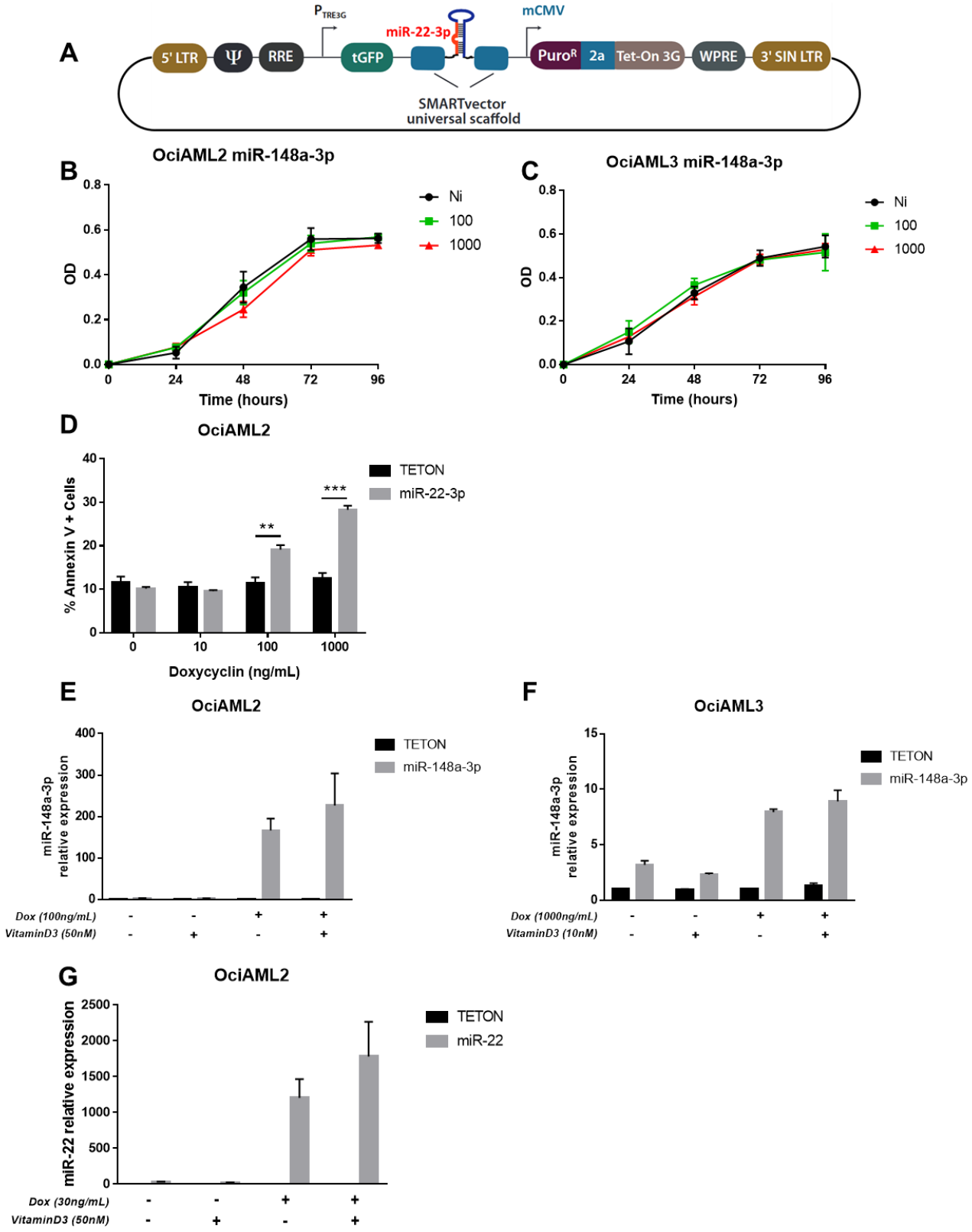
Supplementary Figure 7 : **XBP1s-inducible expression in OciAML2, OciAML3 and HL60**

OciAML2 (A ; B), OciAML3 (C ; D), and HL-60 (E ; F) control (TETON) or expressing XBP1s are respectively treated with 15ng/mL, 8ng/mL or 60ng/mL of doxycyclin during 6 days to chronically induced XBP1s expression. The last 3 days, VitaminD3 at 50nM (A) or 10nM (B) or DMSO at 1% (C) is added to induce cell differentiation. **(A ; C ; E)** XBP1s expression is quantified by RT-qPCR and normalized by housekeeping genes HPRT and MLN51. Results represent average from 3 independent experiments +/- Standard Deviation (SD). **(B ; D ; F)** XBP1s protein levels are quantified by western blot. GAPDH is used as loading control.



Supplementary Figure 8 : **Bone-marrow engraftment in NSG mice xenograft with XBP1s expressing cells**

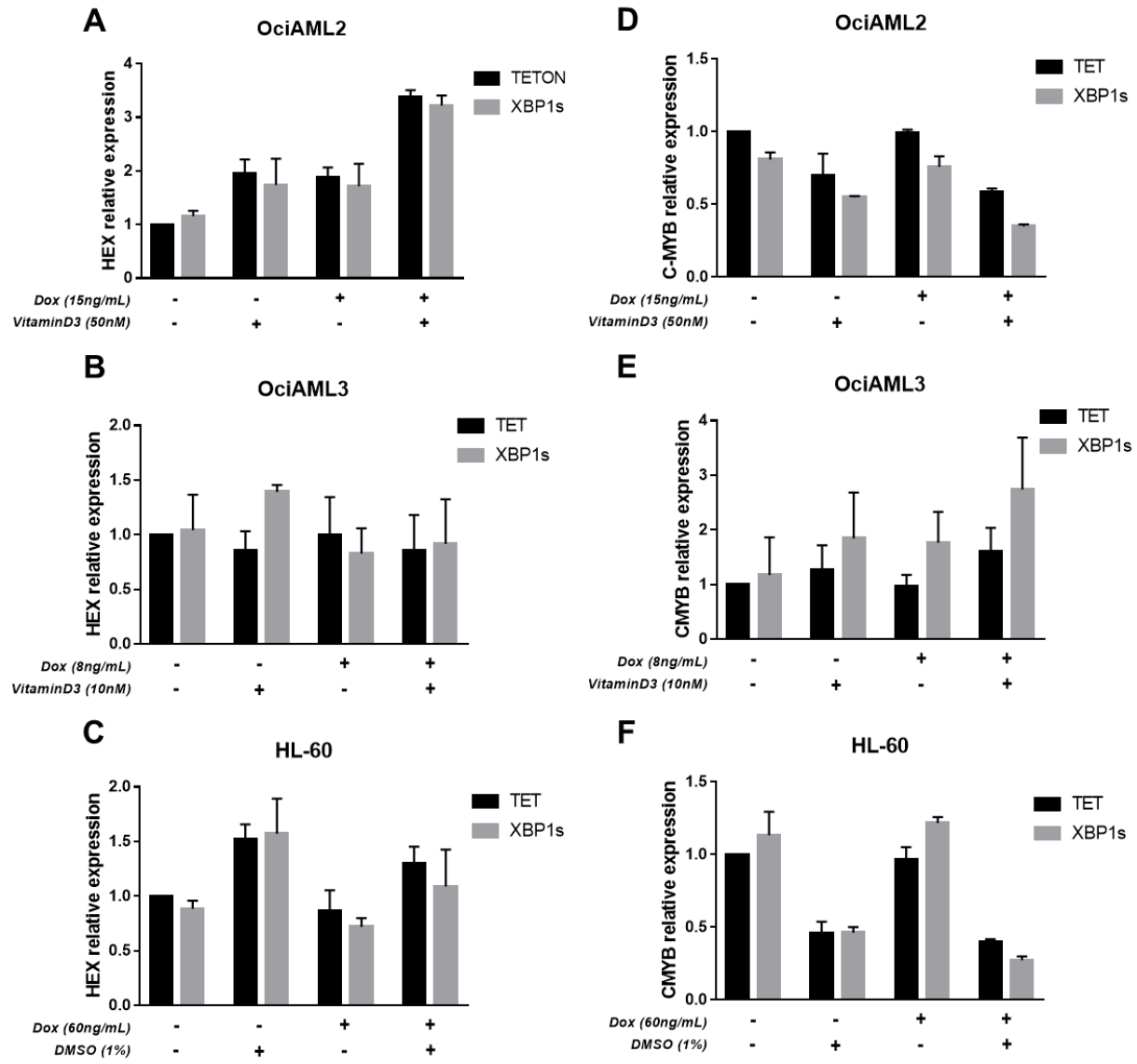
(A ; B) Percentage of $CD45^+$ positive cells is measured in bone marrow (BM) after OciAML2 (A) or OciAML3 (B) xenografts, using flow cytometry. Statistical analysis are performed using Mann-Whitney test ; *$p \leq 0,05$; **$p \leq 0,001$; ***$p \leq 0,0001$.



Legend on the following page

<u>Supplementary Figure 9 :</u> Generation of miR-148a-3p and miR-22-3p inducible models in OciAML2 and OciAML3 cell lines

(A) Schematic representation of inducible miR-148a-3p and miR22-3p model. OciAML2 TETON and OciAML3 TETON are stably transduced with lentivector expressing miR-148a-3p or miR-22-3p, under control of rtTA-Responsive-Elements promotor. **(B ; C)** Cell viability is evaluated using MTS assay in OciAML2 (B) and OciAML3 (C) expressing miR-148a-3p after doxycyclin treatment at 0 (Ni), 100ng/mL (100) or 1000ng/mL (1000) during 96h **(D)** OciAML2 expressing miR-22-3p or not (TETON) are treated with increasing amount of doxycycline during 72h. Apoptosis is measured by flow cytometry using AnnexinV staining. Data represent mean ± SD (n=3). Statistical analysis are performed using unpaired t-tests; *p≤0.05 ; **p≤0.001 ; ***p≤0.0001 . **(E-F)** miR-148a-3p expression is quantified by RT-qPCR after doxycyclin treatment in OciAML2 (E) and OciAML3 (F) in miR-148a-3p expressing cells. qPCR are normalized by housekeeping gene let7 and SNORD44. Results represented average from 3 independent experiments +/- Standard Deviation (SD). **(G)** miR-22 expression is quantified by RT-qPCR after doxycyclin treatment in OciAML2 miR-22 expressing cells. qPCR are normalized by housekeeping gene let7 and SNORD44. Results represented average from 3 independent experiments +/- Standard Deviation (SD).



Supplementary Figure 10 : **HEX and C-MYB expression in XBP1s-expressing cells**

(A-F) HEX (A-C) or C-MYB (D-F) expression is quantified by RT-qPCR and normalized by housekeeping genes HPRT and MLN51 in OciAML2 (A-D) OciAML3 (B-E) and HL-60 (C-F). Results represent average from 3 independent experiments +/- Standard Deviation (SD).

DISCUSSION

A partir d'une étude clinique qui corrèle l'expression de l'isoforme épissée et active de XBP1 à un bon pronostic dans les LAM, nous avons développé un modèle d'expression inductible dans plusieurs lignées différentes, présentant des anomalies cytogénétiques différentes. Ces modèles nous ont permis de moduler l'expression de XBP1s. Nous avons observé que :

- L'activation soutenue de XBP1s induit une réponse apoptotique dans les cellules de leucémie aiguës myéloïdes *in vitro* et *in vivo*,
- L'activation modérée de cette même voie permet de potentialiser les effets de l'aracytine *in vitro* et *in vivo* dans le modèle OciAML3, résistant à l'aracytine.
- L'activation chronique de XBP1s induit un déblocage de la différenciation myéloïde *in vitro* et *in vivo*.

Grâce à ces modèles, nous avons également réalisé un miRnome, un séquençage des ARN, ainsi qu'un séquençage de ChIP, qui nous ont permis de mettre en évidence deux cibles transcriptionnelles directes de XBP1s :

- L'induction de l'expression du long ARN non codant MIR22HG, précurseur du microARN miR-22-3p mature résulte d'une activation transcriptionnelle directe par XBP1s.
- L'induction du micro-ARN miR-148a-3p résulte d'une fixation de XBP1s au niveau de son promoteur putatif.

Nous avons également confirmé que ces deux micro-ARNs sont des médiateurs des effets anti-leucémiques de XBP1s :

- La surexpression de miR-22-3p, par des méthodes de transfections transitoires ou d'expression inductible *in vitro*, est responsable de l'induction de l'apoptose dans les cellules leucémiques. De plus, à des niveaux d'expression plus modérés, il est également responsable d'une induction de la différenciation myéloïde. Enfin, sa répression par des antagomiR est liée à une chimiorésistance dans les OciAML3.
- La surexpression de miR-148a-3p n'est pas lié à une modification des paramètres de survie ou de réponse au traitement. Cependant, il permet une induction de la différenciation myéloïde *in vitro* et *in vivo*.

Afin de caractériser au mieux la cascade moléculaire induite par XBP1s, nous avons recherché les cibles de ces micro-ARNs :

- miR-22-3p réprime l'expression de l'oncogène SIRT1, et va médier des phénotypes apoptotiques et induire une chimiosensibilisation à l'Aracytine. De plus, miR-22 est

également impliqué dans la répression de c-MYB, et permet une levée d'inhibition sur la différenciation des blastes leucémiques.

- miR-148a-3p réprime l'expression du facteur de transcription HEX, et induit lui-aussi un déblocage de la différenciation des blastes leucémiques.

I. Activation de XBP1s dans les Leucémies Aigües Myéloïdes

L'activation de l'UPR a déjà été rapporté dans différents types de leucémies, comme les Leucémies Myéloïdes Chroniques, exprimant la protéine de fusion BCR-ABL [359], ou encore dans les Leucémies Myéloïdes Promyélocytaires, exprimant la protéine de fusion PML-RARα [361]. Cependant, dans ces cas précis, l'activation de l'UPR résulte de l'activité de la protéine oncogénique. L'analyse clinique de Schardt généralise ces résultats, en détectant l'ARN épissé de XBP1 par RT-PCR compétitive dans 18% des patients atteints de LAM, et ce, indépendamment du sous-type de LAM considéré [362]. Une autre étude a également évalué l'expression de XBP1s dans 22 échantillons de LAM par PCR quantitative, et ici encore, les auteurs observent des clusters d'activation de XBP1s qui pourrait ségréger les patients en 3 sous-catégories : négatifs pour XBP1s, $XBP1s^{low}$ et $XBP1s^{high}$ [308]. Cependant, aucune corrélation entre l'expression de XBP1s et les différentes anomalies génétiques n'ont été réalisées pour cette dernière étude. Nous avons constitué une cohorte de 58 patients, en respectant la distribution des anomalies génétiques dans la population, et nous avons évalué l'expression de XBP1s par RT-qPCR. L'analyse des données cliniques, bientôt en notre possession, devrait nous permettre d'étudier plus particulièrement l'activation de XBP1s dans ces pathologies, mais à l'heure actuelle, la question de l'expression de XBP1s chez certains patients reste sans réponse. De plus, la détection de XBP1s dans notre cohorte corrèle avec l'expression de DNAJB9, une cible transcriptionnelle de XBP1s bien caractérisée (coefficient de Pearson = 0.79), qui sous-entend l'expression de la protéine XBP1s fonctionnelle. Associé aux données cliniques, nous pourrions corréler le pronostic de ces patients avec l'expression de XBP1s et confirmer les données de Schardt, afin de proposer XBP1s comme un biomarqueur prédictif de la réponse à la chimiothérapie conventionnelle.

Bien que XBP1s ne soit pas le seul marqueur utilisé pour mettre en évidence l'activation de l'UPR (CHOP, BiP, Calréticuline) dans l'étude de Schardt, l'activation des voies ATF6 et PERK n'est pas montrée directement. D'autres études ont par ailleurs relié l'activation de l'UPR à des effets bénéfiques dans la LAM ; le Bortézomib est actuellement

en essai clinique dans le traitement des LAM en rechute. Cependant, la plupart des inducteurs de l'UPR, comme des inhibiteurs de BiP (BMTP-78), ont montré des effets très délétères dans des modèles animaux [316]. L'activation spécifique d'une seule voie permettrait de limiter ces effets délétères. Dans ce contexte, nos résultats suggèrent que l'activation spécifique de XBP1s pourrait être bénéfique. De plus, des screening de molécules commencent à mettre en évidence des activateurs de IRE1, avec des effets plus spécifiques sur l'épissage de XBP1, qu'il serait intéressant de tester dans le cadre de la LAM [320,321]. Cependant, ces molécules en sont à une étape de caractérisation fonctionnelle et ne sont pas encore étudiées dans un contexte cancéreux.

II. XBP1s et stress génotoxique

Nos résultats montrent que l'expression spécifique de XBP1s induit l'apoptose dans différentes lignées de LAM. Jusqu'à présent, XBP1s était plutôt présenté comme un facteur pro-survie, associé à l'UPR adaptatif par l'activation transcriptionnelle de nombreuses chaperonnes, ou encore permettant une résistance à l'hypoxie [364]. Cette dichotomie peut s'expliquer par les différences de modèles utilisés. En effet, la plupart de ces études sont menées sur des modèles de perte de fonction de XBP1s, dans un contexte d'UPR généralisé. Nous nous sommes concentrés sur l'activation d'une seule voie, en l'absence d'un stress du RE global. La modulation de l'expression de XBP1s par les doses de doxycycline utilisées nous a cependant permis d'exprimer XBP1s à des taux comparables à ceux d'un stress du RE modéré, et induit par un traitement à la tunycamine ou la thapsigargine. De même que la tunycamine induit l'apoptose dans les cellules leucémiques, l'induction spécifique de XBP1s récapitule ces effets, à la fois *in vitro* et dans des contextes plus physiologiques de xénogreffes sous-cutanées. Classiquement, l'induction de l'apoptose par la voie IRE1/XBP1s a été attribuée à IRE1, par le biais de deux mécanismes : le RIDD, qui permet la dégradation de répresseurs de la caspase 2 et 8, mais également par l'activation de la voie JNK, qui active les protéines pro-apoptotiques BAX et BAK [365]. Cependant, nos résultats suggèrent que XBP1s pourraient également prendre part au processus d'UPR terminal, grâce entres autres, à l'activation transcriptionnelle de cibles non-codantes comme le micro-ARN-22.

De plus, nous avons pu observer que l'activation modérée de XBP1s sensibilise la lignée leucémique OciAML3 à l'aracytine. Dans la littérature, les traitements chimiothérapeutiques ont été rapporté comme des inducteurs du stress du RE [366,367]. Cependant, dans nos

modèles, nous n'observons aucune induction de l'UPR à la dose d'aracytine utilisée (résultats non montrés). En effet, la dose d'aracytine utilisée induit relativement peu d'apoptose, afin d'observer au mieux une potentialisation du traitement après induction de l'expression de XBP1s. Probablement pour cette raison, nous n'observons pas d'activation de la réponse UPR en réponse à l'aracytine. De façon intéressante, nous avons testé d'autres traitements de chimiothérapie tels que le bortézomib (inhibiteur du protéasome), la vinblastine (poison du fuseau mitotique), ou encore la staurosporine (inhibiteur pan-kinase), dont l'efficacité ne semble pas affectée par l'expression de XBP1s. En effet, ces résultats suggèrent une action de XBP1s spécifiquement sur les drogues génotoxiques *via* une modulation de la réponse aux dommages à l'ADN. Dans la littérature, XBP1s a effectivement été relié aux mécanismes de réparation de l'ADN. En effet, il induit l'expression d'un cluster de gènes impliqués dans les mécanismes de réparation de l'ADN (DNA Damage Response ou DDR) comme le gène suppresseur de tumeur BRCA1 ou encore RAD51 [354]. Chez la levure, il est directement impliqué dans la détection des cassures-doubles brins [353]. De plus, la perte de XBP1 est associée à une augmentation des foci γH2AX et a une diminution de l'activation des mécanismes de réparation, traduisant une incapacité de la cellule à détecter les éventuelles cassures de l'ADN [354]. Dans le cas de traitements chimiothérapeutiques induisant des dommages à l'ADN, l'augmentation de XBP1s pourrais permettre une meilleure détection des nombreuses cassures générées par le traitement, et de fait, l'induction de l'apoptose de par l'incapacité de la cellule à réparer l'ensemble de ces dommages. Dans ce sens, miR-22-3p apparait comme un candidat pertinent. Dans la littérature, plusieurs exemples démontrent l'implication de miR-22 dans la réponse aux dommages à l'ADN [368]. De façon générale, miR-22 induit une instabilité génomique : il réprime l'expression de MDC1 un composant du DDR (DNA Damages Repair), provoque une inhibition de la recombinaison homologue, et favorise l'entrée des cellules en sénescence [368]. Dans le Myélome Multiple, il est associé à la répression de la DNA ligase III, impliquée dans la survie des cellules cancéreuse [369].

Dans le cadre ses leucémies aiguës myéloïdes, miR-22 a été caractérisé comme un micro-ARN suppresseur de tumeurs ; l'étude d'un miRnome dans des cellules de patients a démontré que miR-22 est sous-exprimé de manière significative dans la LAM par rapport à des cellules issues de donneurs sains [170]. De plus, il est impliqué dans la répression d'oncogènes tels que FLT3, dont la mutation ITD (Internal Tandem Duplication) est présente dans 25% des LAM [170]. L'étude spécifique de l'axe XBP1s/miR-22-3p dans ce sous-type de LAM de mauvais pronostic semble donc particulièrement pertinent. D'après nos résultats,

l'expression de XBP1s dans des lignées cellulaires porteuses de la mutation FLT3-ITD, comme les MOLM-14 ou les MV4-11, induit également de l'apoptose. Il serait intéressant de regarder l'impact de l'activation de XBP1s sur l'expression de FLT3, associée éventuellement avec des inhibiteurs, comme l'AC220.

Actuellement, l'intérêt clinique des ARN non codant se situe dans le développement de signatures diagnostiques et pronostiques. En effet, les micro-ARNs sont susceptibles d'être détectés dans les fluides corporels, tels que la salive, le sérum ou les urines, et sont sécrétés sous forme d'exosomes [370]. L'étude du profil des exosomes a révélé que miR-22 fait partie des micro-ARNs circulants les plus représentés à travers différents séquençages réalisés à partir d'échantillons de sérums humains [371]. De plus, miR-22 a déjà montré un intérêt de biomarqueur aussi bien diagnostique que prédictif de la réponse au traitement. L'augmentation des taux sériques de miR-22 est associé au développement des cancers de l'œsophage et du pancréas [372,373]. Dans le cancer du poumon non à petites cellules, l'augmentation de miR-22 a été corrélé à une résistance au Premetrexed ainsi qu'à une agressivité accru de la maladie [374]. Plus récemment, miR-22-3p a été quantifié dans le sérum de patient atteint de LAM. Les auteurs observent une diminution des taux sériques de miR-22 en cas de maladie. De plus, les taux les plus faibles corrèlent avec les formes de moins bon pronostic. Après chimiothérapie, le ratio s'inverse : les patients avec des taux sérique élevé de miR-22 sont statistiquement les patients qui atteignent la rémission complète [375]. Dans le cas de l'activation de XBP1s, miR-22 pourrait donc être le reflet de l'expression de XBP1s dans les cellules leucémiques, et servir de biomarqueur prédictif de réponse à la chimiothérapie. Pour l'heure, nous avons corrélé l'expression de MIR22HG à celle de XBP1s dans des cellules de patients, et bien que la corrélation soit positive, il faudrait également réaliser ces corrélations pour le micro-ARN mature.

Jusqu'à présent, miR-22-3p, et plus particulièrement MIR22HG, n'a jamais été décrit comme une cible directe de XBP1s. Certaines publications le décrivent comme un micro-ARN exprimé suite à un stress [376], mais jamais comme un acteur de l'UPR. De façon intéressante, nous avons également observé une très bonne corrélation entre l'expression de XBP1s et MIR22HG dans notre cohorte, corroborant ainsi nos résultats *in vitro*. Cependant, nous avons considéré MIR22HG uniquement comme un précurseur de miR-22-3p mature, et c'est cette fonction que l'on retrouve principalement dans la littérature. De plus en plus d'étude s'intéressent à MIR22HG en tant que lncARN. Il apparaît que MIR22HG a été caractérisé comme un compétiteur endogène ou ecRNA (pour endogenous competitor RNA) : en effet, il est décrit comme une éponge à petits ARNs, et cible miR-24-3p, miR-10-

5p et miR-141-3p, conduisant à l'inhibition de la croissance tumorale dans les cancers de la thyroïde, du foi et du carcinome endométrial [377,378,379]. De plus, il interagit avec différentes protéines comme l'oncogène HuR et inhibe la transcription d'oncogènes tels que la β-caténine [380]. Pour toutes ces raisons, MIR22HG est considéré comme un lncARN suppresseur de tumeur et pourrait être également impliqué dans les effets anti-leucémique de XBP1s.

Plus généralement, l'analyse combinée du ChIP-sequencing et du RNA-sequencing a révélé une liste de lncARN directement activés par XBP1s. Or, si les micro-ARNs régulés par XBP1s sont rares, il n'existe à l'heure actuelle aucun lncARN publié en tant que cible transcriptionnelle directe de XBP1s. Cette catégorie d'ARN non codants commence à être étudié dans le cadre des cancers et certains sont mêmes utilisés comme biomarqueur diagnostique, à l'image de PCA3 dans le cancer de la prostate [381]. Dans la LAM, ils ont été relié aux processus de différenciation, survie, et réponse au traitement [382]. L'étude des lncARN régulés par XBP1s pourrait donc représenter une approche novatrice et implémenter nos connaissances sur les effecteurs non codants de cette voie.

La recherche des cibles de miR-22, parmi celles déjà connues, a permis de mettre en évidence une interaction directe entre ce microARN et le messager codant la sirtuine 1. Dans nos modèles, la surexpression de miR-22-3p inhibe l'expression de SIRT1, au niveau protéique. A l'inverse, la transfection d'un antagomiR-22 restaure le niveau protéique de SIRT1. La sirtuine 1 (SIRT1) est une déacétylase nucléaire de type III, d'abord caractérisée en tant qu'histone déacétylase, mais de nombreuses cibles différentes des histones sont aujourd'hui décrites, comme des facteurs de transcription (p53, Ku70, FOXO…)[383,384,385]. SIRT1 a été majoritairement décrite comme un facteur de réponse aux dommages à l'ADN. Elle permet une déacétylation des histones au point de cassure, et engendre ainsi une compaction locale de la chromatine. Elle déacétyle également des facteurs impliqués dans la réparation de ces cassures, et permet d'augmenter leur efficacité [386]. Dans les neurones, SIRT1 a été montrée comme interagissant physiquement avec les acteurs de la réparation notamment la HDAC de classe I HDAC1, favorise et stabilise son recrutement au site de cassures doubles brins [387]. De façon intéressante, l'inhibition de SIRT1 par interférence à l'ARN dans nos modèles induit de l'apoptose. Cependant, l'inhibition pharmacologique de SIRT1 par un traitement à l'EX-527 n'induit pas d'apoptose, mais potentialise l'effet d'un traitement à l'aracytine. Cette différence de phénotype peut s'expliquer par le fait que la transfection de siARN provoque la disparition physique de la protéine SIRT1, quand un

inhibiteur réduit seulement son activité catalytique, laissant sous-entendre la possibilité de partenaires protéiques impliqués, non-déterminés à ce jour.

De façon générale, la sirtuine 1 permet la survie des cellules en favorisant la réparation de l'ADN. Dans un contexte physiologique, elle est essentielle au maintien de l'intégrité du génome, mais dans le cadre de la LAM, elle a été retrouvé surexprimée et caractérisée comme un oncogène, à nouveau dans les LAM FLT3-ITD, ce qui confirme l'intérêt de l'étude de l'axe entier XBP1s/miR-22-3p/SIRT1 dans ce sous-type de LAM [388]. Nous avons montré que l'inhibition pharmacologique de SIRT1 par le EX-527 potentialise les effets de l'aracytine. L'utilisation de l'EX-527 nous permet d'être plus spécifique dans l'inhibition de SIRT1. En effet, chez les mammifères, 7 protéines sirtuines ont été décrites, et présentent de fortes homologies. Bien que leur expression soit tissu-spécifique, voire même organite-dépendant (les SIRT3-4-5 sont mitochondriales), la forte homologie entre les différentes isoformes rend difficile le ciblage de l'une d'entre elles. Une précédente étude montrait déjà que l'inhibition pharmacologique de SIRT1 avait un effet bénéfique sur un traitement à l'aracytine. Cependant, l'inhibiteur utilisé, le Tv-6, est également décrit comme un inhibiteur de SIRT2. L'avantage du EX-527 réside également dans son utilisation sur des modèles murins. En effet, cet inhibiteur n'a montré que de très faibles toxicités annexes, bien que les injections dans les modèle murins n'aient pas été faites dans la circulation générale mais par voie intrathécale [389]. Il semble donc important de récapituler les effets observés *in vitro* par des expériences *in vivo* associant un traitement EX-527 à l'aracytine, bien que les méthodes d'administration de l'inhibiteur soient encore à mettre en place.

III. XBP1s et différenciation myéloïde

L'implication de XBP1s dans l'hématopoïèse normale n'est plus à démontrer : il est nécessaire à la lymphopoïèse B, puisqu'impliqué dans la différenciation et la maturation des lymphocyte B. Il est également crucial dans la myélopoïèse, plus particulièrement dans la maturation des éosinophiles et des cellules dendritiques [291,297,356]. De façon surprenante, son étude dans les hémopathies malignes reste quasi-inexistante, mis à part les pathologies touchant les lymphocytes B comme le Myélome Multiple.

Nous avons constaté que l'expression modérée mais chronique de XBP1s dans des lignées cellulaires de LAM permettait une progression des cellules leucémiques dans la différenciation, marquée par l'augmentation de l'expression du marqueur de différenciation pan-myéloïde CD11b, et ceci aussi bien *in vitro* qu'*in vivo*. Cette augmentation du marqueur

membranaire CD11b s'accompagne *in vitro* de variations morphologiques caractéristiques, comme l'acquisition d'un noyau poly-lobulaire, un cytoplasme plus important, une forte densité vésiculaire ainsi que des excroissances membranaires. Cependant, ces seules informations ne suffisent pas à caractériser les cellules obtenues. La différenciation monocytaire est également marquée par l'expression de facteurs de transcriptions caractéristiques, comme PU.1 et IRF8, qui augmentent, et CEBPα, qui diminue [50,83]. L'évaluation de l'expression de ces FT lors d'une activation de XBP1s pourrait donner des indications quant au stade de différenciation atteint. De façon intéressante, une étude révèle que l'expression de XBP1s est augmentée dès le stade GMP (Granulocyte Monocyte Progenitor), donc très en amont de la différenciation terminale [297]. Nos résultats suggèrent également que l'implication de XBP1s pourrait intervenir plus précocement dans ce processus. L'utilisation de la lignée cellulaire HL-60, caractérisée comme une LAM de type M2, c'est-dire bloquée au stade GMP, semble confirmer cette hypothèse. En effet, dans cette lignée également, la surexpression de XBP1s induit une augmentation du marquage CD11b. Cependant, l'utilisation de lignées cellulaires ne constituent pas une preuve suffisamment robuste pour conclure sur ce point.

In vitro, l'activation de XBP1s augmente les effets de la Vitamine D3 pour les lignées OciAML2 et OciAML3. A l'image des traitements à l'aracytine, les doses utilisées pour ces expériences sont très faibles, afin de mettre en évidence une potentialisation ou un effet synergique. Dans le cadre des thérapies par différenciation dans la LAM, la VitaminD3 a été très largement étudié, hors LAP. Cependant, ce traitement a montré de forte toxicité, due à une hypercalcémie sanguine, engageant parfois le pronostic vital des patients. Le problème majeur soulevé dans ce cas est la dose administrée, qui est supra-physiologique [390]. L'expression de XBP1s pourrait donc représenter un facteur de tolérance accru à la vitamine D3, qui permettrait de diminuer les doses, jusqu'à un seuil bien plus tolérable par les patients.

Les xénogreffes des modèles OciAML2 et OciAML3 ont également permis de montrer que l'induction de XBP1s permet d'augmenter spontanément l'expression du marqueur CD11b *in vivo*, à la différence des cellules *in vitro* qui nécessitent l'ajout d'un agent différenciant comme la vitamine D3 ou le DMSO. Cependant, ces analyses ne sont valables que pour le compartiment médullaire, et ne sont pas généralisables aux cellules circulantes ainsi qu'aux cellules de la rate (résultats non montrés). Ces résultats suggèrent l'importance du microenvironnement médullaire dans la réponse médiée par XBP1s, microenvironnement pourtant très pauvre dans le contexte de la souris NSG. En effet, ces

souris sont caractérisées par une immunodéficience très forte, sans lymphocytes B, T, NK, et très peu de macrophages et de cellules dendritiques. Elles sont également marquées par une déficience en cytokines, étant mutées pour le récepteur IL2Rγ [391]. Cependant, les contacts intercellulaires avec les cellules de la moelle osseuse, comme les précurseurs osseux ou les cellules endothéliales, ont été montrées comme impliqué dans la différenciation hématopoïétique, grâce à l'activation de la voie Notch notamment [28,29]. Il serait intéressant de phénotyper plus précisément les cellules greffées résidant dans la moelle, ce qui permettrait d'apporter des éléments de réponse à cette dichotomie entre la moelle et le reste des organes hématopoïétiques.

L'analyse du miRnome et du ChIP-sequencing réalisé sur les OciAML3 a mis en évidence une activation transcriptionnelle directe de miR-148a-3p. Ce micro-ARN a déjà été décrit comme cible directe de XBP1s dans un modèle de différenciation adipocytaire murin [272], et nos résultats confirment cette régulation dans un modèle humain de LAM. Contrairement à miR-22-3p, issu d'un intron de MIR22HG, miR-148a-3p possède sa propre unité transcriptionnelle. Il est produit avec son complément, miR-148a-5p, que l'on retrouve également augmenté par XBP1s dans les analyses de miRnome, et dans les mêmes taux que miR-148a-3p. Cependant, le promoteur de miR-148a n'a jamais été caractérisé à proprement parler. Les analyses de ChIP révèlent une fixation de XBP1s sur un promoteur putatif situé à environ 1000 paires de base. Il serait intéressant de cloner cette séquence et d'utiliser un système rapporteur « promoter-less » afin de conclure sur la capacité promotrice de cette séquence.

Dans la littérature, miR-148a-3p a été majoritairement relié à des processus de différenciation. Dans la différenciation des adipocytes et des plasmocytes, il réprime l'expression de facteurs pro-apoptotiques tels que PTEN et BIM, et promeut la différenciation *via* la répression de BACH2 et MITF [272,392]. Enfin, plus récemment, miR-148a-3p a été relié à la différenciation des macrophages, ainsi qu'à leur polarisation en macrophage de type M1 pro-inflammatoire [276]. De façon intéressante, l'expression de miR-148a-3p est diminuée dans les patients atteint de LAM, et il est associé à une meilleure survie sans rechute spécifiquement dans les groupes de LAM de haut risque et de risque intermédiaire [393]. MiR-148a-3p apparaît alors comme un micro-ARN suppresseur de tumeur dans les LAM, mais pas seulement [394]. Il inhibe également la prolifération des cellules cancéreuses par la répression de DNMT1 dans le cancer de l'œsophage [395] et il est impliqué dans la suppression de la transition épithélio-mésenchymateuse dans le cancer pancréatique, par l'inhibition de Wnt1 [396]. Nos résultats confirment l'effet suppresseur de

tumeur de miR-148a-3p dans un contexte de LAM. En effet, miR-148a-3p est un médiateur des effets différenciant de XBP1s et participe à un déblocage de la différenciation myéloïde *in vitro* et *in vivo*. Par ailleurs, l'analyse de la composition de la moelle osseuse des souris xénogreffées montrent une diminution des cellules cancéreuses suite à la surexpression de miR-148a-3p. Il serait intéressant de conclure si cette différence de cellularité induit une amélioration de la survie des souris.

Afin de déterminer les cibles impliquées dans cette cascade moléculaire, nous avons réalisés des analyses *in silico*. Ces analyses nous ont conduits à nous intéresser à HEX (ou HHEX pour Hematopoietically-expressed Homeobox). L'interaction entre miR-148a-3p et HEX a déjà été rapportée dans 2 modèles différents de cellules β pancréatiques et des HeLa. Cependant, bien que nous ayons confirmé la diminution de HEX après induction de XBP1s et miR-148a-3p par western blot, il nous faut encore conclure sur l'aspect direct ou indirect de cette interaction dans nos modèles grâce à l'utilisation d'un système rapporteur tel que l'ARNm de la luciférase couplée au 3'UTR de HEX. De plus, nous devons confirmer qu'une partie des effets observés sur la différenciation sont bien dus à la répression de HEX. Pour cela, nous pourrons utiliser des antagomiR-148a-3p, à transfecter directement dans les cellules et observer les effets sur l'expression de HEX. Il est également envisageable d'utiliser un nouveau modèle d'expression inductible permettant la surexpression d'un miR-148a-3p muté dans le « seed ». L'ensemble de ces éléments nous permettra de confirmer l'axe de régulation XBP1s/miR-148a-3p/HEX dans un contexte de LAM.

HEX est un facteur de transcription initialement caractérisé dans le compartiment hématopoïétique [397], mais rapidement relié à un rôle crucial dans la mise en place des tissus endodermiques au cours du développement embryonnaire. Le KO de HEX est létal à E10.5, dû à des défauts de développement de l'encéphale, de la thyroïde et du foie [398]. Dans le système hématopoïétique, HEX est fortement exprimé dans les CSH et les progéniteurs multipotents, et son expression diminue au cours de la différenciation hématopoïétique, particulièrement dans les érythrocytes et les lymphocytes T [399]. En effet, l'activation soutenue de HEX dans les thymocytes (précurseurs de lymphocyte T thymiques) suffit à induire un lymphome [400]. Dans la moelle, la surexpression de HEX dans des CSH provoque un blocage de la différenciation myéloïde, et suffit à induire une leucémie promyélocytaire dans un modèle murin (LAM de type M2) [401]. Il est même impliqué dans une translocation chromosomique rare dans les LAM, la translocation t(10 ;11)(q23;p15), qui provoque l'expression de la protéine de fusion NUP98/HEX. Dans ce cas, la protéine Nucléoporin98 est fusionnée avec le domaine de liaison à l'ADN de HEX. A l'image de la surexpression de

HEX, l'expression de la protéine de fusion conduit au blocage de la différenciation myéloïde associé à des capacités d'auto-renouvèlement aberrantes. Plusieurs études ont également relié l'expression de HEX aux capacités souches des CSH : la perte de HEX est liée à une diminution des capacités de repeuplement dans des expériences de transplantations en séries [402,403]. L'ensemble de ces éléments tendent à laisser penser que la répression de HEX par l'axe XBP1s/miR-148a-3p pourrait donc être particulièrement intéressante à étudier dans les CILs (Cellules Initiatrices de Leucémie) ; dans le cas de la LAM, ces cellules souches cancéreuses sont suffisantes à initier la maladie lorsqu'elles sont transplantées dans des souris immunodéficientes. Elles sont décrites comme le pendant cancéreux des CSH ; ce sont des cellules quiescentes, donc résistantes à la chimiothérapie, avec des capacités d'auto-renouvèlement, ainsi que des capacités de différentiation partielle [404]. L'activation de XBP1s/miR-148a-3p pourrait donc affecter ces cellules dormantes, en diminuant HEX, affectant ainsi leurs capacités d'auto-renouvèlement.

A nouveau, nous avons mis en évidence l'implication miR-22 dans la différenciation médiée par XBP1s, soulignant ici l'importance particulière de ce micro-ARN dans les phénotypes liés à XBP1s. Par ailleurs, miR-22 a été associé à la différenciation hématopoïétique, et plus particulièrement la différenciation myéloïde. En effet, l'expression de miR-22 est augmentée lors de la différenciation des monocytes, par le facteur de transcription PU.1 [405].

A l'image de miR-148a-3p, nous avons généré un modèle miR-22 inductible dans les lignées OciAML3, OciAML2 et HL-60. Cependant, la caractérisation des phénotypes liés à la surexpression de miR-22 étant plus avancé dans le modèle OciAML2, elle est la seule lignée présentée pour cet axe. De façon concordante avec les transfections réalisées sur le OciAML3, l'induction de miR-22-3p induit de l'apoptose dans la lignée OciAML2. De plus, la surexpression de miR-22 entraîne une potentialisation du traitement à la Vitamine D3. De la même manière que pour les traitements à l'aracytine, il serait intéressant de transfecter un antagomiR-22 afin d'observer l'impact de la répression de miR-22 sur la différenciation voir sur l'apoptose.

Nous avons relié l'expression de miR-22-3p à la répression du facteur proto-oncogénique c-MYB. Cependant, nous n'avons pas encore déterminé la nature de l'interaction entre miR-22 et c-MYB. Une précédente étude corrèle l'expression de miR-22-3p à la diminution de l'ARNm de c-MYB dans des cas de cancers ovariens [406]. Cependant, l'analyse par RT-qPCR de l'expression de c-MYB dans les différents modèles ne montre pas de variation de son ARNm, alors sue son niveau protéique diminue en western blot, ce

qui suggère une inhibition de sa traduction. Afin de verrouiller notre démonstration, il nous faudra utiliser les mêmes outils que pour la validation de miR-148a-3p, c'est-à-dire un système rapporteur de type luciférase couplé au 3'UTR de c-MYB afin de déterminer si cette liaison est directe ou pas, et de préférence, réaliser ce type de vérification dans nos trois modèles.

C-MYB est un facteur de transcription, exprimé majoritairement dans le système hématopoïétique [59]. Une activation constitutive de c-MYB provoque un blocage des cellules hématopoïétiques à un stade de progéniteurs. A l'inverse, une diminution de c-MYB active la différenciation monocytaire, au détriment des érythrocytes et des lymphocytes [60]. En effet, c-MYB engendre une répression de PU.1, et de fait bloque spécifiquement la différenciation granulo-monocytaire [161]. C-MYB a également été montré comme un activateur transcriptionnel de FLT3, suggérant à nouveau que l'étude de l'activation de l'axe XBP1s/miR-22 dans les LAM FLT3 mutée serait particulièrement porteuse. De plus, c-MYB n'est pas seulement un répresseur de la différenciation myéloïde. Il est également impliqué dans la survie et la prolifération des progéniteurs hématopoïétiques [59]. La diminution de c-MYB suite à l'activation de l'axe XBP1s/miR-22 pourrait donc être un des mécanismes mis en jeu à la fois dans l'induction de l'apoptose mais également dans l'induction de la différenciation.

IV. Limites et perspectives

L'ensemble de nos résultats confirment les données cliniques observées par Schardt *et. al.* Nous avons développé des outils moléculaires qui nous ont permis d'évaluer l'impact de l'expression de XBP1s *in vitro*, mais également de confirmer les phénotypes observés dans des modèles de xénogreffes orthotopiques. Cependant, la caractérisation complète des axes XBP1s/miR-22/c-MYB ainsi que XBP1s/miR-148a-3p/HEX n'est pas totalement terminée. Les régulations des micro-ARNs sur leurs cibles présumées doivent être encore investiguées, notamment pour conclure sur l'aspect direct ou indirect de ces régulations. Dans le cas de l'axe XBP1s/miR-22/c-MYB, la caractérisation n'a été réalisée que sur les OciAML2, et doit être généralisée à d'autres modèles cellulaires, ainsi que sur des expériences *in vivo*. De plus, la chimiosensibilisation médiée par XBP1s n'a pu jusqu'à

présent être étudié que dans la lignée OciAML3, qui est la seule lignée chimiorésistante *in vitro* et *in vivo*. Il existe cependant des lignées de LAM, comme les HL-60, connues pour être chimiorésistante dans un contexte *in vivo,* qui pourrait venir confirmer l'effet de XBP1s sur la réponse au traitement [407].

De façon générale, l'utilisation de lignées cellulaires constituent une première limite dans notre travail. Bien que nous nous soyons attachés à généraliser nos résultats dans différentes lignées, il serait intéressant de travailler sur des cellules primaires issues de patient *ex vivo*, et moduler l'expression de XBP1s directement dans les blastes. Les cellules immunitaires, et particulièrement les cellules myéloïdes primaires, sont connues pour être difficile à transfecter [408]. Cependant, de plus en plus de stratégies de nucléofection permettent d'envisager la transfection d'ADN plasmidique, voire directement d'ARNm [409,410]. De telles techniques permettraient d'augmenter l'expression de XBP1s dans des cellules primaires, et d'observer les effets sur l'apoptose, la réponse au traitement et la différenciation, ainsi que sur les cascades d'activation en aval de XBP1s, et que nous avons pu mettre en évidence au cours de ces travaux. De plus, la comparaison de l'expression de XBP1s entre les échantillons d'un même patient, au diagnostic et à la rechute pourrait également représenter un bon indicateur de la sensibilité de ces cellules à la chimiothérapie.

L'activation de XBP1s dans les cellules leucémiques nous a permis de mettre en évidence les effets pléiotropiques de ce facteur de transcription, effets qui suggèrent que XBP1s est un suppresseur de tumeur dans la LAM : il inhibe la survie des cellules blastiques, va permettre une meilleure réponse au traitement, et va également promouvoir leur avancement dans la différenciation myéloïde. Dans le cadre de la recherche de thérapie contre la LAM, l'activation de cette voie semble donc prometteuse. Cependant, le ciblage moléculaire des différents effecteurs, y-compris XBP1s lui-même, reste à l'heure actuelle, très compliqué. En effet, activer XBP1s *in cellulo* nécessite l'activation de IRE1 en amont. Bien que quelques molécules soient caractérisées comme activatrices de IRE1, les effets sur l'épissage de XBP1s sont toujours relativement modérés et nécessitent une dimérisation de la RNase en amont. De même, l'utilisation de thérapies basées sur les micro-ARNs sont encore à ce jour à un stade expérimental. Nous avons cependant mis en évidence que l'utilisation de l'EX-527, un inhibiteur hautement spécifique de SIRT1, permet de récapituler la sensibilisation à l'aracytine. De plus, cette molécule est déjà testée sur des patients, dans le cadre d'essai clinique pour la maladie de Huntington et présente très peu d'effet secondaire, même aux doses les plus élevée [411,412]. C-MYB a également été proposé comme cible thérapeutique dans les LAM. En effet, il apparaît comme une cible

thérapeutique de choix : il est majoritairement exprimé dans le compartiment hématopoïétique et, bien que souvent retrouvé surexprimé dans cette pathologie, les mutations l'affectant restent très rares. Enfin, il est à la fois impliqué dans le blocage de la différenciation, la prolifération et la survie des cellules cancéreuses. L'utilisation du mebendazole, un antiparasitaire, provoque la dégradation de c-MYB par le protéasome. Non seulement, cette molécule est bien tolérée, mais elle montre une grande spécificité d'action dans les blastes par rapport à des cellules hématopoïétiques issues de sang de cordon *in vitro* tout en améliorant significativement la survie de souris xénogreffées [413].

CONCLUSION

Les Leucémie Aigües Myéloïdes sont des hémopathies malignes génétiquement très hétérogènes mais caractérisées par 3 grandes « hallmarks » : un blocage de la différenciation des cellules cancéreuses, une survie anormale de ces progéniteurs malins et également une résistance à la chimiothérapie dans les cas de rechute. Par l'utilisation d'un modèle gain-de-fonction, permettant l'expression inductible de XBP1s dans des lignées cellulaires de LAM, j'ai montré que l'activation spécifique de cette voie permet de cibler ces trois grandes caractéristiques. Une activation aigüe de XBP1s conduit à l'induction de l'apoptose. De plus, une activation plus modérée permet non seulement de rétablir une chimiosensibilité à l'aracytine, mais également de promouvoir la différenciation myéloïde. Ces résultats ont été établi dans des modèles *in vitro* mais également dans des modèles plus physiologiques de xénogreffes orthotopiques. Ils démontrent un rôle anti-leucémique de XBP1s et représentent une première indication fonctionnelle permettant d'expliquer la corrélation entre l'expression de XBP1 et un bon pronostic chez les patients atteints de LAM, quand la plupart des études lui attribue un rôle pro-tumoral. De plus, ces résultats permettent de lier XBP1s à l'hématopoïèse cancéreuse, et non plus seulement à l'hématopoïèse générale.

Afin de caractériser la cascade moléculaire mis en jeu, nous nous sommes concentrés sur les cibles non-codantes de XBP1s, qui restent malgré tout assez peu caractérisées. Nous avons pu mettre en évidence une nouvelle cible transcriptionnelle directe, le lncARN MIR22HG, précurseur du micro-ARN miR-22-3p. Nous avons également confirmé l'activation transcriptionnelle directe du micro-ARN miR-148a-3p. Enfin, à travers l'étude des phénotypes liés à l'expression de ces micro-ARNs, nous avons pu mettre en évidence l'importance qu'occupe ces cibles non codantes dans les effets liés à XBP1s.

L'ensemble de ces résultats sont un premier pas dans la compréhension des effets de l'activation de XBP1s dans les LAM. De plus, ils permettront également de proposer de nouvelles pistes de biomarqueurs, voire de cibles thérapeutiques, nécessaire à l'amélioration de la prise en charge de cette pathologie qui demeure, pour certains patients, dans une impasse thérapeutique.

ANNEXES

cancers

MDPI

The PERK Branch of the Unfolded Protein Response Promotes DLL4 Expression by Activating an Alternative Translation Mechanism

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Abstract: Delta-like 4 (DLL4) is a pivotal endothelium specific Notch ligand that has been shown to function as a regulating factor during physiological and pathological angiogenesis. DLL4 functionsas a negative regulator of angiogenic branching and sprouting. Interestingly, *Dll4* is with *Vegf-a* one of the few examples of haplo-insufficiency, resulting in obvious vascular abnormalities and in embryonic lethality. These striking phenotypes are a proof of concept of the crucial role played by the bioavailability of VEGF and DLL4 during vessel patterning and that there must be a very fine-tuning of DLL4 expression level. However, to date the expression regulation of this factor was poorly studied. In this study, we showed that the *DLL4* 5′-UTR harbors an Internal Ribosomal EntrySite (IRES) that, in contrast to cap-dependent translation, was efficiently utilized in cells subjected to several stresses including hypoxia and endoplasmic reticulum stress (ER stress). We identified PERK,a kinase activated by ER stress, as the driver of *DLL4* IRES-mediated translation, and hnRNP-A1 asan IRES-Trans-Acting Factor (ITAF) participating in the IRES-dependent translation of DLL4 duringendoplasmic reticulum stress. The presence of a stress responsive internal ribosome entry site in the DLL4 msRNA suggests that the process of alternative translation initiation, by controlling the expression of this factor, could have a crucial role in the control of endothelial tip cell function.

Keywords: DLL4 (delta like ligand 4); angiogenesis; IRES (internal ribosome entry site); hypoxia; endoplasmic reticulum stress; UPR (unfolded protein response); PERK (PKR-Like endoplasmic reticulum kinase)

1. Introduction

Coordinately expressed and regulated genes control most physiological processes. This is the case for angiogenesis, the process of the expansion of existing blood vessel mainly by sprouting newbranches from pre-existing blood vessels and leading to the outgrowth of new capillaries to form a new functional vascular network.

Angiogenesis is critical for many physiological processes such as embryonic development, wound healing, or vessel penetration into avascular regions and in pathological states including retinopathy,chronic inflammatory disorders (e.g., psoriasis or rheumatoid arthritis) and of course solid tumor

development. Angiogenesis depends on the highly coordinated actions of a variety of pro-angiogenic regulators, the most prominent and best characterized being Vascular Endothelial Growth FactorA (VEGF-A) and Fibroblast Growth Factor 2 (FGF-2) which were among the first pro-angiogenic molecules to be identified [1].

Among the stimuli responsible for the up-regulation of pro-angiogenic factors, hypoxia has beenof particular interest because of its role in cancer progression. It is clearly established that VEGF-A and FGF-2 are strongly expressed in hypoxic tissues, allowing the recruitment of new blood vessels from surrounding territories to ensure their needs of oxygen and nutrients. Hypoxia contributes to angiogenesis by transcriptionally activating several angiogenic factors as well as their receptors thus facilitating the recruitment of endothelial cells to the site of hypoxia, but VEGF-A and FGF-2 expressionis also up-regulated under hypoxic conditions [2,3] and after ER stress [4] through Internal Ribosome Entry Site (IRES)-mediated translation [5]. After tissue reoxygenation, the expression of angiogenic factors decreases. This elegant negative feedback mechanism is a key event in the regulation of blood vessel growth.

However, the vascular response to angiogenic factors is also dependent on other regulatory mechanisms. Several studies demonstrate that one such regulator essential for tumor neovascularization is the NOTCH ligand DLL4, which is one of three delta-like ligands in the mammalian genome [6]. DLL4 is an important component of the NOTCH pathway, which is critical for embryonic vascular development and arterial specification. It is weakly expressed in adult tissues, but markedly induced in murine and human tumor vasculature [7–9]. Using different experimental models and a variety of genetic and pharmacologic approaches, several studies report that the DLL4/NOTCH pathway is a critical negative regulator of tumor angiogenesis, acting to restrain excessive VEGF-induced vascular sprouting and angiogenesis. The DLL4 pathway regulates sprouting and branching behaviors by influencing the formation of vascular 'tip cells'—specialized endothelial cells at the leading edge of vascular sprouts [10–16]. The tip cells which express high levels of VEGFR-2, VEGFR-3 and platelet-derived growth factor-B, are characterized by their cellular protrusions or filopodia that sense the local environment and migratory behavior to guide the growth of new blood vessels towards the source of angiogenic growth factors [17]. VEGFR2 signaling induces the expression of the Notch ligand DLL4 on the surface of the tip cell membrane allowing the suppression of tip-cell features in adjacent stalk cells via DLL4/NOTCH-mediated lateral inhibition [18]. Then the endothelial stalk cells follow the polarized migration of tip cells and proliferate in order to form new blood vessels.

During this process, endothelial cells are exposed to gradient of oxygen [19]. Indeed, since lumen formation of new vessel has not occurred, blood flow is not present, thus endothelial cells from these new blood vessels are under hypoxia, especially the tip cells due to their position furthest away from the circulating blood. It was demonstrated that *Dll4* haploinsufficiency causes embryonic lethality and reduces tumor growth due to defects in the development of the vasculature [7,20]. These striking phenotypes resulting from heterozygous deletion of *Dll4* indicate that vascular development may be very sensitive to subtle changes in *Dll4* expression. Interestingly VEGF-A and DLL4 are the only proteins for which the heterozygosity results in a lethal embryonic phenotype and obvious vascular abnormalities, highlighting the essential and unique role of both proteins during angiogenesis [7,21,22]. Furthermore, several data indicate that VEGF-A and DLL4 are coordinately expressed and that VEGF activates DLL4 expression via VEGFR2 signaling [15,18,23,24]. Moreover, in vitro, hypoxia caninduce transcription activation of both *Vegf-a* and *Dll4* in endothelial cells [25]. Finally, endothelial expression of DLL4 was demonstrated to be significantly associated with VEGF-A in many cancers including glioma, colon, nasopharyngeal and lung cancers [26–29]. Taken together, these data indicate a potential co-regulation of these two genes.

It is well known that DLL4 expression is regulated by transcriptional and post-transcriptional (i.e., 3′-end processing) mechanisms, but the translational regulation of DLL4 messenger has notyet been studied. In this study, we sought to further investigate the role of stress responses in DLL4 expression regulation. We have identified an Internal Ribosome Entry Site (IRES) in the 5′-UTR of DLL4

mRNA which is activated under hypoxic and ER stress conditions. Further, we have identified PKR-like ER-associated protein kinase (PERK), a kinase activated during ER stress which phosphorylates the eIF2α subunit and impairs the generation of the ternary complex Met-tRNAi-eIF2-GTP, as the mainregulator of DLL4 IRES-mediated translation and hnRNPA1 as an IRES Trans Acting Factor regulating DLL4 IRES-mediated translation during stress.

2. Results

2.1. The Human DLL4 Transcript Contains a Functional IRES Which is Active in Different Cell Types

Strong conservation of non-coding exonic sequences during vertebrates' evolution often means involvement in post-transcriptional regulation of gene expression [30]. Interestingly, *DLL4* 5′-UTR shows fairly high conservation, with more than 70% mRNA sequence identities between 14 mammal species (Figure S1A). This indicates that this non-coding region may contain functional RNA structuresor regulatory sequences important for the translation of the *DLL4* mRNA.

Indeed, according to the prediction, the 320-nucleotide-long human *DLL4* 5′-UTR is highly structured (Figure S1B) due to a high percentage of G and C residues (more than 70%). Analysis by the MFold prediction algorithm revealed that the full human *DLL4* 5′-UTR form a free energy structure of less than -150 kcal/mol, but also that the first 85 bases of the human DLL4 mRNA might form a very stable secondary structure ($\Delta G = -37.2$ kcal/mol) (Figure S1B). Secondary structural features of the mRNA 5′-untranslated region (UTR) are important for translational regulation by affectingthe recruitment and positioning of the ribosome at a favorable initiation codon [31,32], and both thermal stability and cap-to-hairpin proximity affect translational efficiency, particularly when the predicted hairpin stability is below -25 kcal/mol [33]. These structural elements act as strong barriersto scanning ribosomes in the 5′-UTR of mRNAs and are incompatible with the conventional scanninginitiation model. In general, the corresponding mRNAs have evolved nonconventional mechanisms to initiate translation, including IRES elements, which are specialized RNA regulatory sequences governing cap-independent translational initiation. A typical example is the Ornithine Decarboxylase(ODC) mRNA, which possesses, in the cap proximal part, a very stable stem-loop structure highly inhibitory of cap-dependent translation [34] but also an IRES element allowing an efficient translationof this mRNA [35]. Thus, we first investigated whether the *DLL4* 5′-UTR contained any IRES activity. In this aim, a classical bicistronic reporter plasmid was constructed by the insertion of a cDNA corresponding to the *DLL4* 5′-UTR (nts 1 to 320) between two reporter gene sequences, the first encoding Renilla Luciferase which is strictly dependent upon cap-dependent translation, and the second encoding Firefly Luciferase which is dependent upon the presence of an IRES for its translation (Figure 1A) [36,37]. Twenty-four hours after transfection into HUVEC, HeLa and NIH-3T3 cells, Renilla and Firefly activities were measured and the LucF (Firefly)/LucR (Renilla) ratios were calculated as an index of IRES/Cap-dependent translation (Figure 1B–D). The EMCV and VEGF constructs containing IRES were used as positive controls [4,38], and the FGF-1A construct was used as a negative control since although it contains an IRES, its activity is cell type-dependent [39]. Our results showed that *DLL4* 5′-UTR contained a putative IRES with an activity comparable to that of *VEGF-A* in the three cell lines tested (Figure 1B–D). To rule out the possibility of the presence of a cryptic promoter in the *DLL4* 5′-UTR, which could also cause Firefly activity, we cloned the *DLL4* 5′-UTR in the Tet-Off bicistronic system [2].In this system, the bicistronic cassette is under the control of a doxycycline-repressible promoter (Figure 1E). Thus the LucF/LucR ratio is expected to be stable and independent of the CMV-driven expression level if expression of the second cistron is IRES dependent. In contrast, if there is an intercistronic cryptic promoter, LucF expression will be independent of the CMV promoter, and thus the LucF/LucR ratio will increase proportionally to the repression of CMV promoter by doxycycline.The individual two *VEGF* IRESes, known to be promoterless, and the full length 5′-*VEGF-A* 5′-UTR, known to contain a promoter, were used as controls [2]. The results clearly show that the LucF/LucR

ratio increased with the inactivation of the CMV/Tet promoter after doxycycline treatment when the full *VEGF-A* 5′-UTR was present between the two cistrons. In contrast, the ratios remained unchanged when the individual *VEGF* IRESes or the *DLL4* 5′-UTR were tested, demonstrating the absence of cryptic promoter in the intercistronic region. Altogether these results indicate that the *DLL4* 5′-UTR possesses an IRES that can initiate cap-independent translation.

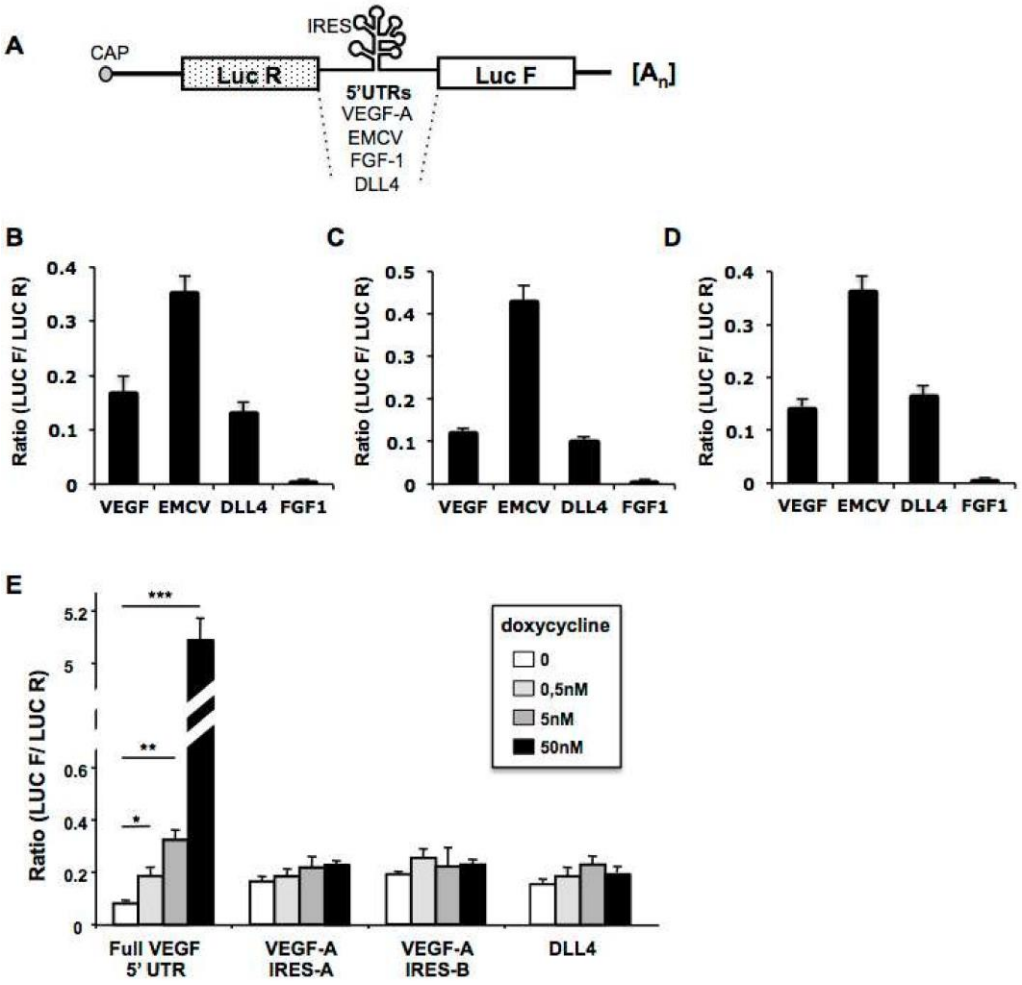

Figure 1. *DLL4* 5′-UTR contains an internal ribosome entry site. (**A**) Schematic representation of bicistronic constructs. IRESs cloned within the inter-cistronic region were either viral (*EMCV*) or cellular (*VEGF-A* IRESA, *VEGF-A* IRESB, *FGF-1*, *DLL4*), (**B–D**) Analysis of the *DLL4* IRES activity in transiently transfected (**B**) HeLa, (**C**) HUVEC or (**D**) NIH3T3 cells. 48h after transfection IRES activitywas determined by calculating the LucF/LucR ratio. *DLL4* IRES activity was compared to that ofthe cellular IRES-A of *VEGF-A*, the viral *EMCV* IRES or the *FGF-1A* IRES, known to be highly tissue and cell line specific. (**E**) HeLa Tet off cells were transfected with TET sensitive bicistronic constructscontaining the full 5′-untranslated region of *DLL4*. At 2 h prior to transfection, cells were treated with
0.5 nM, 5 nM or 50 nM doxycycline (Dox). Forty-eight hours after transfection, luciferase activities were measured as described. As positive controls, the *VEGF-A* full 5′-UTR (containing a cryptic promoter)was introduced in the intercistronic region and only the *VEGF-A* IRES A or B as negative controls (sequences without cryptic promoter). Data are means ± SEM from 3 independent experiments in duplicates, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.2. *DLL4 IRES Activity is Stimulated by Hypoxia*

Given that DLL4 is mainly expressed by the tip cells, localized at the leading edge of vesselsprouts in an unfavorable hypoxic microenvironment, we next tested the effect of hypoxia on the *DLL4* IRES

activity (Figure 2). Bicistronic constructs were transfected in HeLa and HUVEC cells. As expected, under hypoxic conditions HIF1α expression increased and Eukaryotic Initiation Factor 4E-Binding Protein 1 (4E-BP1) was dephosphorylated (shift to a band of lower apparent molecular weight in western blot) (Figure 2A,C). In the meantime we observed the inhibition of cap-dependent translation(decreased LucR activity) and no effect on LucF activity driven by *EMCV* IRES, while the *DLL4* IRES-driven expression of LucF increased in both cell types after hypoxia (Figure 2B,D). Hypoxic stress resulted in a 2.5-fold stimulation of *DLL4* IRES activity whereas *EMCV* IRES-mediated translation (LucF activity) remains stable in the two cell types (Figure 2B,D).
These results confirm the presence of a bona fide IRES element in the 5′-UTR of DLL4 mRNA, which is activated under hypoxia.

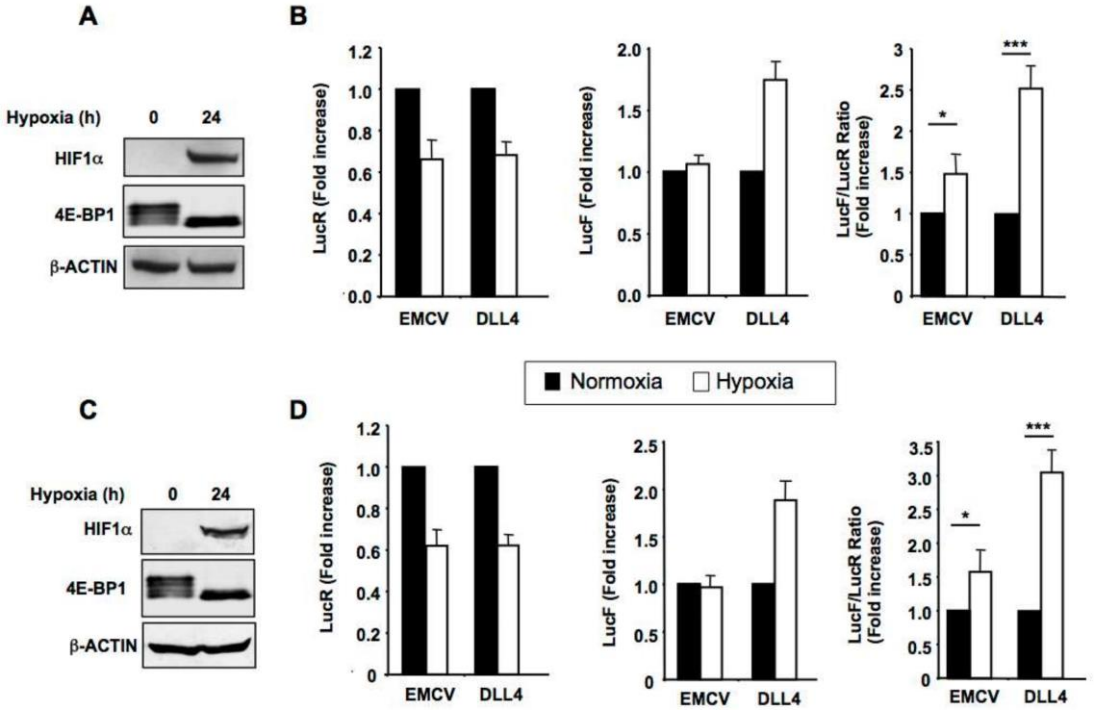

Figure 2. The *DLL4* IRES is activated by hypoxia. HeLa (**A**,**B**) or HUVEC endothelial cells (**C**,**D**) cells were transfected by bicistronic constructs containing either the *EMCV* or the *DLL4* IRES and submitted to 24 h hypoxia. (**A**,**C**) Hypoxia was confirmed through verifying both expression induction of HIF-1αand 4E-BP1 dephosphorylation (lower band visible at 24 h of hypoxia) by western blotting. β-ACTINwas used as a loading control. (**B**,**D**) Relative luciferase activities LucR, LucF or LucF/LucFR ratio (fold increase) under normoxia (black bars) and hypoxia (white bars) in HeLa (**B**) or HUVEC (**D**) cells.Results represent the means of three independent experiments (±SEM), * $p < 0.05$, *** $p < 0.001$.

2.3. The DLL4 IRES is Stimulated Following ER Stress

Many cellular stresses, including hypoxia, can activate ER dependent pathways by inducing an accumulation of misfolded/unfolded proteins within the ER [4,40]. In order to investigate whether the *DLL4* IRES responds to ER stress, we transfected HeLa cells with constructs containing the *EMCV*, or the *DLL4* IRES and treated them for 4 h with increasing amounts of dithiothreitol (DTT) a well-known ERstress inducer (Figure 3). To confirm ER stress activation we verified the increased levels of both *XBP1* splicing by RT-PCR and phospho-eIF2α (p-eIF2α) by western blotting after DTT treatment (Figure 3A). By comparing the ratio of luciferase reporter activities (LucF/LucR) between treated and control cells,we found that the relative *DLL4* IRES activity was around 5-fold greater in cells treated with 8 mM DTT for 4 h versus control cells (Figure 3B). On the contrary, and as previously described, the *EMCV*IRES was not activated under the same conditions [4,40]. As expected, triggering of ER stress resulted in a decrease of LucR expression in a dose-dependent manner after DTT, given its inhibitory effect on cap-dependent

translation [4,40]. Interestingly, whereas the *EMCV* IRES-mediated expression of thesecond cistron encoding LucF also diminished dose-dependently, the LucF expression driven by the *DLL4* IRES increased after DTT treatment (Figure 3B). These results demonstrate that the *DLL4* IRES isactivated by ER stress. Furthermore, during ER stress, DLL4 cap-independent translation is increasedwhen global cap-dependent translation is repressed.

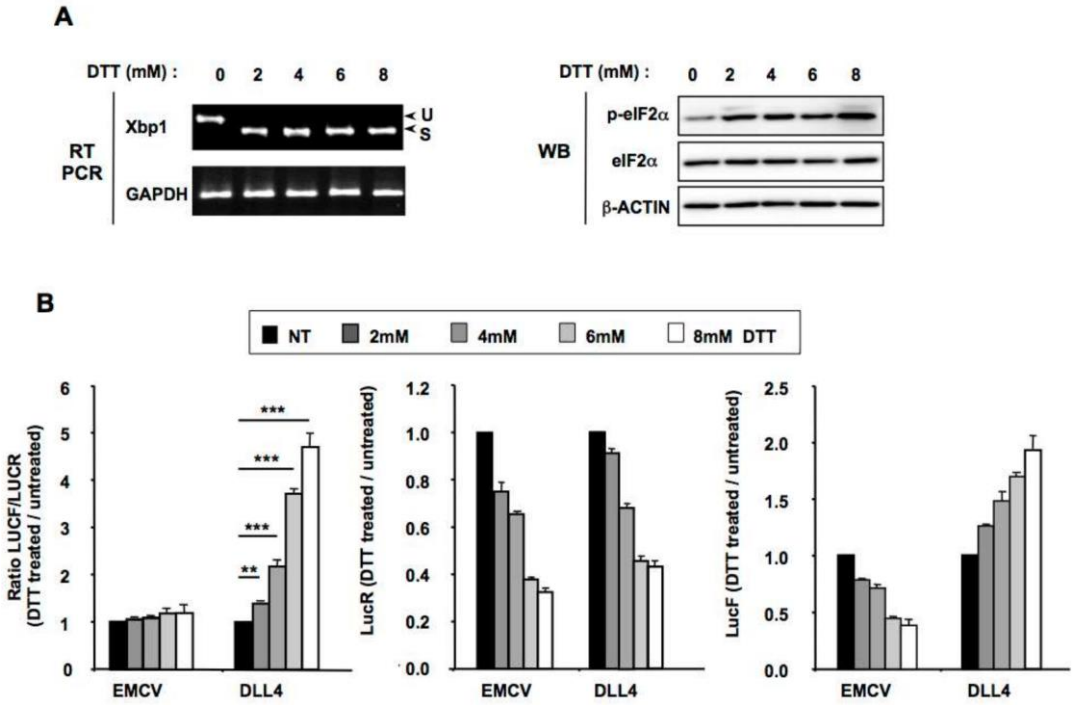

Figure 3. The *DLL4* IRES is activated by ER stress. HeLa cells were transfected by bicistronic constructs containing either the *EMCV* or the *DLL4* IRES and treated or not for 6 h with increasing concentrations of DTT. (**A**) ER stress induction was verified by monitoring both cytoplasmic *XBP1* splicing by RT-PCR and eIF2 phosphorylation by western blotting (**B**) Relative luciferase activities (LucR, LucF or LucF/LucR ratio) after ER stress induction by treatment with increasing amounts of DTT for 6 h. IRES activities were determined by calculating the LucF/LucR ratios and are expressed as fold change versus untreated cells.The results represent the means of three independent experiments (±SEM), ** $p < 0.01$, *** $p < 0.001$.

2.4. PERK Kinase is Required for DLL4 IRES-Mediated Translational Upregulation During ER Stress in Vitro

In response to ER stress, cells activate the physiological unfolded protein response (UPR) triggered by the activation of three ER transmembrane sensors: PKR-like ER-associated protein kinase (PERK), Activating Transcription Factor-6 (ATF6) and Inositol-Requiring Enzyme-1 (IRE1) [41,42]. To investigate the pathways involved in *DLL4* IRES activation during ER stress, we down-regulated the expression of these three ER stress sensors in HeLa cells. Transient downregulation of PERK, ATF6 and IRE1 by siRNA interference was confirmed by western blotting or semi-quantitative RT-PCR (Figure 4A). After DTT treatment, the phosphorylation of eIF2α was, as expected, diminished in cells transfected with PERK siRNA as was XBP-1 splicing after transfection of IRE1 siRNA (Figure 4B). We then co-transfected the bicistronic constructs with the respective siRNAs and calculated the ratio of IRES activities (LucF/LucR) between cells treated or not with DTT. The down-regulation of the three ER-stress transducers had no effect on *EMCV* IRES activity after DTT treatment (Figure 4C). On the other hand, ER stress-induced stimulation of the *DLL4* IRES was only affected after PERK down-regulation, suggesting that PERK, but not IRE1 or ATF6, is required for the control of *DLL4* IRES activity.

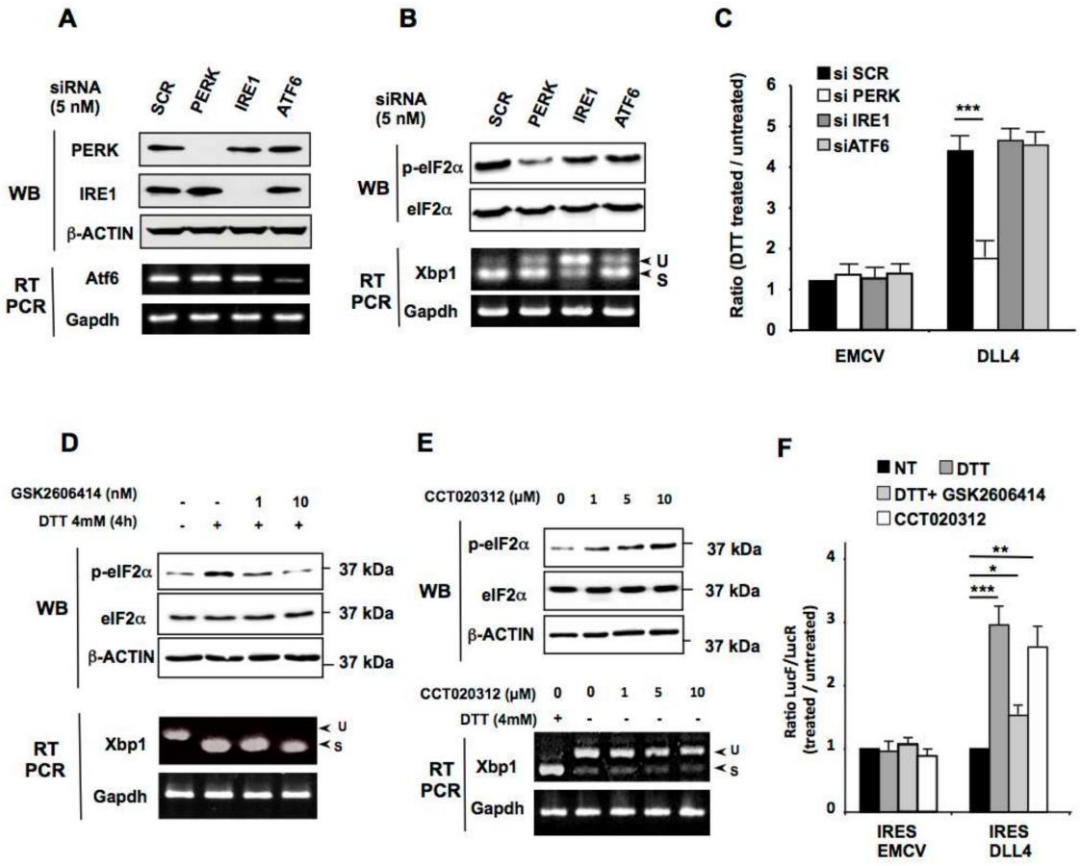

Figure 4. Role of three UPR sensors in *DLL4* IRES translation activation by ER Stress. (**A**) HeLa cells were transfected with PERK, ATF6, IRE1 or control (Scr) siRNA. The knockdown efficiency of the targeted transcripts was determined by western blotting with PERK, IRE1 and β-ACTIN antibodies, and by semi-quantitative RT-PCR against ATF6 and GAPDH as a control. (**B**) Western blot analysis of phosphorylated eIF2α and total eIF2α and RT-PCR analysis of *XBP1* splicing performed after transfection of HeLa cells either with siRNA specific for PERK, ATF6, or IRE1 or with control siRNA (Scr), after treatment with DTT. (**C**) Relative IRES activities in HeLa cells treated with DTT/control after co-transfection with siRNA specific for PERK, ATF6 or IRE1, or with control siRNA (scr) and DLL4 or EMCV bicistronic vectors. (**D**) Western blot analysis of phosphorylated eIF2α and total eIF2α and RT-PCR analysis of *XBP1* splicing performed after transfection of HeLa cells with DLL4 or EMCV bicistronic vectors, after treatment with DTT and increasing concentration of the PERK inhibitor GSK2606414. (**E**) Western blot analysis of phosphorylated eIF2α and total eIF2α and RT-PCR analysis of *XBP1* splicing performed after transfection of HeLa cells with DLL4 or EMCV bicistronic vectors, after treatment with increasing concentration of the PERK activator CCT020312. (**F**) Relative IRES activities in HeLa cells treated with DTT/control after transfection with DLL4 or EMCV bicistronic vectors and treatment with PERK activator (CCT020312) or inhibitor (GSK2606414). The results represent the means of three independent experiments (±SEM), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Similar results were obtained after both specific pharmacological PERK activation with CCT020312 or inhibition with GSK2606414 during ER stress (Figure 4D–F). As expected, PERK inhibition by GSK2606414, which had no effect on the *XBP1* splicing efficiency, prevented DTT-inducedeIF2α phosphorylation (Figure 4D), whilst PERK activation by CCT020312, which does not initiate cytoplasmic *XBP1* splicing as opposed to DTT, stimulated eIF2α phosphorylation (Figure 4E). Consistently, the inhibition or activation of PERK affected the *DLL4* IRES (Figure 4F), whereas no effect on *EMCV* IRES activity was observed. Taken together, these results indicate that PERK activation is sufficient to stimulate *DLL4* IRES activity.

To complement the pharmacological approach, we used an already described inducible tetracycline/leucine zipper-based dimerization system enabling artificial activation of the PERK

pathway [4,40]. In this model the intraluminal ER sensor domain of PERK is replaced by a c-Jun leucine zipper fused to a HA tag (PERK-LZ; Figure 5A). Thus, the addition of increasing concentrations of doxycycline induces specific expression and subsequent dimerization and activation of PERK-LZ as visualized by concomitant expression of the PERK-LZ (HA) expression and phosphorylation of eIF2α (p-eIF2α) (Figure 5B). These cells were then used to confirm that selective PERK pathway activation was sufficient to stimulate *DLL4* IRES activity. Forty-eight hours after the addition of 1 µg/mL doxycyclineto the culture medium, cells were transfected with the bicistronic vectors containing the *EMCV*, and *DLL4* 5′-UTR IRES, and luciferase activities were measured 24 h later. An increase in IRES activity was seen with the *DLL4* 5′-UTR constructs but not with the construct containing the *EMCV* IRES (Figure 5C),further demonstrating that the PERK pathway is sufficient for *DLL4* IRES activation during ER stress.

Finally, to investigate whether PERK alone is sufficient to induce *DLL4* IRES activation or insteadif signaling downstream PERK is required, we evaluated the effect of ATF4 (Activating Transcription Factor 4) down-regulation by siRNA on ER stress-induced *DLL4* IRES activation. ATF4 regulates the transcription of a number of genes involved in stress response and cell survival and, in contrast to most transcripts, the translation of ATF4 is enhanced as a consequence of increased phosphorylationof eIF2α. siRNA-mediated ATF4 knockdown impaired neither eIF2α phosphorylation nor PERK activation (shown by supershift in immunoblots of total PERK) (Figure 5D) and had no effect on the stimulation of *DLL4* IRES after induction of ER stress by DTT (Figure 5E). This indicates that activationof the PERK pathway, independently of ATF4, is sufficient to stimulate *DLL4* IRES activity.

To investigate the potential role of eIF2α phosphorylation in this process we transfected bicistronic vectors in mouse embryo fibroblasts (MEFs) derived from either wild-type mice or from eIF2α knock-in mice that have a homozygous mutation precluding eIF2α phosphorylation (S51A). As expected, no phosphorylation of eIF2α was observed in mutant MEFs after ER stress induction by DTT (Figure 5F) while this treatment efficiently induced a comparable *XBP1* splicing in both S51A and WT MEFs (Figure 5F). The ratio of Firefly to Renilla luciferase between ER stress inducers treated and control cells remained stable with bicistronic vector containing the *EMCV* IRES and was significantly increased with the *DLL4* IRES only in WT MEFs but not in S51A MEFs (Figure 5G). Taken together these results independently confirm that translation from *DLL4* IRES is stimulated by PERK during ER stress, and demonstrate that phosphorylation of eIF2α is required.

2.5. hnRNP A1 Modulates DLL4 IRES-Mediated Translation

IRES-dependent translation efficiency is controlled by RNA-binding proteins known as IRES trans-acting factors (ITAF). Subcellular relocalization of ITAFs plays a crucial role in the modulationof IRES-dependent translation efficiency [43]. Indeed, many RNA-binding proteins are able to shuttle between the nucleus and the cytoplasm. For example, it has been reported that cytoplasmic relocalization of ITAFs, such as hnRNPA1, may either activate or inhibit IRES activity when accumulating in the cytoplasm [44,45]. Interestingly, many stresses like UVC, osmotic shock or ER stress induce cytoplasmic hnRNPA1 relocalization [44–46]. Moreover, it was demonstrated that this hnRNPA1 cytoplasmic accumulation, during osmotic stress, requires eIF2α phosphorylation [47].

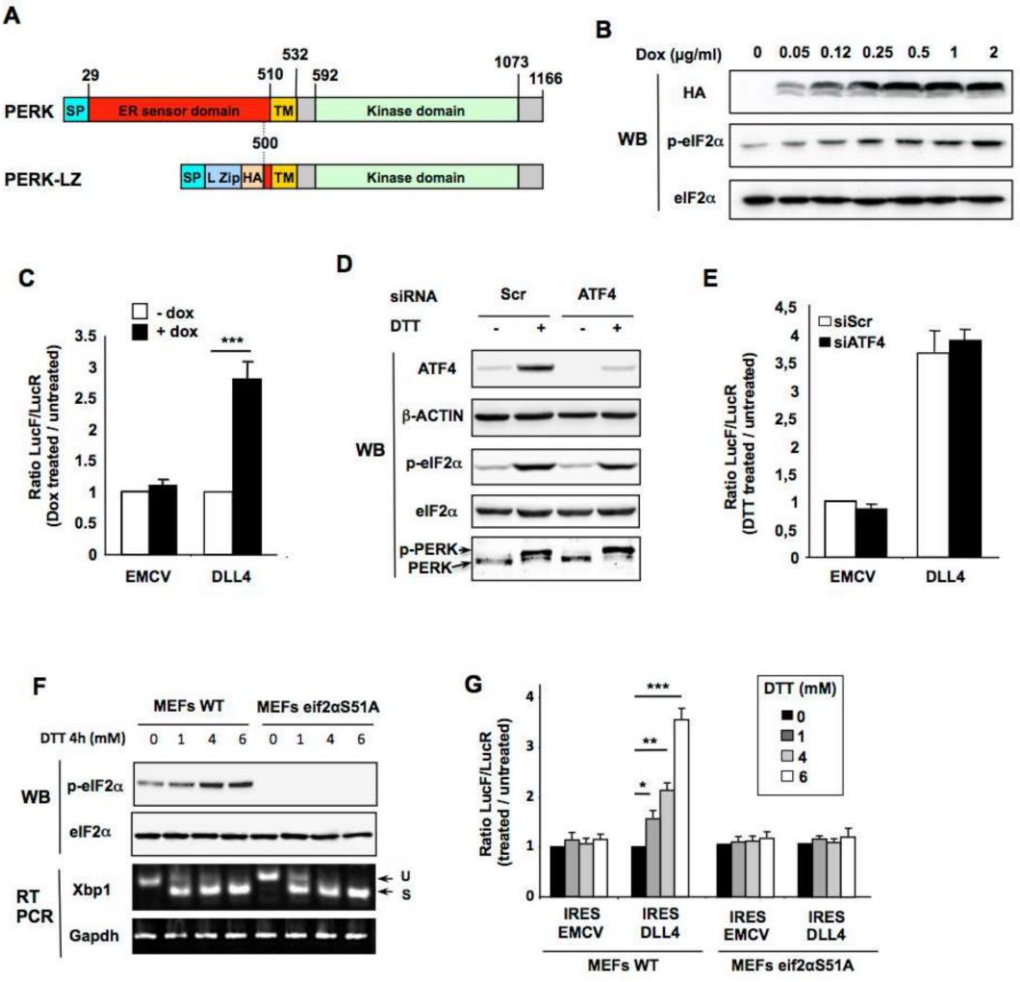

Figure 5. PERK kinase activity stimulates *DLL4* IRES-mediated translation in a phosphorylated eIF2α–dependent manner. (**A**) Linear schematic representation of PERK and PERK-LZ showing the locations of the major functional domains (SP = Signal Peptide, L zip = Leucine Zipper, HA = HA Tag).Numbers indicate amino acid positions. (**B**) Western blot analysis of doxycycline-induced-PERK-LZ (HA) expression and eIF2α phosphorylation after 48 h treatment with increasing amounts of doxycycline. (**C**) Relative IRES activities in PERK-LZ expressing HeLa cells treated with 1 µg/mL doxycycline /control after transfection of EMCV or DLL4 bicistronic vectors. Means ± SEM are shown,
*** $p < 0.001$. (**D**) Western blot analysis of ATF4, total and phosphorylated eIF2α, as well as total PERK in HeLa cells, after transfection with ATF4 or scramble (Scr) siRNA and treatment with DTT. β-ACTIN was used as a loading control. (**E**) Relative IRES activities in HeLa cells treated with DTT/control, after cotransfection with the EMCV or DLL4 bicistronic vectors and with either siRNA specific for ATF4 or control siRNA (Scr). Results represent the means of three independent experiments ± SEM.
(**F**) Wild-type (WT) and eIF2αS51A MEFs transfected with the bicistronic LucR-IRES-LucF vectors and treated with increasing concentrations of DTT. ER stress induction was verified by monitoring eIF2αphosphorylation by western blot and cytoplasmic *XBP1* splicing by RT-PCR. (**G**) Relative IRES activities were determined as previously described in WT (left) and eIF2αS51A (right) MEFs. Results represent the means of three independent experiments ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Thus we evaluated whether hnRNP A1 could play a role on the activation of DLL4 cap-independent translation after ER-stress-mediated eIF2α phosphorylation. After verification of extraction efficiency of cytosolic and nuclear proteins in each fraction (Figure S2), we analyzed the nuclear and the cytosolic level of hnRNPA1 in DTT treated cells by western blotting experiments (Figure 6A). Results showed

that hnRNPA1 level in the cytoplasm increased after 6 h of treatment with increasing concentrations of DTT, while the amount of hnRNPA1 in the nuclei decreased (Figure 6A),and the total hnRNPA1 level was not affected by the DTT treatment. To decipher the role of hnRNPA1 on the ER stress-mediated induction of *DLL4* IRES activity, cells were co-transfected with the bicistronic constructs and either with scrambled or hnRNPA1-specific siRNA and ER stress was induced by DTT treatment. hnRNPA1 knockdown was efficient but did not impair eIF2α phosphorylation after induction of ER stress by DTT (Figure 6B). While hnRNAP1 expression inhibition had no effect on *EMCV* IRES activity, it reduced *DLL4* IRES activity during DTT-induced ER stress, compared to scramble siRNA transfected cells (Figure 6C). The data presented are consistent with a model in which *DLL4* IRES activity is governed, at least in part, by the cellular IRES trans-acting factor hnRNPA1.

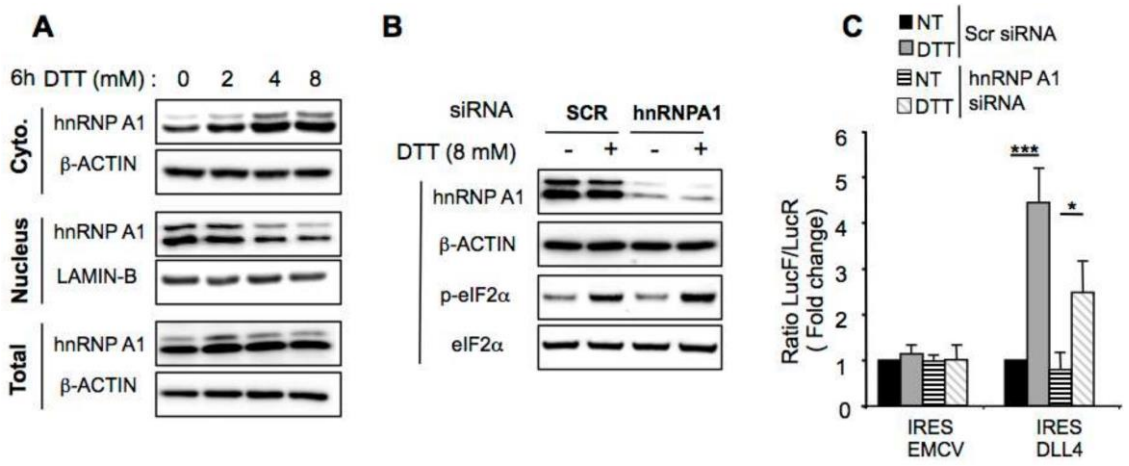

Figure 6. hnRNPA1 modulates *DLL4* IRES activity during ER stress. (**A**) Western blot analysis of hnRNPA1 in HeLa nuclear, cytoplasmic or total extract after treatment with increasing concentrationsof DTT. β-ACTIN was used as a loading control for cytoplasmic and total extracts and LAMIN-B for nuclear extract. (**B**) Western blot analysis of hnRNPA1, total and phosphorylated eIF2 in HeLa cells, after transfection with hnRNPA1 or scramble (Scr) siRNA and treatment with DTT. β-ACTIN was used as a loading control. (**C**) Relative IRES activities in HeLa cells treated with DTT/control, after cotransfection with the EMCV or DLL4 bicistronic vectors and with either siRNA specific for hnRNPA1or control siRNA (Scr). Results represent the means of three independent experiments ± SEM. * $p < 0.05$,
*** $p < 0.001$.

3. Discussion

Translational initiation is a well-established and crucial regulatory step of gene expression, allowing reprogramming of protein synthesis in response to environmental changes. Under stress conditions, the maintenance of routine translation machinery would be deleterious. Hence the synthesis of "housekeeping" proteins is paused in stressed cells whereas the translation of a pool of proteins necessary for the adaptive stress response is maintained, via alternative mechanisms of translational initiation. To bypass the stress-mediated inhibition of cap-dependent translation, more than 100 mammalian mRNAs harbor internal ribosome entry site (IRES) elements in their 5′-UTRs that mediate internal initiation of translation. These mRNA include many mRNA encoding proteins strongly involved in angiogenesis like VEGF-A, FGF-2, FGF-1A, VEGF-C, PDGF or TSP1 [48]. In thisstudy we show that a *bona fide* IRES element is present in the 5′-UTR of *DLL4* mRNA encoding the DLL4 protein that regulates angiogenesis during development, but also in pathological conditions, such as cancer [16,20,26,49]. Interestingly, as has already been shown for the *VEGF* and *FGF-2* IRESs [2,4], the *DLL4* IRES is activated under stress conditions including hypoxia and ER stress. This suggests that DLL4 remains efficiently translated during stress despite the substantial global inhibition of cap-dependent protein translation. We have also shown the significance of the PERK kinase in regulating stress-induced IRES-dependent translation of DLL4. Taken together our data suggest thatDLL4 is translated under ER stress conditions despite phosphorylation of the major PERK substrate, eIF2α. It was

previously demonstrated that PERK signaling is crucial for determining the growth andangiogenesis of specific tumors [50,51]. For example, UPR induced by glucose deprivation increasesVEGF expression in human tissues in a PERK-dependent manner [52]. Moreover, tumors derived from K-Ras– transformed Perk−/− MEFs were found to be smaller than those derived from MEFs with an intact integrated stress response, because targeting PERK signaling disrupts angiogenic signals and prevents the appropriate organization and maturation of functional vessels [51].

This finding has biological relevance because several components involved in the same mechanism are translated in an IRES-dependent manner, providing selective co-regulation under stress conditions (Figure 7). Indeed, during tumor progression, the stress area encompasses both the tumor and its microenvironment. The growing neovessels that are inefficiently perfused, and more particularly TIP cells which are furthest away from the circulating blood, are also subjected to stress due to the unfavorable environment (hypoxia, glucose or amino acid starvation, acidosis). These adverse conditions are known to induce ER stress leading to eIF2α phosphorylation and thus to activation of a network of genes dependent on ER stress. Many genes whose product are involved in angiogenesis, including *Vegf-a*, *Fgf-2*, *Hif1α* and *Dll4*, and expressed either by tumoral cells or by the microenvironment, contain IRES element allowing the maintenance of an efficient translation of these mRNAs under stressful conditions while cap-dependent initiation is compromised (Figure 7). A number of other reports have demonstrated that IRES-dependent translation is driven by ER stress [4,40,53–55]. However, our results clearly show the link between the UPR, more precisely PERK/eIF2α signaling, and IRES-dependent translation of angiogenesis related genes. The physiological relevance of IRES-dependent translation mechanisms of non-viral mRNA is poorly documented in the literature and the precise mechanism of how stress signals downstream of eIF2α phosphorylation are transduced to IRES elements remains unknown.

It was previously largely described that upstream open reading frames (uORFs) are key regulators of mRNA translation upon eIF2α phosphorylation. In the case of stress-induced eIF2α phosphorylation, it was proposed that after having translated the uORF, recharging of the ribosome with active initiation factors (including the ternary complex eIF2.GTP.met-tRNA) is the limiting step for reinitiating translation [56]. This model account for the observed increased translation efficiency of the downstream open reading frame when the intercistronic region is longer. The hypothesis is that when the region to be scanned is long, the ribosome would have enough time to reacquire reinitiation factors before encountering the downstream initiation codon. Nevertheless, *DLL4* 5′-UTR which is highly GC rich, doesn't contain uORF.

Moreover, secondary structures, which could be stabilized by specific protein binding, could slow down scanning of the 43S pre-initiation complex and by this way increase translation reinitiation efficiency [57]. All the cellular IRESs already characterized used the so-called "land and scan" mechanism to initiate translation. The 40S subunit associates with the IRES upstream of the initiation codon, and then scan the mRNA in a 5′-to-3′ direction until start codon recognition occurs [58–60]. The presence in IRES elements of extensive secondary structure, representing a significant barrier slowing down the ribosome scanning, could explain the need of IRES mediated translation under stress conditions when eIF2α is phosphorylated. We can also postulate that the presence of pause sites within the IRES sequence scanned by the ribosome, will allow it to reacquire active initiation factors and to initiate efficiently the translation at the downstream AUG. This hypothesis is supported by the fact that for *EMCV* IRES which is insensitive to ER stress, there is good evidence that the 40S ribosomal subunits associated with initiation factors bind directly to the initiating AUG without anyscanning requirement [61]. Indeed, it was demonstrated that eIF2 was necessary for *EMCV* mRNA translation [62] and that either treatment stimulating PKR (RNA-dependent protein kinase R, one of the four known eIF2α kinases) or activation of PKR alone suffices to block EMCV translation [63,64]
confirming that *EMCV* IRES activity was inhibited upon eIF2α phosphorylation.

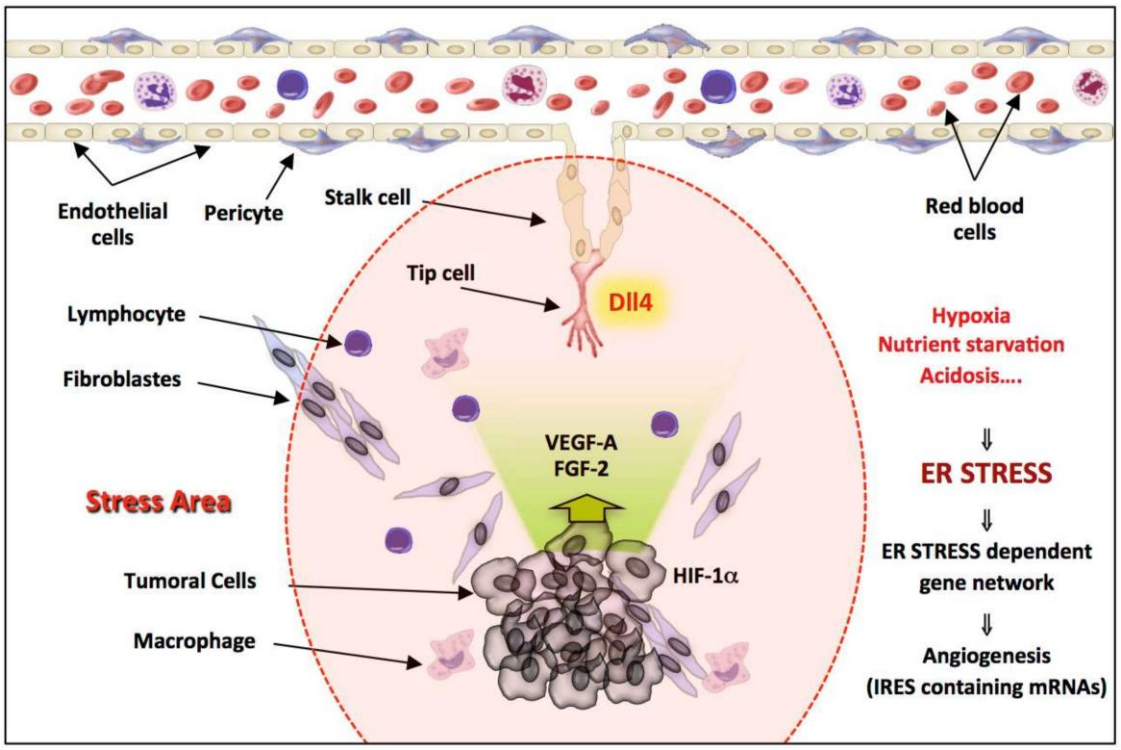

Figure 7. Schematic model of the network of gene expression co-regulation by stress during tumoral progression. During tumor progression, the stress zone encompasses the growing tumor, but also its microenvironment. The neo vessels and more particularly the Tip cells, present at the extremity and which guide the neo vessels towards the tumor, are located in this unfavorable microenvironment. Hypoxia, nutrient starvation and acidosis will irremediably induce the accumulation of unfolded protein in the reticulum of cells located in this area, leading to ER stress and UPR activation.Thus, in addition to transcriptional regulations, the activation of the PERK pathway will induce the co-regulation of an UPR dependent gene network containing IRES elements, revealing a translational regulon in which the synthesis of a cohort of angiogenic related genes is activated in response to ER stress. The fine-tuning of gene expression allows an efficient control of angiogenesis, which is a highly regulated process.

Emerging evidence has shown that the ribosome itself can play a crucial role in the specialized translation of specific subsets of mRNAs harboring specific *cis*-regulatory elements. Ribosomal biogenesis involves hundreds accessory factors and requires transcriptional and post-transcriptional steps which are timely and spatially regulated. It is thus not surprising that alteration of ribosome biogenesis is associated with dysregulation of translational efficiency. During hypoxic stress for example, cells maintained viability by restricting ribosomal biogenesis, themost energy-demanding cellular process [65]. We can hypothesize that cap-independent translation through the use of IRES elements is a mechanism, which is favored by low ribosome content during stress conditions. In the same vein, recent evidence suggests that ribosomal proteins themselves may function to recognize specific IRES elements. For example RPS19 and RPL11 regulate IRES-dependent translation of BCL-2-associated athanogene (*BAG1*) and cold shock domain containing protein E1 (*CSDE1*) [66].
Both the ribosomal RNA and proteins are heavily modified in mature ribosomes leading to ribosome heterogeneity that could be an important mechanism regulated during stress conditions.It was demonstrated that both 2′-O-methylation and hypo-pseudouridylation of rRNA is associated with impaired IRES-dependent translation [67,68], and that ribosomes bound to the HCV IRES have a different methylation pattern from ribosomes bound to host mRNAs, indicating a role of methylated ribosomal proteins in IRES-mediated translational control [69]. Moreover, in mouse and human cells

expressing a hypomorphic form of the pseudouridine synthase dyskerin, the translation of some IRES-containing mRNAs was impaired [68,70,71]. Likewise, yeast expressing a catalytically inactive form of Cbf5, the homolog of human dyskerin, are also deficient in IRES dependent translation initiation [72]. However, it was also shown that dyskerin depletion increases VEGF mRNA internal ribosome entry site-mediated translation [73], indicating that cell type or genetic and environmental factors most certainly could influence the degree of implication of dyskerin in the translation of IREScontaining mRNA. Given that hypoxic stress decreases dyskerin expression level [74], it would be of great interest to investigate the effect of ER stress or hypoxia on both ribosomal protein expression or rRNA modifications including 2′-O-methylation and pseudouridylation. Moreover,it will also be interesting to investigate whether ribosomal proteins promote specialized translation of IRES-containing mRNAs, either directly or through ITAFs. Indeed, many studies showed that ITAFs are able to stabilize the adequate RNA conformation allowing ribosome recruitment [75]. ITAFs are responsible for sensing changes in cellular metabolism and influence IRES activity. Moreover, subcellular distribution of many of them is modulated by stress [43]. Thus, both the expression level and localization of ITAFs could finely regulate the expression of IRES containing mRNAs. The results presented in this study suggest that the hnRNP A1 is an ITAF that increases the *DLL4* IRES activity in stress conditions, and that this regulation is dependent on the nucleo–cytoplasmic relocation of hnRNP A1 upon ER stress. The same mechanism was already described for the cap independent translation of the transcription factor SREBP-1 (sterol-regulatory-element binding protein 1) during ER stress [75]. Nevertheless, we did not observe a complete inhibition in IRES-mediated translation after knockdown of hnRNP A1. The main reasons are the knockdown efficiency of hnRNP A1 by the siRNA (hnRNP A1 protein is abundant in the cell) and the potential presence of other ITAFs. Further investigation wouldbe necessary to completely understand IRES-mediated translation of *DLL4* mRNA.

4. Materials and Methods

4.1. Plasmid Constructions and Viral Production

The ER transmembrane and cytoplasmic domains of the human PERK protein were cloned fromhuman cDNA using the PERKHALZ and PERK reverse primers (Table 1). Signal peptides and leucine zipper (LZ) peptides were merged with the PERK domains using the primers shown in Table 1.

Table 1. Oligonucleotide sequences.

Primer Name	Sequence 5′→3′
PERKHALZ	CAGCTCAAGACGCGTTTCGAATACCCATACGATGTTCCTGACTATGCGAGATTCCTCGACAACCCACA
PERK rev	TTCTCGAGTATCGATTTACTAATTGCTTGGCAAAGGGC
SP	AAACTAGTGCCATGGCTCCGGCCCGGCGGCTGCTGCTGCTGACGCTGCTGCTGCCCGGCCT
SP-LZ 1	CACTTTCTCTTCCAGGCGCGATGTGCTGGTACTTCCAAAAATCCCGAGGCCGGGCAGCAGCAGCGT
SP-LZ 2	CGCGCCTGGAAGAGAAAGTGAAGACCCTCAAGAGTCAGAACACGGAGCTGGCGTCCACGGCGAGC
LZ	TTCGAAACGCGTCTTGAGCTGCGCCACCTGCTCGCGCAGCAGGCTCGCCGTGGACGCCAGCTC XBP1-
F	CTGGAACAGCAAGTGGTAGA
XBP1-R	CTCCTCCAGGCTGGCAGG
DLL4+1-F	AAACTAGTGCTGCGCGCAGGCCGGGAACACG
DLL4 ATG-R	AAAACCATGGCCCCTCGGGCGTCGCTCTCTC
GAPDH-F	CAAGGTCATCCATGACAACTTTG
GAPDH-R	GTCCACCACCCTGTTGCTGTAG
ATF6-F	GGGAGACACATTTTATGTTGTGTC
ATF6-R	GGTTTGATTCCTCTGCTGATCTCG

Sequences were confirmed by DNA sequencing. Purified PCR products were digested with KpnIand XhoI restriction enzymes and inserted into the pTRIPz-TRE-Tigh plasmid (Open Biosystems, Dharmacon Lafayette, CO, USA) to generate the pL-TRE-PLZ expression vectors. Viruses were

produced by transfecting HeLa cells (5.0×10^4) with the JetPEI transfection reagent (Polyplus Transfection, Illkirch, France), according to the manufacturer's instructions.
Constructs containing the *VEGF-A*, *FGF-1A* and *EMCV* IRESs have been previously described [4,37]. *DLL4* 5′-UTR was amplified by PCR using Phusion High-Fidelity (New England Biolabs, Evry, France) polymerase using the primers DLL4+1-F and DLL4 ATG-R. PCR product wassubcloned into the pCR™-Blunt shuttle vector (Thermo Fisher Scientific, Dardilly, France), which was sequenced and then cloned into a bicistronic vector using the SpeI and NcoI restriction sites to give the pCRD4L vector. Constructs containing the *VEGF-A* IRES A, B and the full *VEGF* 5′-UTR (namely pTCRVL) upstream of a tetracycline responsive promoter have been previously described [2]. After digestion of the PCRD4L by XbaI and PacI, the cassette LucR-5′-DLL4-LucF was inserted in the vectorpTCRVL also digested by XbaI and PacI, thus replacing the VEGF sequence by the DLL4 one.

4.2. Cell Culture and Transfection

HeLa cells, NIH3T3 and both wild-type and eIF2αS51A MEF cells (kindly provided by P. Fafournoux from INRA, Unité de nutrition Humaine, France) were cultivated in DMEM media (Sigma Aldrich, Saint-Quentin Fallavier France) supplemented with 10% FCS, 1% glutamine and antibiotics. Cells were transfected using the JetPEI transfection reagent (Polyplus Transfection), according to the manufacturer's instructions. 24 hours after transfection with the bicistronic constructs, cells were treated with DTT, the PERK activator CCT020312 (Merck Millipore, Fontenay sous Bois, France) or the PERK inhibitor GSK2606414 (Merck Millipore) for various concentrations, and were then harvested. For hypoxia, cells were incubated at 37 °C at 1% O_2.
Human Umbilical Vein Endothelial Cells (HUVEC) pooled from 6 donors were prepared by digestion of umbilical veins with 0.1 g/L collagenase A (Roche, Meylan, France) and cultivatedin the specific Endothelial Cell Growth Medium 2 (PromoCell, Heidelberg Germany). Cells were transfected using the JetPEI-HUVEC transfection reagent (Polyplus Transfection), according to the manufacturer's instructions.

4.3. Total RNA extraction and RT-PCR

Total RNA was purified using the TRI Reagent solution (Applied Biosystems, Thermo Fisher Scientific, Dardilly, France)). Reverse transcription was carried out with 1 µg of total RNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Dardilly, France) with randomhexamers, according to the manufacturer's instructions. The resulting cDNA was amplified by PCR for 30 cycles using Phusion High-Fidelity DNA Polymerase, 2× mix (New England Biolabs) and specific primers (Table 1).

4.4. Western Blot Analysis

Western blotting was performed as previously described [76]. PERK, IRE1, ATF4, eIF4E-BP1, eIF2α and p-eIF2α were immunodetected using rabbit anti-human monoclonal antibodies (Cell Signaling, Danvers, MA, USA, dilution 1:1000) as the primary antibody, and peroxidase-conjugated sheep anti-rabbit (Cell Signaling Technology (Leiden, The Netherlands), dilution 1:5000) as secondary antibody. HA-tagged proteins and HIF-1α were detected using the mouse monoclonal antibodies clone HA-7 (Sigma Aldrich) and Clone 54/HIF-1α (BD Biosciences, Le Pont de Claix, France)respectively, and peroxidase-conjugated horse anti-mouse (Cell Signaling Technology, dilution 1:5000)as secondary antibody.
Protein signals were normalized using either total eIF2α or an anti-β-actin monoclonal antibody (AC-15, Sigma-Aldrich (Saint-Quentin Fallavier France), dilution 1:10,000). Signals were detected using the Clarity chemiluminescence kit (Bio Rad, Marnes-la-Coquette, France). Nuclear and cytoplasmic extracts were obtained by using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Dardilly, France) according to the manufacturer's protocol.

4.5. RNA Interference

RNA interference-mediated gene knockdown was achieved by transfecting 5 nM of ATF6-, IRE1α-, PERK-, and ATF4-targeting or duplex control siRNA (ON-TARGETplus SMARTpool, Dharmacon, Lafayette, CO, USA) into HeLa cells using Interferin (Polyplus Transfection), according to themanufacturer's

protocol. After 48 hours, cells were co-transfected (3 μg plasmids and 5 nM siRNA) with Lipofectamine 2000 (Invitrogen, Cergy-Pontoise, France), and harvested 24 hours later for protein and RNA analyses.

4.6. Luciferase Activity

For reporter vectors, luciferase activities were performed using Dual-Luciferase Reporter Assay (Promega, France). The quantification of Renilla and Firefly Luciferase activities was achieved 48 h after transfection with a luminometer (TriStar^2 LB 942 Modular Multimode Microplate Reader, Berthold, Versailles, France), according to the manufacturer's instructions.

4.7. Statistical Analyses

All experimental data are expressed as mean ± SEM and statistical significance was evaluated using Student's *t*-test. Differences were considered significant at values of $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

5. Conclusions

In conclusion, DLL4 has evolved an IRES element that allows the enhancement of its translationunder ER stress, a condition known to be activated during tumor development when the expression of this protein is crucial. The presence of IRES elements in many genes involved in angiogenesis and expressed in stress conditions including *Fgf-2*, *vegf-a* or *Hif1* α (Figure 7) and *Dll4* suggest that IRESs function as cis-acting regulons during ER stress. In addition, we have shown thesignificance of the PERK kinase in regulating stress-induced IRES-dependent translation of this mRNA. These observations suggest that PERK could be a useful druggable target to control angiogenesis, possibly even locally, following an ischemic disorder or in cancers.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/11/2/142/s1, Figure S1: (**A**) Partial alignment of the DLL4 mRNA 5′-untranslated region of several species. Conserved nucleotides are shown in red. Main *DLL4* AUG translation initiation codon(on the right) is framed. (**B**) Example of RNA structure prediction by the RNAFold software (http://rna.tbi.univie.ac.at/cgibin/RNAWebSuite/RNAfold.cgi) of the first 85 nucleotides of the *DLL4* 5′-UTR and of the full *DLL4* 5′-UTR. Figure S2: Western blot analysis of β-ACTIN and LAMIN-B in HeLa nuclear and cytoplasmic extracts after treatment with increasing concentrations of DTT. β-ACTIN was mostly detected in the cytoplasmic fraction while LAMIN-B is exclusively present in the nuclear fraction.

Author Contributions: Conceptualization, H.L., S.P. and C.T.; Methodology, L.V.D.B., C.S. and L.M.; Formal Analysis, M.J., C.P., S.P., L.M., and C.T.; Investigation, M.J., C.P., L.M., H.L. and C.T.; Writing—OriginalDraft Preparation, S.P., and C.T.; Writing—Review & Editing, L.M., S.P., H.L. and C.T.; Supervision, S.P., H.L. and C.T.; Funding Acquisition, C.T.

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Conflicts of Interest: The authors declare no conflict of interest.

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Translational Regulations in Response toEndoplasmic Reticulum Stress in Cancers

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Abstract: During carcinogenesis, almost all the biological processes are modified in one way or another. Among these biological processes affected, anomalies in protein synthesis are common in cancers. Indeed, cancer cells are subjected to a wide range of stresses, which include physical injuries, hypoxia, nutrient starvation, as well as mitotic, oxidative or genotoxic stresses. All of these stresseswill cause the accumulation of unfolded proteins in the Endoplasmic Reticulum (ER), which is a major organelle that is involved in protein synthesis, preservation of cellular homeostasis, and adaptationto unfavourable environment. The accumulation of unfolded proteins in the endoplasmic reticulum causes stress triggering an unfolded protein response in order to promote cell survival or to induceapoptosis in case of chronic stress. Transcription and also translational reprogramming are tightly controlled during the unfolded protein response to ensure selective gene expression. The majority of stresses, including ER stress, induce firstly a decrease in global protein synthesis accompanied by the induction of alternative mechanisms for initiating the translation of mRNA, later followed by a translational recovery. After a presentation of ER stress and the UPR response, we will briefly present the different modes of translation initiation, then address the specific translational regulatorymechanisms acting during reticulum stress in cancers and highlight the importance of translationalcontrol by ER stress in tumours.

Keywords: translation initiation; ER stress; unfolded protein response (UPR); IRES; uORF

1. Introduction

Over the years, eukaryotic cells have evolved different mechanisms to deal with stressfulenvironments. Under stress conditions, eukaryotic cells activate adaptive pathways to restore cellularhomeostasis and to save energy. Given that cells consume a large amount of their available energy for the process of translation and for protein folding, it is not surprising that most stresses cause an inhibition in global protein synthesis. Indeed, under stress conditions, the maintenance of routine translation machinery would be deleterious. Hence the synthesis of "housekeeping" proteins is paused in stressed cells, whereas the translation of a pool of proteins necessary for the adaptive stressresponse is maintained, via alternative mechanisms of translational initiation. This level of regulation

is particularly important in stress conditions, as it enables a rapid change in the protein synthesis level both quantitatively and qualitatively to obtain a response that is relevant to the type of stress being induced.

This is particularly true in cells with high growth rates and elevated metabolic requirements suchas cancer cells, which are exposed to environmental stresses because of inadequate vascularisation causing hypoxia, acidosis and nutrient starvation. All of these stresses have been reported to cause the accumulation of unfolded or misfolded proteins in the lumen of the endoplasmic reticulum (ER) and induce ER stress because the folding capacity of the ER is limited. ER stress triggers activation of the unfolded protein response (UPR), an adaptive reaction mediated by three molecular sensors present on the membrane of endoplasmic reticulum: Activated Transcription Factor 6 (ATF6), Inositol-Requiring Enzyme 1 (IRE1) and PKR-like ER kinase (PERK). UPR activation in cells alters both transcriptional and translational programs to coordinate adaptive and/or apoptotic responses.

Indeed, UPR aims to restore cellular homeostasis and to promote cell survival by inhibiting protein synthesis, improving protein folding ability, increasing the degradation of unfolded proteins. However, when damages are irreversible after intense and prolonged activation, UPR induces cell death. At the moment, the molecular determinants of the transition from survival to death are still unknown. Even if PERK kinase activation, which leads to phosphorylation of eukaryotic translation initiation factor-2α (eIF2α), is required for the global translation reprogramming during ER stress, involvement of IRE1 in translational regulation has also been established.

It should be noted that the regulation of translation is extremely complex, with many interconnected mechanisms. Our aim here is not to go into the details of the various translation initiation modes, but to give the reader an overview of the translation rewiring upon stress. After a brief presentation of Endoplasmic Reticulum stress and UPR response, a particularly complex mechanism but also well documented in the literature, we will focus on the translational regulatory mechanisms acting during this stress in cancers and highlight the importance of translational control in stress conditions.

2. Endoplasmic Reticulum Stress Signalling in Cancer

The endoplasmic reticulum (ER), which accounts for more than 50% of the cell's membranes in certain cells, is the site of synthesis and modification of secreted and membrane-related proteins (up to 50% of all proteins in certain cell types) [1,2]. It represents, therefore, an important hub where proteins undergo very strict quality control ensuring that only properly folded proteins progress down the secretory pathway. Thus, all situations leading to an alteration of the ER function, including the accumulation of excess unfolded proteins in the lumen of endoplasmic reticulum, lead to ER stress. Given the harmful impact of unfolded proteins, it is crucial that cells adapt to an imbalance betweenthe ER's folding capacity and unfolded proteins accumulation. The physiological response caused bythe accumulation of misfolded or unfolded proteins is commonly called unfolded protein response (UPR) [2,3].

During cancer development and progression, cells are under a wide range of stresses, which includechanges in oxygen levels (hypoxia), acidosis, nutrient starvation, disrupted calcium homeostasis, genotoxic or oxidative stresses. All of these stresses induce an accumulation of unfolded proteins within the reticulum, thus activating the UPR. In addition, cells with high proliferation rate, such as cancer cells, have to sustain a high rate of protein synthesis and massive protein flow through ER, leading to accumulation of misfolded protein in the ER, perturbation of ER homeostasis and finally toER stress [4]. It has also been shown that genetic alterations found in cancers (translocations, mutations, aneuploidy, etc.) could be linked to the establishment of chronic ER stress [5–7]. ER stress and UPR are, therefore, the focal point of a large number of endogenous or exogenous cellular stresses.

2.1. The Unfolded Protein Response

In mammals, UPR is triggered by activation of three ER transmembrane sensors: PERK, ATF6,and IRE1 [3,8,9]. The luminal part of these proteins integrates the information coming from the ER

lumen whereas their cytoplasmic part interacts with the effectors and mediates the signalling cascades (Figure 1). In absence of stress, the ER resident protein chaperone BiP interacts with the luminal domain of the three effectors and keep them in an inactive state. Upon accumulation of unfolded proteins in the ER lumen, BiP will act as a protein chaperone. Indeed, BiP has a relatively low but very broad affinity for hydrophobic regions of proteins, enabling it to recognise and bind a wide range of misfolded proteins exposing hydrophobic segments. As a consequence of its binding to the misfolded proteins, BiP is released from ATF6, IRE1, and PERK, leading to their activation [3,8,9].

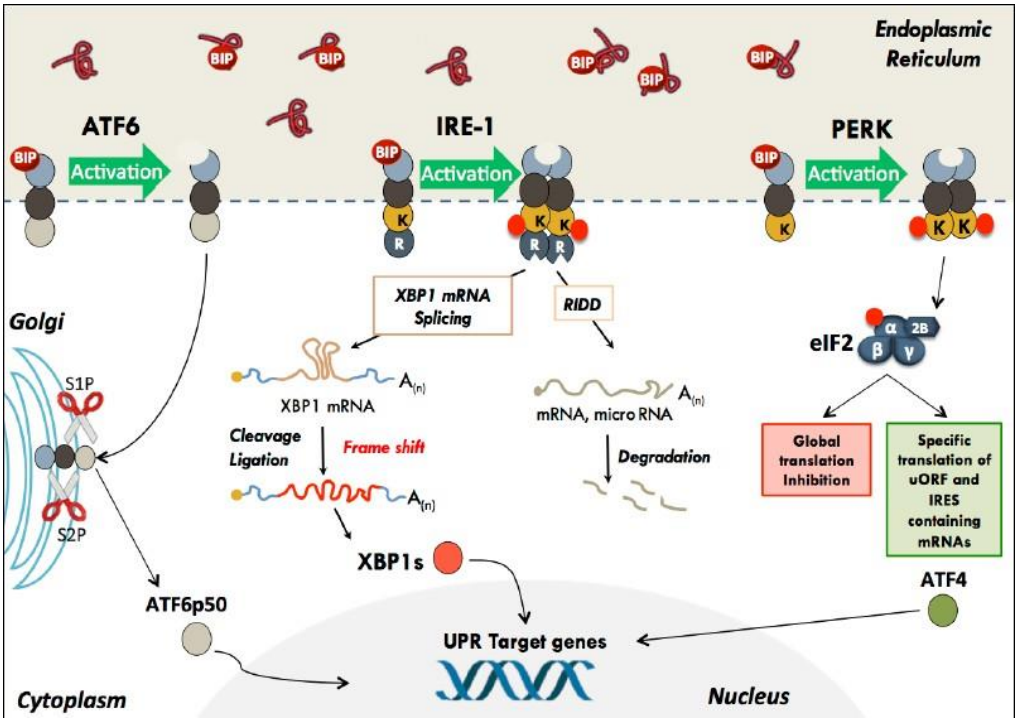

Figure 1. The different UPR effectors and their modes of action. In the basal state, the three UPR effector transmembrane proteins (PERK, ATF6, and IRE-1) are maintained inactive through their interaction with the protein chaperone BiP. The accumulation of poorly folded polypeptides in the ER lumen results in dissociation of BiP and activation of UPR. –I- PERK dimerises and phosphorylates the eIF2α subunit, leading to a global translation initiation inhibition. Specific mRNA subsets, containingcis-acting elements in their 5'UTR, such as uORF and IRES, escape translational inhibition triggered by eIF2 phosphorylation. –II- IRE-1 initiates an unconventional splicing of XBP-1 mRNA, as well as the degradation of some RNAs (this mechanism has been called RIDD for Regulated Ire1-DependentDecay) –III- ATF6 traffics to the Golgi where proteolysis liberates its transcription factor amino-terminal domain, which is nuclearised and activates the expression of target genes.

Each of the three activated pathways will contribute, sometimes in a redundant way, to the stress response. Indeed, activation of these 3 sensors, namely IRE1, ATF6, and PERK, induce protective feedback mechanism essential to restore ER homeostasis through translation inhibition, to reduce protein synthesis, enhancing degradation of misfolded proteins and transcriptional regulation of specific stress target genes.

More generally, it seems that many chaperone proteins, residing in the endoplasmic reticulum, may be involved in the regulation of the activation/inactivation of UPR sensors. First of all, the involvement of different proteins of the PDI family (Protein Disulfite Isomerase) has beenhighlighted. PDIA5 has been described to interact with the ATF6 protein by modifying its conformation and consequently facilitating its export from ER [10]. Similarly, PDIA6 binds the disulfide bridges ofthe active IRE1 and PERK proteins and promotes their inactivation [11]. More recently, an original

mechanism for activating IRE1 has been identified. The chaperone protein HSP47 directly binds the luminal domain of IRE1 and dislodges the BiP protein [12]. This mechanism promotes the activationof the IRE1 protein. Thus, HSP47 deficiency sensitises the cells to endoplasmic reticulum stress. Through screening of different IRE1 protein partners, this study revealed that other proteins such as mitochondrial ATPase Atp5h or phosphatase PP2a, are able to regulate IRE1 activity [12]. It has also been proposed that unfolded proteins may bind IRE1 thus promoting its oligomerisation and potentially modifying its activity [13].

The activation of the three UPR sensors, therefore, seems finely regulated and a better understanding of the mechanisms involved in these regulations could eventually allow each ofthese pathways to be specifically modulated.

However, after activation of these three pathways, UPR fosters cell survival in response to stressin the following three ways: (i) blockade of protein translation to re-establish homeostasis; (ii) positive regulation of molecular chaperones to promote protein folding (iii) up-regulation of signalling pathways responsible for targeting ER misfolded proteins to degradation after ubiquitination. In addition,to promoting cellular survival, UPR can however also induce apoptosis under chronic or unresolvedER stress [14,15].

2.2. ATF6

ATF6 is a basic Leucine Zipper transcription factor (bZIP) with two isoforms, ATF6α and β. ATF6β is a distant homologue of ATF6α, and both are ubiquitously expressed. These two isoforms differ in their transactivation domain. As a result, a modulation of transcriptional activity is observedbetween the two proteins and is lower in the case of ATF6β [16]. The individual Knock-Out (KO) of each of these genes does not cause embryonic lethality [17]. In contrast, the double KO ATF6α/$\beta^{-/-}$ triggers an embryonic lethality at E8.5, suggesting that some mutual compensations may be established between the two isoforms [17]. Unlike the other two sensors (PERK and IRE1), ATF6 is both a sensorand a direct effector of the UPR. Indeed, during stress, release of BiP unmasks a *Golgi* Localisation Signal (or GLS) in its C-terminal intra-luminal part [18]. ATF6 is then addressed to the Golgi and processed by two proteases (S1P and S2P) into an active ATF6p50 transcription factor [19].Thus activated, ATF6p50 is nuclearised and participates in the transcription of stress response geneswhose promoter contains UPRE (Unfolded Protein Response Element) or ERSE (ER Stress–Response Element) nucleotide motifs elements [19]. It activates specific transcriptional programs involved in

(i) ER folding capacities enhancement by activating chaperone proteins [20,21], and (ii) increased protein turnover through the Endoplasmic Reticulum Associated Degradation system (ERAD) by upregulating genes such as EDEM (ER Degradation Enhancing alpha-Mannosidase like protein) or HERP (Homocysteine-responsive ER-resident ubiquitin-like domain member 1 Protein) [17]. ATF6 also activates expression of several transcription factors such as CHOP (C/EBP homologous protein) and XBP1(X-Box Binding Protein 1) [22,23].

ATF6 has been associated with cancer development, however its role in tumours has not been fully elucidated yet. In chronic myeloid leukemia, ATF6 drives cell survival upon imatinib treatment [10]. Some evidences also showed that ATF6 plays an important role on cell dormancy in rapamycin-treated tumours [24]. All together, these findings shed light on the potential role of ATF6 in chemoresistance.

2.3. IRE-1

IRE1 is the most conserved UPR sensor in eukaryotic cells, and is also the only one that has an embryonic lethal knockout phenotype at E12.5, resulting from a defective placental vascularisation [25]. The mammalian genome encodes two IRE1 isoforms, IRE1α and IRE1β. The first one is ubiquitously expressed while IRE1β expression is restricted to intestinal epithelial cells [26] and airway mucous cells [27].

IRE1 is a bifunctional protein, characterised by two cytoplasmic catalytic domains in its carboxy-terminal part: a serine/threonine kinase domain fused to an endoribonuclease domain (RNAse). During endoplasmic reticulum stress, protein dimerisation/oligomerisation triggers trans-

autophosphorylation of the kinase domains, thereby inducing a conformational change leadingto the allosteric activation of the RNase domain [14]. IRE1 activates several downstream intracellular signalling pathways through its RNAse activity and through its kinase activity. Indeed, it has been reported that the kinase domain is able to recruit the protein TRAF2 (TNF receptor-associated factor 2). The IRE1/TRAF2 complex then interacts with ASK1 (apoptosis signal-regulating kinase 1) to activatethe JNK, c-Jun N-terminal kinase thus activating the pro-apoptotic ASK1/JNK (c-Jun N-terminal kinase pathway) [28,29].

The IRE1 endoribonuclease activity was first described for its role in cytoplasmic splicing of XBP1 (X-Box Binding Protein 1) mRNA. Once activated, IRE1 initiates the non-conventional XBP1 splicing by cleaving the mRNA at two sites in a conserved stem-loop structure folded sequence located in the open reading frame [30]. The excised sequence, whose length differs depending on the species,is composed of 26 nucleotides in humans.

Then, the cleaved mRNA is processed by the tRNA ligase RTCB [31]. This unconventional splicing results in a frame-shift that allows the expression of an extended protein encompassing the transactivation domain of the transcription factor: XBP1s (s for spliced). The proteins XBP1s and XBP1u (u for unspliced), therefore, only differ by the presence or absence of the transactivation domain located in the C-terminal part, which also influences the stability of the proteins.

XBP1 splicing by IRE1 is a co-translational mechanism [32–34]. The nascent XBP1 protein possesses a highly hydrophobic domain (HR2), which enables the RNA/protein complex to be addressed to the endoplasmic reticulum membrane. More recently, it has been proposed that this relocalisation could happen indirectly through the recognition of the HR2 domain by the SRP (Signal Recognition Particle), which addresses the XBP1 mRNA to the IRE1/SEC61 complex [32]. A translational pause site would also facilitate this step [33].

Once activated, XBP1s induces the transcription of target genes for stress response by binding toUPRE or ERSE sequences in the promoter, but ChIP-seq studies have also shown that the role of XBP1s can extend to other cellular processes such as differentiation [35].

The endoribonuclease activity of IRE1 has been implicated in an additional process. For some other RNA targets, including both non-coding and coding RNA, the endonucleolytic cleavage by IRE1, sensitises these transcripts to the action of cytoplasmic exonucleases, triggering RNA decay. This mechanism, first described in Drosophila, is termed RIDD for Regulated IRE1-Dependent Decay [36]. Currently, only few direct targets have been characterised, however transcriptomic and bioinformatic studies seem to reveal a much broader spectrum of action [37,38]. Among the characterised RNA targets are 4 microRNAs (miR-17, 96, 125b, 34a) [39] and some mRNAs including PER1 [40], SPARC [41], BLOS1 [42], and DR5 [43]. Prediction of the RNA targets is relatively complex because no consensus sequence has been clearly defined even though an "XBP1-like" stem loop structure can be found at the cleavage site [44].

It remains to be determined how IRE1 selects between XBP1 splicing and RIDD. Given that bothdimers and IRE1 oligomers have been detected during ER stress [45–47], it is likely that the RIDD mechanism can be influenced by IRE1's oligomerisation status. Indeed, in addition to its intra-luminal dimerisation domain, IRE1 also contains a cytoplasmic oligomerisation domain leading to the formation of clusters that can be visualised as foci at the ER level during UPR [47]. While some studies showedthat a high oligomerisation is correlated with activation of the RIDD [48], other experiments revealedan opposite effect [47]. Therefore, the precise impact of IRE1 oligomerisation status on XBP1 splicingand RIDD awaits further clarification.

IRE1 mutations can be found in cancers however the biological significance remains to be determined [15]. For instance, in glioblastoma, IRE1 has been widely investigated and shown to contribute to cancer progression by different mechanisms such as promotion of angiogenesis, tumour invasion and also inflammation [49]. Whereas, another study reported a negative regulation of invasion by IRE1 in a glioblastoma model [50].

Interestingly the main downstream effector of IRE1, XBP1 has also been found mutated in cancer [51]. Despite recent efforts to investigate the RIDD branch, XBP1 remains the most described target of IRE1

and thus the most studied in cancer, so far. In triple-negative breast cancers, upon hypoxia,XBP1 cooperates with HIF1a to promote tumour growth and foster relapse by activating pathways such as angiogenesis and glucose metabolism [52]. However, the pro-tumoural or anti-tumoural roleof XBP1 in cancer is discussed and is probably context-dependent. In Multiple Myeloma, for instance,a high expression of XBP1s correlates with a lower response to thalidomide-based treatment [53] while a high XBP1s expression correlated with a better response to Bortezomib-based chemotherapy [54]. Moreover, two inactivating mutations of XBP1 have been characterised in multiple myeloma patients and are responsible for resistance to bortezomib treatment [55,56]. Therefore, further investigations are required to understand the role of the canonical and non-canonical IRE1J's pathways in cancers.

2.4. PERK

PERK is found in all metazoans and has the same domain organisation as IRE1; both proteins share a structurally- and functionally-related intra-luminal sensor domain [57] but a different cytoplasmic domain. The PERK monomer (encoded by the EIF2AK3 gene for eukaryotic translation initiation factor 2-alpha kinase 3) is located inside the ER membrane. The intra-luminal N-terminal part of PERK binds to BiP, which prevents its dimerisation, while its cytoplasmic C-terminal region contains a serine/threonine protein kinase domain. During the UPR, BiP is released from PERK, thereby allowing dimerisation and trans-autophosphorylation at Thr-982, which endows PERK with its catalytic activity [58,59]. Activated PERK, in turn, phosphorylates the subunit of eukaryotic initiation factor eIF2α at serine
51. The final consequence of PERK activation is the decrease in protein synthesis by rapid and potent inhibition of global translational initiation.
Other PERK substrates have been identified: (i) Nrf2 (Nuclear factor (erythroid-derived 2)-like
2) transcription factor, which is a master regulator of redox homeostasis and whose stability is increased after PERK-dependent phosphorylation of the threonine 80 leading to increased Nrf2 nuclear import [60]. (ii) FOXO3, one of the Forkhead transcription factor family members which regulates a set of genes that contribute to cellular homeostasis and whose activity is increased after its PERK-dependent phosphorylation of serines 261, 298, 301, 303 and 311 [61]. (iii) Multiple DAG (diacyglycerol) species, which are important second messengers [62].
A key point of the present review is the PERK-eIF2α UPR signalling pathway, which is the moststudied and, therefore, the best characterised. It's however important to note that three additional eIF2α kinases have been discovered in mammalian cells which can be activated by different stresses: PKR, GCN2, and HRI. PKR is activated by long double-strand RNA and thus senses viral double-strandedRNA in infected cells [63–65]. GCN2 (general control nonderepressible 2) is activated by amino acid starvation through binding to uncharged tRNAs [66], or by UV irradiation [67,68]. Heme-regulated inhibitor (HRI) is known to get activated in various stresses, such as heme deficiency or heat shock inerythroid cells [69].
Much evidence showed an essential role of PERK in cancer. The PERK-ATF4 pathway induces autophagy in MYC-induced lymphoma and support the transformation process and tumourgrowth [70,71]. Furthermore, the PERK pathway has been reported to trigger a multidrug resistance phenotype in different tumour types through the PERK/Nrf2/MRP1 axis [72]. PERK is also closely linked to the anti-oxidative response. Whereby, by limiting oxidative DNA damages, PERK has been shown to enhance tumour growth [60]. The PERK-ATF4 axis has also been associated with metastasis through the transcription of matrix metalloproteinases [73]. Preclinical use of PERK inhibitors has shown great efficiency in pancreatic cancers [74]. However, the complexity of the downstream network activated by PERK suggests that the effect of PERK activation needs to be addressed in a context-dependent manner.

3. Translational Regulation: Dealing with Endoplasmic Reticulum Stress

Many studies have supported the idea that the UPR requires translational reprogramming, in which protein synthesis is globally repressed and is accompanied by the preferential synthesis of a specific subset of mRNAs whose protein products are required for responding to ER stress [45,75,76].

Indeed, while IRE1-XBP1 and ATF6 are well known to elicit a transcriptional response, the PERK pathway mainly induces an overall translational shutdown response by phosphorylating eIF2α, but it also enables increased translation of many stress-related genes including the transcription factor ATF4, which mediates a secondary transcriptional response. Although global translation inhibition allows cellular resources to be preserved, an efficient synthesis of some factors is necessary to cope with the consequences of stress. Mammalian mRNAs whose expression is known to escape translational inhibition triggered by eIF2α phosphorylation contain specific features in their 5′ untranslated region including uORF or IRES. After a brief reminder of the basics of mRNA translation initiation, we will develop some examples of RNA whose translation is upregulated during ER stress in more detail in the following paragraph. We will also discuss studies demonstrating the involvement of IRE1 RNase activity in translational regulations during reticulum stress.

3.1. Canonical Cap-Dependent Translation Initiation

Translation is a high demanding energy process, which needs to be rationalised upon stress conditions. The most well-known stress-relative pathways have been described to play critical roles in the regulation of the initiation step. In a nutshell, the general cap-dependent translation is turned off under stress conditions while the translation of some specific mRNAs is maintained or activated. Therefore, in order to understand the specific alternative routes of translation involved during stress,it is essential to understand the fine-tuning of the canonical cap-dependent translation initiation (Figure 2A).

Ribosomes were identified as the link between mRNA and proteins in the 1950s, while the m7G-cap discovery came later with a first description in viruses in 1975 [77]. During those years, many efforts were made to identify this structure in eukaryotic mRNA and eventually led to the characterisation of the cap structure in HeLa and mouse myeloma cells [77]. The cap consists of a methylated guanine (m7G), which is engaged in an unconventional 5′ to 5′ triphosphate linkage to the mRNA. This structure plays an essential role in the mRNA stability and the regulation of translationinitiation [77].

In order to interact with the cap, start scanning and initiate translation, the 40S small ribosome subunit (SSU) needs to be loaded. This priming is orchestrated by key actors of the translation, calledeukaryotic initiation factors (eIFs): eIF3, eIF1/1A. In brief, eIF3 is binding the SSU in order to allow itsrecruitment at the cap. On the other hand, eIF1 and eIF1a are regulating the tRNA binding by stabilizing the preinitiation complex (PIC) in an open conformation. A third member of the eIFs family, eIF2 binds the SSU within a complex that also includes the methionine RNA transfer (Met-tRNAi), hence creating the ternary complex. The association of eIF2 and Met-tRNAi is allowed when eIF2 is loaded with a GTP. Therefore, the eIF2-GDP recycling into eIF2-GTP is a critical, rate-limiting, and highly regulatedstep, which is catalysed by the guanine exchange factor, eIF2B. At this stage, the SSU is comprisedof eIF3, eIF1/1A and the ternary complex (eIF2-GTP-Met-tRNAi) thus forming the 43S pre-initiation complex [78–81].

The 43S PIC complex has the ability to bind the m7 GTP-cap through the heterotrimeric eIF4F complex composed of three non-identical subunits: the cap-binding protein eIF4E, the DEAD-box RNA helicase eIF4A, and the large “scaffold” protein eIF4G. This interaction recruits the PIC on the mRNA and the ATP-dependent scanning of the 5′UTR is initiated. When the first AUG enters the P-site of the SSU, the perfect codon/anti-codon matching triggers irreversible GTP hydrolysis in the ternary complex. Cooperative events are also required to fully complete the AUG recognition such asthe release of eIF1 and 2. Then the binding of the 60S subunit to the 40S is catalysed by eIF5B-GTP andenables the first elongation step [78].

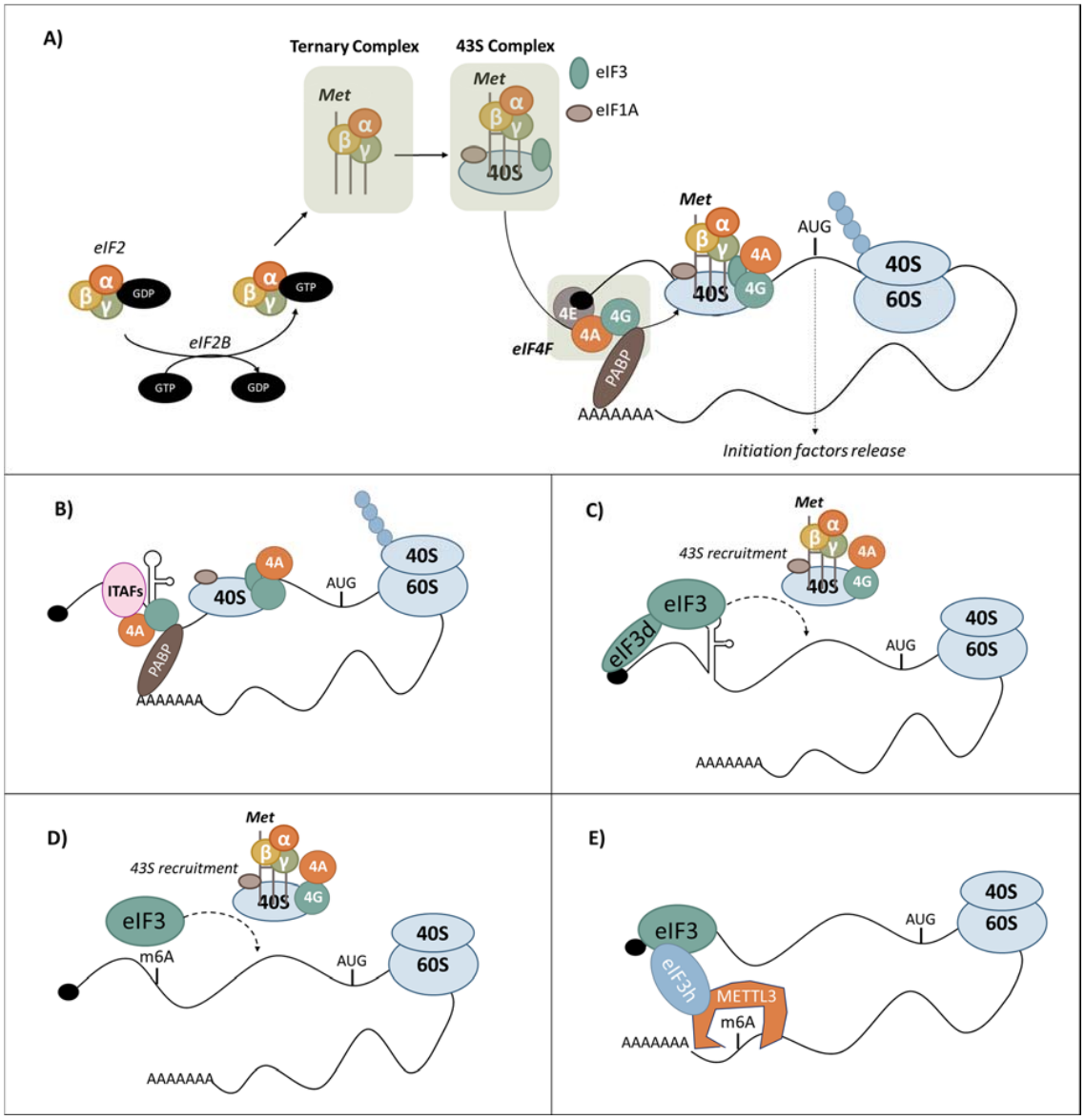

Figure 2. Currently known processes of translation initiation (**A**) Cap-dependent mechanism of translation. The eukaryotic initiation factor 2 (eIF2)-GDP is recycled in eIF2-GTP by the enzyme eIF2B.eIF2-GTP binds the methionine transfer RNA (Met-tRNAi) in order to form the ternary complex which integrates the 43S complex comprising the 40S ribosome subunit, eukaryotic initiation factors (eIF3, eIF1/1A) and the ternary complex. 43S is recruited to the mRNA through the m7G cap by interacting with the eIF4F complex (eIF4E, eIF4A, eIF4G) and 43S scans the 5^{J}UTR until the first starting codon. The codon/anti-codon interaction triggers the release of initiation factors and the recruitment of the 60S, and then elongation can start. (**B**) Internal ribosome entry sites (IRES)-mediated translation initiation.The IRES directly recruits ribosomes, thereby bypassing the requirement of the mRNA 5^{J} cap structure.
(**C**) The binding of the cap by the eIF3d subunit in presence of the stem-loop in the 5^{J} UTR can bypass the canonical eIF4E translation and initiate an eIF3d-directed cap-dependent mRNA translation. (**D**) A single 5^{J} UTR-located N(6)-methyladenosine m(6)A can promote cap-independent mRNA translation initiation, through direct interaction with eIF3 which is sufficient to recruit the 43S complex and initiatetranslation even in the absence of the cap-binding factor eIF4E. (**E**) METTL3 enhances translation of mRNA containing m(6)A in its 3^{J}UTR through interaction with eIF3h.

Although the 5JUTR plays a central role in the translation initiation process, it is important to mention that the 3JUTR is also involved in the regulation of translation initiation. Indeed, the poly(A)-binding protein (PABP) interacts with the cap through eIF4G and eIF4B, leading to the circularisation of the mRNA. A deregulation of this interaction can compromise translation [79]. Furthermore, circularisation of mRNA brings the 3JUTR binding regulators, such as micro-RNA or RNA binding proteins, next to the 5JUTR, giving them the ability to modulate the translation initiation step [81]. A key example is the mechanism of action of the Bicoid protein, which regulates the translation of mRNA during the development of Drosophila. Bicoid suppresses translation of caudal mRNA at the anterior of the embryo by binding the 3JUTR and an eIF4E-related protein, whichcompete with eIF4E in the binding of eIF4G [82].

3.2. Translational Control PERK-Mediated under ER Stress

Due to its central role during the recruitment of the initiator tRNA, the alpha subunit of eIF2, eIF2α, is one of the main targets for translation inhibition in the case of cellular stress and. as indicatedpreviously represents the main PERK substrate. The phosphorylation of eIF2α on serine then induces overall translational impairment but mRNAs encoding stress response proteins must be able to escape global translation repression induced by PERK-mediated eIF2α phosphorylation, and many mechanisms have been developed to do so.

3.3. Selective mRNA Translation during eIF2 Phosphorylation:

3.3.1. Regulation by uORF

Upstream Open Reading Frames: uORFs

uORFs represent one of the major regulatory motifs present in the 5J UTR, which have been found to be involved in translational regulation under stressful conditions. In this context the repression imposed by uORFs on the initiation of translation of the main ORF is relieved, thus allowing the production of specific proteins in response to stress. The mode of action of each uORF appears dictated by its initiation codon context, secondary structure and coding capacities [83,84]. Moreover, the overall regulation of a given mRNA will depend on the specific combination and organisation of uORFs in its5J terminal region [85].

The precise mechanisms by which eIF2α phosphorylation regulates the relative translation initiation efficiencies at uORFs and at their downstream protein-coding sequences remain not entirelyclarified and may be diverse depending on the nucleotide sequence and relative organisation of the different translation initiation regions [85]. For some genes which exhibit preferential translation during ER stress, such as ATF4 and C/EBPβ it has been proposed that during stress the lowered eIF2-GTP level resulting from eIF2α phosphorylation on Ser51 induces a reduction of the eIF2-GTP-met-tRNAi ternary complex intracellular concentration. This would delay the reacquisition of the ternary complex by the 40S ribosome after the translation reinitiation, which follows the completion of uORF translation andallow the scanning ribosome to skip downstream additional inhibitory uORFs sequences. This mode of regulation has been widely accepted by scientists in the field and has been proposed to take place inother stress-regulated genes as well [85]. However, kinetic and stoechiometric data validating this mechanism are still missing. Furthermore, the nucleotide sequence around the start codon of the uORF also influences the efficiency of translation of the downstream sequence and it has been shown that mRNAs which are preferentially translated upon stress frequently contain uORFs with suboptimal Kozak consensus sequences [86]. The phosphorylation of eIF2α on Ser51 may induce conformationalchanges, which impair initiation at AUG codons with suboptimal sequence context [87]. A decreasedrecognition of uORFs due to their unfavourable Kozak context has been proposed to be at least in partinvolved in the increase of expression of GADD34 [88] and CHOP [89,90] during ER stress.

It is currently assumed that the translation of uORFs is detrimental to the expression of the downstream coding sequences. These uORFs may exert their repressive effect by diverse, non-exclusive, mechanisms such as direct competition for translation initiation, translation elongation stall [89], and increased ribosome release for the translated mRNA [88]. In addition, uORFs could contribute tothe inhibition of downstream CDS expression through mechanisms unrelated to mRNA translation initiation per se, such as increased mRNA decay [85,91]. However, a recent elegant research work demonstrated that most of the uORFs may actually remain translated during stress [92] and in addition that translational activation of the main coding sequence may involve at least in part specific translation initiation factors such as eIF2A [92]. Therefore, additional studies are still needed to fully elucidate the precise mechanisms of translational activation during ER stress.

A recent study illustrated well the relevance of investigating uORF in cancers. From different tumour samples, a screen based on a multiplex identifier-tagged deep sequencing, revealed 404 uORFand two loss-of-function mutations in uORF in EPHB1 and MAP2K6. By luciferase- assay, the authors confirmed that the observed mutations lead to an increase of translation of the downstream reporter.In parallel, whole exome sequencing allows one to identify another 53 deleting mutations in uORF suggesting that uORF-associated mutations can contribute to rewire the translation in cancers [93].

uORFs Translation upon ER Stress

Some of the proteins that were shown to be upregulated through an uORF-dependent mechanism are known to play a central role in determining cell fate following stress induction. The ER chaperone BiP/Grp78, a master regulator of ER stress signalling, contains 2 uORFs encoded from non-canonical initiation codons and the translation of these ORFs, which involves at least in part the translation factor eIF2A, was found to be necessary for sustained BiP expression during stress and, therefore, general modulation of the stress signalling response [92]. The activating transcription factor 4 (ATF4) regulates the expression of a variety of cytoprotective genes acting in particular on amino acid metabolism and oxidative stress damage and which participate in the recovery of the cell from the injuries, which induced the stress response. ATF4 is however also involved in the activation of the transcriptionof CHOP (C/EBP Homologous Protein)/GADD153/DDIT3 [22,94,95], a transcription factor which when expressed to high levels can activate the transcription of a set of genes promoting caspase activation and cell death. On the other hand, ATF4 and CHOP both up-regulate the expression of GADD34 [96,97], an activatory subunit of the protein phosphatase 1 complex, which is involved in the dephosphorylation of α and resumption of translation. Moreover, the combined action of the ATF4 and CHOP transcription factors at the promoters of a set of genes involved in protein synthesishas also been found to contribute to the re-induction of global translation [98]. Reactivation of proteintranslation following stress may have opposing effects on cell viability depending on the cellular context. Whereas resumed translation will participate in cell recovery after stress-associated injurieshave been repaired, increased protein translation can also lead to cell death resulting from ATP depletion and oxidative stress [98]. Therefore, the precise and timely-controlled expression of factorssuch as ATF4, CHOP, and GADD34 regulating global translation as well as repair of cell damage or entry into apoptosis is essential to the cell in order to adequately cope with the stress response.

Interestingly, the mRNAs coding for these different proteins have all been found to contain functional uORFs that inhibit their translation under normal conditions. Upon stress induction however the inhibition imposed by the uORFs on ATF4, CHOP, and GADD34 is strongly abrogated and this mostly contributes to the re-expression of these different factors in the cell. Two uORFs are presentin the 5^{J} region of ATF4. The most downstream uORF overlaps with the ATF4 coding sequence and inhibits its translation in normal conditions and low eIF2α phosphorylation [99,100]. A unique uORFencoding a specific peptide motif able to stall translation elongation and to inhibit CHOP translation in the absence of stress has also been identified in its mRNA [90]. The mRNA encoding GADD34 contains two uORFs. The most 5^{J} proximal sequence acts as a slight attenuator of GADD34 translation whereas the second uORF strongly inhibits downstream protein translation by likely promoting release of theribosome from the mRNA after the uORF STOP codon has been reached [88,101]. The recognition and translation of the

uORF may however in some instances be required for the up-regulation of proteins during stress. This is the case for the C/EBPβ transcription factor, which is expressed as three different isoforms produced from translation events occurring at differentinitiation codons. The shortest C/EBPβ isoform (LIP: liver-enriched inhibitory protein) has been reported to interact as an heterodimer with the CHOP transcription factor and to be essential to its nuclear relocalisation and the activation of its target genes, resulting in the induction of apoptosis [102]. The highly increased production of LIP during sustained endoplasmic reticulum stress involves the translation of an uORF allowing the ribosome to skip the initiation codon of the following main C/EBPβ isoform (LAP: liver-enriched activatory protein) and start translation from the LIP initiation codon [103].

Additional proteins involved in the metabolic recovery of cells and whose expression is inducedduring stress through an uORF-dependent manner have been identified. For example, the aminoacyltRNA synthetase EPRS is involved in the charging of tRNAs with glutamine and proline residues, therefore restoring the available glutamyl- and prolyl-tRNA intracellular pools. Two inhibitory uORFs initiated at non-canonical codons (CUG and UUG) have been identified in the EPRS mRNA and shown to reduce EPRS translation under normal conditions [104]. This inhibition is relieved during stress, leading to the re-accumulation of EPRS in the cell. The amino acid transporter Cat-1, which mediatesthe uptake of the essential amino acids arginine and lysine, is induced during ER stress by different complementary mechanisms [105]. In particular, the translation of an uORF has been shown to play an essential role in the re-expression of Cat-1 by an original mechanism which involves the modificationof the folding and reactivation of a cryptic IRES element in the 5′UTR of Cat-1 mRNA [106].

The currently available data strongly emphasise the essential role played by the upstream ORFsin the translational control of genes involved in the recovery from cell injuries and in the regulation ofthe stress response itself. Whatever the mechanisms operating, the uORF-dependent regulation of translation appears tightly controlled by the intracellular levels of phospho^{51}-eiIF2α, which can be rapidly modulated during the stress response. It therefore represents a highly sensitive and responsive mean to regulate specific protein expression under stress conditions. Mutations affecting the activity of uORF and consequently the translation of the downstream ORF have been reported in different cancermodels [83]. It is likely that uORFs mutations could as well affect the expression of stress-regulated factors and favour some cancer-associated processes but this has still not been reported to date.

3.3.2. Cap-Independent Translation RegulationIRES-

Dependent Translation Initiation

In the early 80s, the cap-dependent mechanism was believed to be the only possible mechanism of translation initiation in eukaryotic cells. An alternative route that bypasses the initial cap-recognition, allowing ribosomal recruitment to internal locations in mRNA, termed internal ribosome entry sites (IRESs) was however revealed more than 30 years ago (Figure 2B) [107–109]. The IRESs were first discovered in the late 1980s in studies on poliovirus [108,110], whose characteristics are incompatible with cap-dependent translation initiation and encephalomyocarditis virus (EMCV) [109]. Indeed, poliovirus mRNA is naturally uncapped and the virus itself interferes with the cellular translation by proteolytic degradation of the cap-binding proteins, implicating that poliovirus mRNAs must be translated by cap-independent mechanisms. Many groups confirmed this notion for example by showing, through means of mutagenesis, that an internal sequence in the 5′UTR of poliovirus RNA was responsible for its cap-independent translation and this sequence could also confer cap-independent translation to heterologous mRNAs [108–111]. During the same years, a cellular mRNA, encoding glucose-regulated protein 78 (GRP78)/immunoglobulin heavy-chain binding protein (BiP), was found to be translated at an increased rate in poliovirus-infected cells, while cap-dependent translation was inhibited [107], showing for the first time that translation initiation by an internal ribosome-binding mechanism was used by eukaryotic mRNAs [112,113]. Since that time, many viraland cellular IRESs have been reported and recorded in the IRESite database [114]. More recently, the existence of IRES elements in cellular mRNAs was investigated using a high throughput strategy,which highlighted the

existence of thousands of sequences, allowing cap-independent translation, and showed that 10% of mRNAs could potentially be translated by a cap-independent mechanism [115]. Interestingly, many IRES-containing mRNAs encode proteins that are involved in proliferation, differentiation, and apoptosis and can be translated when the overall cellular protein synthesis is inhibited upon different stress conditions, including ER stress, apoptosis, viral infection, nutrient starvation and hypoxia [116] generating ongoing interest in the field of protein translation and its regulation.

Despite a growing list of IRES-containing mRNAs, the mechanism of internal initiation is still poorly understood. Given that many IRESs have been identified in conditions of inhibited cap-dependent initiation, the cap-binding protein eIF4E and the scaffolding protein eIF4G do not seem to be required for IRES-mediated translation [107,117], although, it has been reported that,for example, MYCL IRES requires both eIF4E and eIF4G for its translation [117]. Curiously, homologs of eIF4G, such as eIF4G1, eIF4G2 [113,118] and DAP5/p97 [118,119], are shown to be associated with polysomes in poliovirus-infected cells and, at the same time, to be required for the IRES-mediated translation of selected mRNAs following cellular stress [119], implicating that eIF4G proteins are needed in both 5^{J} cap-independent and 5^{J} cap-dependent translational initiation mechanisms. Finally, the activity of the RNA helicase eIF4A seems to be essential for the translation of MYC, MYCN [120] and BIP IRESs [121]. The role of eIF2 has also been investigated for cellular internal initiation. Importantly, IRES-mediated mRNAs translation can operate upon global protein synthesis attenuation induced by eIF2 phosphorylation [116,122–127]. In addition to the involvement of canonical initiation factors, efficient IRES-dependent translation requires auxiliary RNA-binding proteins, known as IRES trans-acting factors (ITAFs; Figure 2B) [128]. The mechanism of ITAF function is not fully understood, but it is generally believed that many ITAFs are required for the stabilisation of IRES conformation. Importantly, the subcellular localisation of ITAFs have been shown to be crucial for their function [128] (described below). Examples of ITAFs include La autoantigen [129] and several heterogeneous nuclear riboproteins (hnRNP) such as hnRNPC1/C2 [130] and hnRNPA1 [123,131].

One of the most relevant examples highlighting IRES importance in cancers is the MYC IRES inmultiple myeloma. Indeed, a point mutation in this specific IRES sequence was identified in 42% of patient bone marrow samples [132]. This mutation enhances the translation of the proto-oncogene MYC suggesting that IRES deregulation could be responsible for overexpression of oncogenes. In silico analysis fail to reveal IRES that need to be functionally tested in-vitro, which render their identification laborious hence the list of IRES is still limited. However, IRESs have already been described in many other mRNAs encoding proteins involved in tumourigenesis and cell survival (Apaf-1, cJUN, AML1/Runx1, EGFR/HER1, BCL2, BCL-XL, XIAP, MYC, MYCN, VEGF-A, P27, P53), suggesting that

IRES-mediated translation play a crucial role in tumour progression and survival [133]. Direct evidence that supports this hypothesis comes from many studies. For example, 3D spheroids culture of ovariancancer cells treated with a PI3K/mTOR inhibitor reveals resistant cells expressing BCL2, which is indeed translated by a cap-independent mechanism in these conditions [134]. IRESs mediated translation also promotes inflammatory breast cancer tumour cell survival and formation of tumour emboli by activating p120 catenin mRNAs expression [135]. Another example concerns the P53 protein, which is also translated by a cap-independent mechanism due to the presence of two IRES residing within the 5^{J}UTR and the coding sequence [136,137]. These IRES, activated in response to DNA damage, binds several ITAFs including DAP5 (or p97 or NAT1) which is a member of the eIF4G proteins,the translational control protein 80 (TCP80), Ribosomal protein L26 (RPL26) and nucleolin [138–140].If these proteins are either over or underexpressed in the cells, or if mutations affect p53 mRNA IRESstructure, p53 protein level can fluctuate [136,141]. Thus, some wild type p53-expressing cancer cells may not express p53 due to an IRES-dependent defective translation demonstrating the key role of IRES-mediated translation initiation in cancer development.

IRES Regulation in Response to Stress

Even if cellular IRESs have been described in a limited, but growing, number of mRNAs, many genes involved in stress response, such as HSPA5, ATF4, HIF1 α, NRF2, FGF-2, VEGF-A, VEGF-C, STAUFEN

or DLL4 are thought to contain IRESs [123,142–151].

Several master regulators of the UPR can be translated by a cap-independent mechanism. Remarkably, the BiP transcript was the first cellular mRNA reported to contain an IRES [112]. Moreover, ATF4 translation is regulated by either uORFs or an IRES. Indeed, an alternatively spliced variant of ATF4, expressed in leukocytes and induced by UPR, is translated by a cap-independent mechanism, which is activated by PERK-mediated eIF2α phosphorylation [152]. The presence of these elements therefore allows these mRNAs to be efficiently translated in stress conditions.

PERK activation also results in an IRES-dependent activation of TP53 isoform translation [153]. It has been demonstrated that two TP53 isoforms, TP53 and TP53/47, are translated by two different IRESs located on the same mRNA [137,154]. In stressful situations, an increase of the TP53/47 isoform-dependent IRES translation results in a cell cycle arrest in the G2 phase, while the fulllength TP53 induces a G1 phase arrest [153,154].

Cellular IRESs are often found in long and GC-rich structured 5ʲUTRs, and are relatively ineffective in directing translation under physiological conditions. However, the precise molecular mechanism of cellular IRES-directed translation in stress conditions when eIF2α phosphorylation is not completely understood.

Stabilisation of secondary structures, for example through interaction with proteins, could slowdown the scanning of the 43S pre-initiation complex and thus promote translation re-initiation efficiency [155]. According to the "land and scan" initiation model used by the already characterised cellular IRESs, the 40S subunit lands at the IRES before scanning from 5ʲ to 3ʲ to the initiation codon [156–158]. Since IRESs are highly structured elements, one hypothesis is that these elements could represent a barrier that would slow down ribosome progression, thus explaining the increase inIRES-dependent translation under eIF2α phosphorylation conditions. In the same way, these structures, potentially bound by proteins, may also be pause sites allowing the ribosome to recruit active initiating factors, thus stimulating translation when eIF2α is phosphorylated.

As previously mentioned, most IRESs, require ITAFs for their regulation [159]. The expression and the activity of these ITAFs can be modulated by UPR. For example, under ER stress, caspase-12 cleavage of the ITAF eIF4G2 (DAP5/p97) produces a fragment known as p86 [160] enhancing the IRES-mediated translation of HIAP2 (human apoptosis protein 2 inhibitor) Apaf-1 and XIAP [161,162] leading to thereduction of apoptosis and allowing the UPR to cope with stress [163].

Subcellular relocalisation of ITAFs plays also a crucial role in the modulation of IRES-dependent translation efficiency [128]. This is the case of hnRNPA1, which is relocalised from the nucleus tothe cytoplasm during ER stress [131]. Moreover, hnRNPA1 cytoplasmic accumulation requires eIF2α phosphorylation [164], and was shown to modulate IRES-dependent translation of SREBP-1a, c-MYCor DLL4 in response to endoplasmic reticulum stress [123,131,165].

Such relocation has also been documented for other ITAFs, including PTB and PCBP1 (poly r(C)1 binding protein or hnRNPE), which work jointly to activate BAG1 IRES (Bcl-2 Associated with Athanogen 1) following chemotoxic stress [166]. Nucleolin is also translocated from the nucleolus to the cytoplasm and activates the VEGF-D IRES-dependent translation in response to heat shock [167]. Interestingly both chemotoxic stress and heat shock are known to activate the UPR.

The Angiogenesis Paradigm

Angiogenesis is critical for many physiological processes, such as embryonic development andwound healing, but also in pathological states including the development of solid tumours.
As previously indicated, more than 100 mammalian mRNAs harbour IRESs in their 5ᴶUTRs.
Interestingly, these mRNA include many mRNA encoding proteins strongly involved in the angiogenic process like VEGF-A, VEGF-C, VEGF-D, FGF-2, HIF1α, DLL4 or TSP1 [123,142,148,167–171].
Angiogenesis depends on the highly coordinated action of a variety of angiogenic regulators, the most prominent and best characterised being Vascular Endothelial Growth Factor A (VEGF-A), FibroblastGrowth Factor 2 (FGF-2) and DLL4. Indeed, DLL4 is with VEGFA one of the few examples of haplo-insufficiency, resulting in obvious vascular abnormalities and in embryonic lethality [172–174]. It was already demonstrated that VEGF-A, FGF-2 and DLL4 IRESs are activated upon stress conditions including hypoxia or ER stress and that these mRNAs remain efficiently translated under

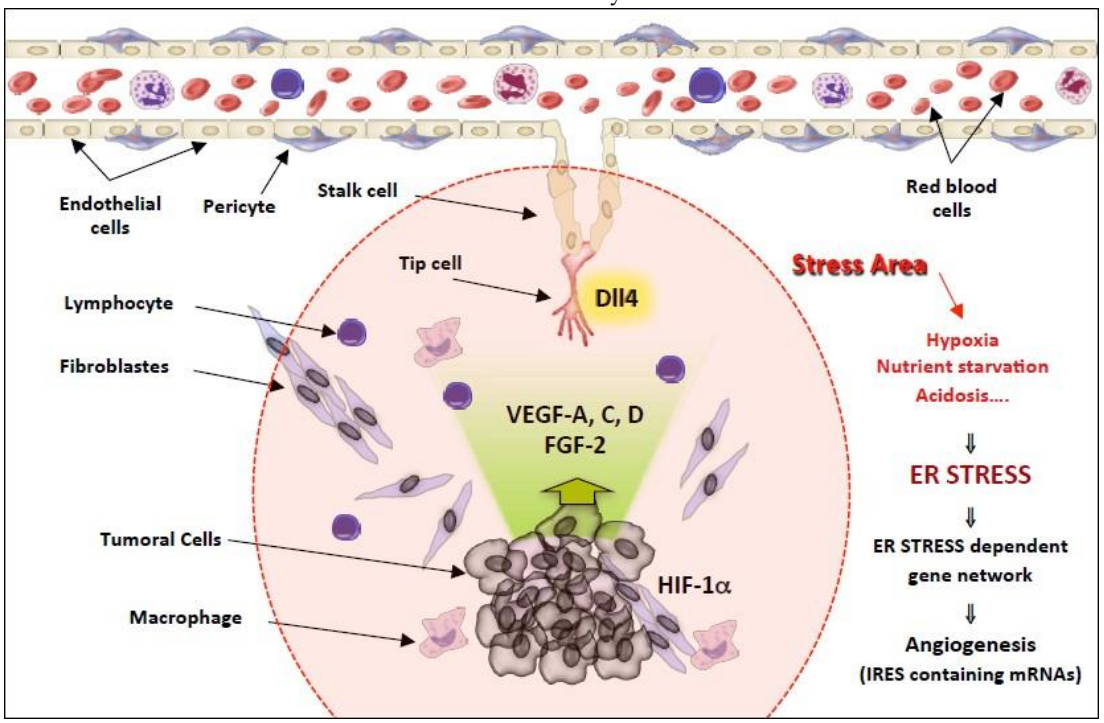

Figure 3. Schematic model of the network of gene expression co-regulation by IRES elements in stressconditions during tumoural progression. During tumour progression, the stress zone encompasses the growing tumour, but also its microenvironment. Both the tumour and the neo vessels, more particularly the Tip cells located at their extremity, which guide the neo vessels towards the tumour, are located in this unfavourable microenvironment. Hypoxia, nutrient starvation, and acidosis will irremediably induce the accumulation of unfolded protein in the reticulum of cells located in this area, leading to endoplasmic reticulum stress and UPR activation. Thus, in addition to transcriptional regulations, the activation of the PERK pathway will induce the co-regulation an UPR-dependent gene network containing IRES elements,revealing a translational regulon in which the synthesis of a cohort of angiogenic master regulator genes including VEGF-A,C,D, FGF-2, DLL4, and HIF1 is activated in response to ER stress. The fine-tuning of gene expression allows for efficient angiogenesis, which is a highly regulated process.

ER stress conditions despite phosphorylation of the major PERK substrate, eIF2α [123,124,142,168]. These results are consistent with the fact that tumours derived from K-Ras-transformed $Perk^{-/-}$
MEFs (mouse embryonic fibroblasts) display less angiogenesis and grow less rapidly than tumours with an intact UPR signalling [175], demonstrating the role of PERK activation in the angiogenic process.
The presence of IRESs in many mRNAs encoding proteins tightly involved in the angiogenic process enables a selective co-regulation of these mRNAs expression under stress conditions (Figure 3). The tumour microenvironment is composed of a set of tumour and stromal cells and extracellular matrix. During tumour

progression, impaired vascularisation causes several stresses including hypoxia, glucose or amino acid starvation or acidosis. These unfavorable conditions are known to induce ER stress, phosphorylation of eIF2α and thus activation of a gene network dependent on this phosphorylation in the stress area surrounding the tumour. Consequently, mRNAs encoding VEGF-A, -C, -D, FGF-2, HIF1A or DLL4 that are expressed by tumour cells or microenvironment (such as DLL4 expressed by TIP cells which are furthest away from the circulating blood are still efficiently translated while cap-dependent initiation is compromised (Figure 3). IRES therefore function as cis-acting regulons during ER stress.

These results demonstrate that for cancer to progress under stressful conditions, it must use alternative translation mechanisms, such as IRES-dependent translation, to promote angiogenesis and thus survival and growth.

3.4. mRNA Translational Control by IRE1 under ER Stress

Even if it is much more anecdotal, several studies have proposed that IRE1 also participates in translation repression during ER stress.

First of all, IRE1 selectively suppresses secretory protein translation by targeting mRNAs through RIDD to alleviate the load on the protein folding machinery. As indicated previously, a few dozens of mRNAs are known to be targeted by IRE1. Even if a consensus cleavage site embedded ina stem loop structure has been identified in vitro [37,176,177], it remains particularly difficult to predict the direct RIDD targets. The question arises of how these RIDD mRNA targets are addressedto the reticulum membrane. Indeed, most but not all these mRNAs encode proteins with signal peptide/transmembrane domains [36]. It was demonstrated that the removal of the signal peptide from a known RIDD target impedes its degradation and, inversely, introduction of a signal peptide to the GFP mRNA is sufficient to favour its degradation by IRE1 [178]. However, the question of the targeting of messenger RNAs that do not code for secreted or membrane proteins remains open. Alternative mechanisms could, for example, involve specific RNA-binding proteins. It is, however, important to mention that mRNAs encoding cytoplasmic proteins are also found at the membrane of the ER [179,180]. In addition, ribosome-profiling experiments combined with subcellular fractionation demonstrated that ER-associated mRNAs encoding both cytosolic proteins and those encoding secreted/membrane proteins display similar ribosome loading densities [181]. These data, therefore, suggest that ER-associated ribosomes would play a major role in the translation of both mRNAs encoding cytoplasmic or secreted proteins. On the other hand, the co-activation of PERK and IRE1 pathways appears to be essential to the RIDD mechanism. Indeed, it has been shown that PERK depletion decreases the degradation of some mRNA targets while artificial translation blockage restores the RIDD. To explain these results, a possible hypothesis is that translation by ribosomes disturbs the stem loop structure recognised and cleaved by IRE1. Thus, the correct recognition of thetarget RNA would require a translation blockade by PERK or eventually a translational pause [182].

In glioma cells, IRE1 has been described to target the mRNA encoding the extracellular matrix protein SPARC. Downregulation of SPARC by IRE1 leads to a modulation of stress fibre formation and enhances migration properties of glioma cells [41]. Another substrate of the RIDD is PER1 mRNA,a circadian clock gene that controls the expression of CXCL3, an important chemokine involved in cancer development [40]. IRE1 has also been reported to protect cells from apoptosis, notably, through the decay of the death receptor 5 (DR5) mRNA. These few examples among the increasing list of RIDD targets highlight the pivotal role of this IRE1 downstream pathway in cancer [43].

IRE1 can also modulate the translation by another mechanism. Indeed, it was demonstrated that overexpression of IRE1β induces 28S rRNA cleavage [183] more efficiently than IRE1α [184]. In this inducible hIRE1β expression model, total protein synthesis was repressed by 30% 1 day after hIRE1βinduction. Thus, the cleavage of 28S RNA could reduce the number of functional ribosomes. As it was demonstrated that reduced ribosome levels impaired the translation of transcripts that are normally efficiently translated and have short and unstructured 5ᴶUTRs in comparison to other transcripts [185],this mechanism could enable the specific modulation of expression of certain messenger RNAs.

Finally, a subset of RNAs including ER-targeted mRNAs, SRP RNA, ribosomal and transfer RNAs were

demonstrated to physically associate with IRE1 in living cells [186]. Moreover, IRE1 interacts with the translocon, the translocon-associated TRAP component, SRP proteins and ribosomal proteins [32].IRE1 also strongly binds 80S ribosomes in vitro [186]. These results show that IRE1 is closely coupled to the translation machinery, but the precise functions of these different interactions are not yet clearly identified.
Although post-transcriptional gene regulation mediated by the IRE1 proteins has received a lot of attention in recent years, our understanding of the role of IRE1 in translational regulation is still in itsearly days.

3.5. mRNA Structures and Modifications Regulate Translation:

3.5.1. eIF3 Recruitment by RNA Structures

The multisubunit initiation factor eIF3 (13 subunits eIF3a-m) plays a central role in the cap-dependent translation initiation through its interaction with eIF4G, which allows the recruitment of the 43S pre-initiation complex [187]. However, eIF3 can also interact with mRNA stem loop structuresin the 5ʲUTR and directly regulate both cap-dependent and independent translation. eIF3 playsan essential role in translation of specific subsets of mRNA [188]. Indeed, Lee et al., reported that eIF3 uses different modes of mRNA stem loop binding to exert positive and negative translation regulation of key proliferative transcription factors such as cJUN and BTG1 [188]. Using PAR-CLiP (photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation) technology, they showed that eIF3 interacts with 3% of the expressed transcripts through direct interactions of mRNAwith the eIF3 subunits a, b, d and g. The binding of the cap by the eIF3d subunit in presence of the stem loop in the 5ʲUTR allows to bypass the canonical eIF4E translation and initiate an eIF3d-directedcap-dependent mRNA translation (Figure 2C) [189].
Different viruses seem to exploit the capacity of eIF3 to initiate translation. The hepatitis C virus harbours an IRES whose direct interaction with eIF3 is critical to induce efficient translationinitiation [190]. The positive strand RNA virus, Barley Yellow Dwarf Virus (BYDV, Genus Luteovirus) employs a cap-independent mechanisms where eIF3 bridges the mRNA 5ʲ and 3ʲUTR to initiate andregulate its translation [191].
There is still limited evidence of a role of this eiF3-dependent translation regulation mechanismunder ER conditions. For example, it was demonstrated that mutations in eIF3k and eIF3l genes enhanced resistance to ER stress in *Caenorhabditis elegans* [192]. Moreover, UV crosslink experimentsreveal that activation of ER stress by thapsigargine treatment results in a marked increase of several eIF3 subunits binding to polyadenylated mRNAs [193]. This work also demonstrates that eIF3 subunits favour the recruitment of selected mRNAs to 40S ribosomes during chronic ER and that this chronicstress renders eIF3 as the key mediator of mRNA recruitment to the PIC [193]. Taken together, even though the molecular mechanisms are still unknown, these data suggest a critical functional role forthese eIF3 subunits in the regulation of cellular responses to ER stress.

3.5.2. m6A-Dependent Translation Initiation

In addition to secondary mRNA structures, specific RNA modifications may have a strong impact on alternative translation mechanisms. For instance, a single N(6)-methyladenosine (m(6)A) residue in the 5ʲUTR promotes cap-independent mRNA translation initiation, through direct interaction with eIF3 which is sufficient to recruit the 43S complex and initiate translation even in the absence of the cap-binding factor eIF4E [194] (Figure 2D). N(6)-methyladenosine modification is the most abundant post-transcriptional mRNA modification [195,196], it exhibits tissue-specific regulation with an enrichment of m(6)A sites near stop codons and in 3ʲUTRs [197,198]. The methylation and demethylation of mRNA adenosine is dictatedby "writers" and "readers" (reviewed by [199]). The intracellular fate of methylated mRNA is underthe control of "readers" which according to their abundance, localisation (nucleus vs. cytoplasm), or the presence of specific RNA binding proteins (RBPs) will determine mRNA decay, stability or translation [187,199]. Thus, three readers—YTHDF1, YTHDF3, and YTHDC2—are heavily involved in translation of m(6)A mRNA [200–202]. YTHDF1 selectively recognises m(6)A 3ʲ UTR modified mRNA, promotes ribosome loading and interacts with different subunits of eIF3 complex to facilitate translation initiation [202]. YTHDF3 promotes protein synthesis in synergy with YTHDF1 and affects methylatedmRNA decay mediated through YTHDF2 ("reader" involved in mRNA decay). However, it is still unclear if the processes require direct interaction between the different YTH proteins or the cooperation of co-factors [201]. While decreasing the abundance of m(6)A 3ʲUTR modified mRNAs, YTHDC2 alsoenhances

their translation efficiency. The authors suggested that YTHDC2 may increase the translationof transcripts, and then destabilise the transcripts after translation has been completed to prevent further differentiation of cells [200].

Interestingly while METTL3 is a m(6)A "writer" in the nucleus, it functions as a potential "reader" when localised in the cytoplasm where it enhances translation of m(6)A mRNA through interaction with eIF3h (Figure 2E). METTL3 has also been proposed to promote oncogene translation through a mRNA looping mechanism [203,204]. In addition, promoter-bound METTL3 can induce m(6)A modification within the coding region of the associated mRNA transcripts, and enhance its translation by relieving ribosome stalling [205].

Although N6-methyladenosine marks on mRNA are preferentially located in the 3ʲUTR [197], diverse cellular stresses can induce a wide redistribution of m(6)A transcriptome marks, resulting in increased numbers of mRNAs with 5ʲUTR m(6)A [194]. For instance, heat shock stress induces preferential m(6)A deposition at the 5ʲUTR of newly transcripts. In the nucleus, under such stress, YTHDF2 preserves 5ʲUTR methylation of stress-induced transcripts by limiting the m(6)A "eraser" FTO activity. This mechanism allows the cap-independent translation of stress mRNA such as Hsp70 [198]. Moreover, it was already reported that m(6)A could recruit eIF3 to induce 48S initiationcomplex formation independent of eIF4E cap binding [194] especially under heat shock conditions when cap-dependent initiation is blocked, and also that m(6)A-based regulation contributed to the translational control of *ATF4* during amino acid starvation [206]. A correlation between increased local m(6)A modification and usage of non-canonical start codons during amino acid starvation wasalso described, which increase further the complexity of translation regulation mechanisms in stress conditions [206]. Even though the reversible m6A RNA methylation process has been now widely described, the cellular functions of this modification remain largely unclear, and further studies will be required to clarify the role of this modification in translational regulation, specifically during ER stress.

4. Conclusions

Protein translation regulation is an essential mechanism to maintain the cell's integrity and enableit to cope with stresses. Protein synthesis is not a single process, but a combination of several differentmechanisms that finely regulate the expression of specific mRNAs in response to stress in order to quickly adapt the cellular proteome.

Upon ER stress and eIF2α phosphorylation by PERK, cancer cells use alternative translation mechanisms that are mediated by cis-acting sequences, such as uORF and IRES, to drive the expressionof specific mRNA subsets involved in the stress response. Even if translational regulation mediated by uORF and IRES during stress response is well documented, these alternative mechanisms of translation are not yet fully understood.

Thus, even if the knowledge of translational regulations has grown considerably during the pastfew years, thanks in part to recent technological advances in profiling genome-wide translation, strong efforts must be made in order to better understand the molecular mechanisms underlying translational control in cancer during the stress response. In addition, we believe that a better understanding of themechanisms allowing the selective translation of specific mRNAs in stress conditions could also leadto the identification of new targets and holds great promise for novel therapeutics in oncology.

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Review

ER Stress and Unfolded Protein Response in Leukemia: Friend,Foe, or Both?

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Abstract: The unfolded protein response (UPR) is an evolutionarily conserved adaptive signaling pathway triggered by a stress of the endoplasmic reticulum (ER) lumen compartment, which is initiated by the accumulation of unfolded proteins. This response, mediated by three sensors-Inositol Requiring Enzyme 1 (IRE1), Activating Transcription Factor 6 (ATF6), and Protein Kinase RNA-Like Endoplasmic Reticulum Kinase (PERK)—allows restoring protein homeostasis and maintaining cell survival. UPR represents a major cytoprotective signaling network for cancer cells, which frequently experience disturbed proteostasis owing to their rapid proliferation in an usually unfavorable microenvironment. Increased basal UPR also participates in the resistance of tumor cells against chemotherapy. UPR activation also occurs during hematopoiesis, and growing evidence supports the critical cytoprotective role played by ER stress in the emergence and proliferation of leukemic cells.In case of severe or prolonged stress, pro-survival UPR may however evolve into a cell death program called terminal UPR. Interestingly, a large number of studies have revealed that the induction of proapoptotic UPR can also strongly contribute to the sensitization of leukemic cells to chemotherapy. Here, we review the current knowledge on the consequences of the deregulation of UPR signaling in leukemias and their implications for the treatment of these diseases.

Keywords: endoplasmic reticulum stress; unfolded protein response (UPR); leukemia; AML; CLL; ALL; CML

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1. Introduction

About one-third of human genes encode secreted or transmembrane proteins as wellas proteins resident of the endoplasmic reticulum, the Golgi apparatus, and lysosomes. Most of these proteins are targeted to the ER. The endoplasmic reticulum is a complex network of membrane-enclosed tubules and vesicles, extending from the nuclear membrane throughout the cytoplasm. ER is the largest organelle of most eukaryotic cells, as its membrane may account for at least 50% of all cell membranes and even more for specialized secretory cell. Its total area is 10–30 times that of the plasma membrane. ER constitutes thefirst compartment of the secretory pathway in which secreted and transmembrane proteins are folded and post-translationally modified [1].

ER is also the most important compartment for intracellular calcium ions (Ca2+) stor-age, which is necessary for the physiological activities of the ER, allowing the maintenanceof the oxidation–reduction potential [2,3]. In its lumen, a set of specialized proteins like chaperones, foldases, glycosylating enzymes, oxidoreductases, and cofactors ensures the correct folding of newly synthesized proteins. By interacting with the exposed hydropho-bic segments present on the newly synthesized proteins or on misfolded proteins, the chaperones (BiP/GRP78, calnexin, GRP94, etc.) act both to complete the folding process and to correct folding errors [4]. After passing the protein quality control checkpoints in the ER, correctly folded proteins traffic via the Golgi to other organelles and/or to the plasma membrane. Despite this optimized environment in the ER luminal domain, the success rate for accurate folding is variable. In case of unsuccessful folding, proteins are released in the cytosol where they become ubiquitinated and targeted to degradation by the proteasome. This rigorous quality control system has been named ERAD for EndoplasmicReticulum-Associated Degradation [5].

In addition, to cope with the perturbations caused by unfolded or misfolded proteins,cells set off an adaptive response called the unfolded protein response (UPR), which aims to restore normal ER functioning [6–9]. This is achieved by (i) lowering the biosynthesis of proteins to reduce accumulation of misfolded proteins in the ER; (ii) increasing the biosynthesis of chaperone proteins; (iii) increasing ER size through membrane synthesis, (i), and (ii) resulting in a boost of ER folding capabilities; and finally (iv) increasing the biosynthesis of ER-associated degradation proteins thus improving the cell's ability to eliminate misfolded proteins. Consequently, "adaptive UPR" limits cell damages and allows cell recovery and survival to a new stressful environment. However, if stress overcomes cell recovery capacities UPR can switch from an adaptive to a "terminal UPR"program triggering cell death [10–12].

Perturbations in the ER stress response such as either chronic ER stress or defects in UPR signaling, have been associated with a number of pathologies: diabetes, atheroscle- rosis, inflammation, stroke, pulmonary fibrosis, several eye diseases, neurodegenerative disorders (including amyotrophic lateral sclerosis, Alzheimer's, Parkinson's or Hunting- ton's diseases), and, of course, cancer [13–16]. The common feature among these seemingly different diseases is a cellular dysfunctioning leading to an accumulation of misfolded proteins in the ER.

With respect to cancer, the role of ER stress response/UPR signaling pathways was mainly studied in primary solid tumors in which a very unfavorable microenvironment mainly originating from inadequate vascularization and characterized by nutrient (e.g., amino acids, glucose) deprivation, hypoxia, acidosis leads to the activation of ER stress inthe highly proliferative and metabolically active cancer cells [17–21]. However, in recent years our current knowledge on the essential functions played by the UPR in leukemia hasalso significantly improved.

In this review, after introducing the Unfolded Protein Response, we will summarizecurrent findings on the involvement of ER stress in the progression of leukemia, and discuss the potential therapeutic effects of UPR activation or repression in these pathologies.

2. The Unfolded Protein Response

In mammals, UPR is triggered by activation of three ER transmembrane sensors: PERK (PKR-like ER-associated protein kinase), ATF6 (Activating Transcription Factor-6),and IRE1 (inositol-requiring enzyme-1) [6,10,22,23]. The luminal part of these proteins integrates the information coming from the ER lumen, whereas their cytosolic part interacts with their effectors and mediates the signaling cascades (Figure 1). In the absence of stress,the ER resident protein chaperone BiP also known as GRP78 (Glucose-regulated protein 78kDa) or HSPA5 (Heat Shock Protein Family A (Hsp70) Member 5) binds to the luminaldomain of the three effectors and keep them in an inactive state. Upon accumulation of unfolded proteins in the ER lumen, BiP will act as a protein chaperone, interact with exposed hydrophobic segments of misfolded proteins, and thus be released from ATF6, IRE1, and PERK, leading to their activation [24,25]. In addition to BiP release, an activation

of IRE1 by oligomerization induced by direct binding of unfolded proteins has also been reported, both in yeast [26] and mammalian cells [27]. Therefore, the relative ratios of three proteins complexes inside the endoplasmic reticulum, namely, those created by interaction between BiP and either unfolded proteins or UPR sensors, as well as those formed by direct interaction between unfolded proteins and the UPR sensors themselves, could contributeto a very precise and dynamic regulation of the UPR [28].

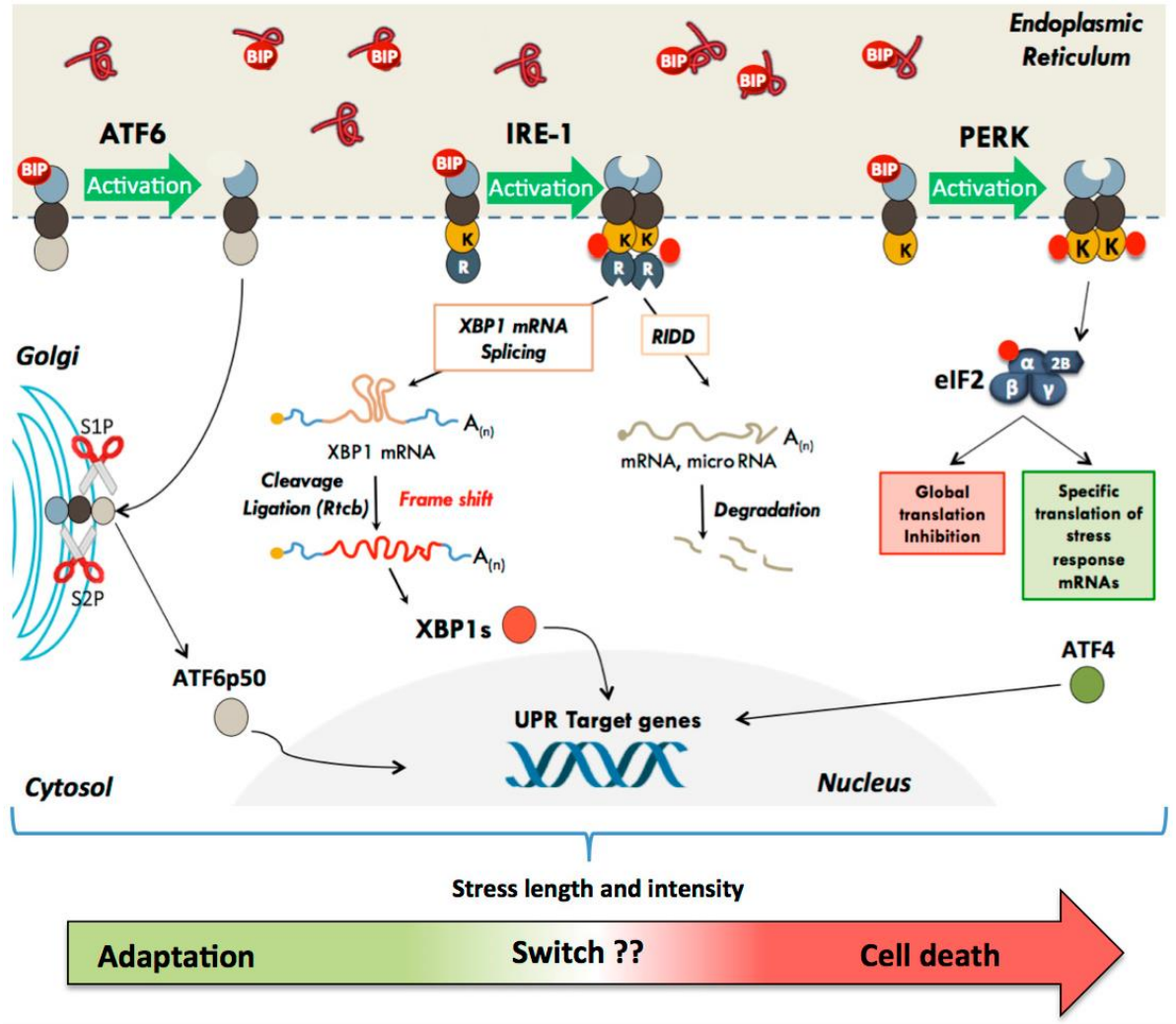


Figure 1. The different UPR effectors and their modes of action. In the basal state, the three UPR effector transmembrane proteins (PERK, ATF6, and IRE-1) are maintained inactive through their interaction with the protein chaperone BiP. The accumulation of misfolded proteins in the ER lumen results in dissociation of BiP and activation of UPR. (1) PERK dimerizes and phosphorylates the eIF2 α subunit, leading to a global inhibition of translation initiation. Specific mRNA subsets, containing cis-acting elements in their 5′UTR, such as uORF and IRES, escape translational inhibition triggered by eIF2 phosphorylation. (2) IRE-1 initiates an unconventional splicing of XBP-1 mRNA. IRE1 α cleaves Xbp1u mRNA within two stem-loop structures, leading to excision of 26 nucleotides. Subsequent ligation of the Xbp1 mRNA by the tRNA ligase RTCB results in a frame shift and allows the translation of the active transcription factor XBP1s, which is imported into the nucleus and activates the expression of target genes. IRE1 α mediates also the degradation of some RNAs (this mechanism has been called RIDD for Regulated Ire1-Dependent Decay). (3) BIP dissociation from ATF6 exposes its Golgi Localization Signal. ATF6 is translocated to the Golgi apparatus where proteolysis releases its transcription factor amino-terminal domain, which is imported into the nucleus and activates the expression of target genes. The UPR has a primary function in adaptive response in order to restore homeostasis and promote cell survival, but depending on the duration and intensity of the stress, a switch can induce cell death to get rid of the damaged cells.

As previously stated, the primary goal of the activated signaling cascades is to reestab-lish ER homeostasis by a two-step process: in a first stage, through the reduction of overall protein synthesis and the degradation of misfolded proteins, and in a second stage through the activation of cellular functions crucial for cell survival [6,10,22].

However, in the absence of protein homeostasis restoration, the adaptive UPR will switch to terminal UPR, which ultimately results in cell death. Cell fate is largely influenced by the intensity and duration of the stress. A long or intense stress leads to the activationof this terminal UPR [10,18]. The regulatory networks, which determine the transition from adaptive to terminal UPR, are complex and not fully understood. Regardless, the molecular events that will direct the cell towards either adaptive or terminal UPR involveto some extent each of the PERK, IRE1, and ATF6 signaling cascades. The contribution ofeach pathway to the execution of the adaptive or terminal UPR may be variable depending on the type of cell and on the nature and extent of damage experienced by the cell. The different UPR signaling cascades are described below.

The Translational Pathway: Activation of the PERK Kinase

Among the three key proteins involved in UPR, PERK (encoded by the EIF2AK3 gene for eukaryotic translation initiation factor 2-alpha kinase 3) is the first to be activated by autophosphorylation. The dissociation of BiP from its luminal domain causes dimerization or oligomerization and trans-autophosphorylation of PERK (threonine 981), thus activating the cytosolic serine/threonine kinase domain (Figure 1). The main substrate of PERKis the alpha subunit of the translation initiation factor eIF2 (eukaryotic Initiation Factor2) [29–32]. The eIF2 factor, which possesses three subunits- α, β, and γ -links the initiatormethionine tRNA (tRNA-Met) to the small ribosomal subunit. The regulatory α subunit contains a serine (ser51) strictly conserved in eukaryotes. By phosphorylating ser51, PERK induces a global inhibition of cap-dependent translation initiation and therefore overall protein synthesis in order to temporary reduce unfolded protein load, until favorable conditions return [33]. Mechanistically, the phosphorylation of the eIF2 α ser51 increasesthe affinity of eIF2 for its own eIF2B guanine nucleotide exchange factor (GEF), which recycles the inactive form of eIF2-GDP into its active form eIF2-GTP. This strong interaction induces sequestration of the eIF2 factor by eIF2B, causing a blockade of active translationpre-initiation complex formation and thus inhibition of translation initiation [34]. This translation inhibition prevents further protein loading in the ER, reduces cell overall metabolism and saves energy to repair the damage caused by the stress [34]. In parallel to cap-dependent translation arrest, translation of specific messenger RNAs exhibiting particular features in their 5′ untranslated region is selectively induced [35]. This is the case of the Activating Transcription Factor 4 (ATF4) mRNA which contains severalupstream open frames (uORFs) in its 5′ untranslated region, preventing translation of the main open reading frame (ORF) in normal conditions. Under stress conditions however,low levels of active eIF2 α allow the ribosomes to reach the main ATF4 ORF and efficiently initiate translation of this transcription factor, which in turn activates the expression of chaperones and of genes involved in amino acid metabolism and resistance to oxidative stress [36,37]. Interestingly, some mRNA whose translation depends on the presence of internal ribosome entry sites (IRESs) in their 5′ untranslated region [35] and coding for stress response proteins are also activated when eIF2 α is phosphorylated (Figure 1) [38–41].The dephosphorylation of eIF2 α is necessary to restore a normal protein synthesis level after stress. This reset to the basal state is achieved by two phosphatases, composedof a single catalytic subunit PP1 (Protein Phosphatase 1) and one of the two regulatory subunits GADD34 (Growth And DNA-Damage inducible protein 34) or CReP (Constitu-tive Repressor of eIF2 α phosphorylation) [42]. In contrast to CReP, which is constitutively expressed, the expression of GADD34 is only induced in response to stress as a negative feedback loop [43]. Indeed, transcription of GADD34 is activated by ATF4 and its trans-lation is, as for ATF4 itself, regulated by a uORF mechanism ensuring proper GADD34 expression despite eIF2 α phosphorylation [44]. Under chronic stress, sustained activation of PERK and thus prolonged expression ofATF4 induce apoptosis by activating CHOP transcription (C/EBP Homologous protein, also known as GADD153-Growth And DNA-Damage inducible protein 153 or DDIT3- DNA-Damage Inducible Transcript 3) [45]. This transcription factor, a member of the CCAAT/enhancer-binding protein (C/EBP) family, plays a central, multifunctional role in the UPR-induced apoptotic process [46]. CHOP can alone or cooperatively with other transcriptional factors function either as a transcriptional activator or repressor. It acts mainly by modulating the expression of various members of the BCL-2 protein family playing either pro-(Bim) or antiapoptotic (Bcl-2, BCL-XL and MCL-1) functions [47]. CHOP can however also induce cell death by many additional, non-exclusive, pathways such as restoration of protein synthesis (via GADD34 activation) which leads to increased proteins load detrimental to the cell ("proteotoxicity") and by increased ROS production (throughupregulation of the ER reductase ERO1 α) [48].

It is interesting to note that eIF2 α is not the only PERK substrate. Indeed, the transcription factor Nrf2 (Nuclear Factor (erythroid derived 2)-like2), which is involved in the response to oxidative stress, is normally maintained in the cytoplasm by association withKeap1. Under stress conditions, PERK phosphorylates Nrf2. This causes a dissociation of the Nrf2/Keap1 complex and allows the import of Nrf2 to nuclear compartment [49]. Nrf2 then bind to ARE sequences (Antioxidant response element) on the promoter of its target genes such as GCLC (Glutamate Cysteine Ligase Catalytic Subunit), HO-1 (Heme oxygenase 1) or NQO1 (NADPH dehydrogenase quinone 1) [50]. Thus, the activation of Nrf2 by PERK helps

in maintaining the redox status of the cell subjected to ER stress.

2.1. The Transcriptional Pathway: Activation of ATF6α and IRE1α

In mammals, the transcriptional response to ER stress involves two families of trans-membrane proteins: the IRE1 and ATF6 proteins (Figure 1). The ATF6 α (activating transcription factor 6 α) transcription factor is a type II trans- membrane protein characterized by a C-terminal luminal domain, sensitive to misfolded proteins, and an N-terminal cytosolic portion containing a leucine zipper DNA binding domain (bZIP) and a transcriptional activation domain. In mammals, two ATF6 proteins, ATF6 α and ATF6 β, are produced form independent genes. Whereas both proteins are ubiquitously expressed, only ATF6 α has proven to be an effective transcriptional activator and its it is currently accepted that only ATF6 α plays a major role in the ATF6-dependenttransduction of UPR signaling [51]. The amount and mode of contribution of ATF6 β to the unfolded protein response remain poorly understood and need to be further in- vestigated [52]. During ER stress, Bip dissociation from the ATF6 α protein allows the exposure of two Golgi localization signals, and migration of ATF6 α from ER to the Golgi apparatus where it undergoes 2 sequential cleavages by the proteins S1P and S2P (Site-1 and Site-2 Proteases) (Figure 1) [25,53]. These cleavages generate a transcriptionally active N-terminal short-lived fragment of 50 kDa called ATF6p50 which translocates into the nucleus to activate the transcription of chaperone and foldase proteins such as BiP, calretic- ulin, calnexin, and protein disulfide isomerases. ATF6p50 also activates the transcription of enzymes such as the calcium pump SERCA (sarco/endoplasmic reticulum Ca2+-ATPase).This ER ATPAse transports calcium ions from the cytosol into the ER and plays a major role in the maintenance of calcium homeostasis which controls many essential cellular processes [54]. ATF6p50 also promotes the expression of different genes involved in lipid biosynthesis, thus participating to the expansion of the endoplasmic reticulum [55]. It alsoupregulates XBP1 (X-box binding protein 1), a transcription factor which acts immediatelydownstream of the third UPR sensor IRE1 (see below). Moreover, ATF6 α can also form het- erodimers with XBP1 and upregulate genes involved in the ERAD pathway like EDEM (ER Degradation Enhancing Alpha-Mannosidase Like Protein 1) or HERPUD1 (HomocysteineInducible ER Protein With Ubiquitin Like Domain 1). ATF6 α gene invalidation induces increased sensitivity to ER stress probably due to impaired induction of chaperone proteins such as BiP or GRP94 (Glucose-regulated protein 94 kDa) [56,57]. However, ATF6 α can also activate the expression of the proapoptotic factor CHOP [58,59], and a very recent work suggested that ATF6 α could play an important role in the decision from adaptive to terminal UPR by modulating early and late CHOP expression kinetics [60]. Therefore, the role played by ATF6 α on cell survival or death appears complex. In addition, the ATF6 α transactivator domain (more precisely the first N-terminal 93 amino acids) has been shown to be responsible for its own degradation by the proteasome [61]. As a result, ATF6 α appears as a powerful transcriptional activator, but with a transient effect. This may contribute to finely tune the UPR machinery.

The third UPR sensor is IRE1 (inositol-requiring protein 1 also known as ERN1 for Endoplasmic reticulum-to-nucleus signaling 1), a 110 kDa protein initially identified in yeast where it is the only ER stress sensor. In mammals this protein is expressed as two isoforms: IRE1 α, which is ubiquitously expressed, and IRE1 β expressed only in the ep- ithelial cells of the digestive system [62–66]. IRE1 α possesses a luminal structure and an activation mode similar to that of PERK. However, in addition to a Ser/Thr kinase enzymatic activity, the IRE1 α cytosolic domain also retains an atypical endoribonuclease (RNAse) activity, which becomes functional after IRE1 α homodimerization under stressconditions [67]. This dimerization is essential for endoribonuclease activation, which is also dependent on IRE1 α phosphorylation status [68]. The IRE1 α RNAse domain catalyzes the excision of a 26-nucleotide sequence within the Xbp1 (X-box binding protein1) mRNAby an unconventional cytoplasmic splicing mechanism independent of the spliceosome (Figure 1) [69]. This cleavage, followed by a ligation step mediated by the RTCB tRNA ligase [70], generates a frame shift in the open reading frame, which leads to the expression of XBP1s (XBP1 spliced), a transcription factor belonging to the ATF/CREB family. The activation of the IRE1 α/XBP1s signaling axis induces the expression of genes encoding proteins of the ERAD pathway (EDEM, HRD1) and factors that modulate protein transloca- tion into the ER and folding, including the protein BiP [53,71]. Importantly, the non-spliced Xbp1 mRNA encodes the protein XBP1u (XBP1 unspliced), which is an inactive form with no transcriptional activity because it lacks the transactivating domain, and is an extremely short-lived protein. Interestingly, however, XBP1u was also found to interact with XBP1sunder ER stress conditions, functioning as a negative feedback regulator [72,73]. IRE1 α's endoribonuclease activity has also been shown to induce rapid and specific degradation of some RNAs by a mechanism called RIDD (Regulated Ire1-Dependent Decay) (Figure 1) [74,75]. Currently, only a limited number of direct targets have been identified and validated, including 4 microRNAs (miR-17, 96, 125b, 34a) [76] and some mRNAs notably PER1 [77], SPARC [78], BLOS1 [79], and DR5 (death receptor 5), but bioinformatic studies coupled with transcriptomic studies suggest a wider spectrum of action [80,81]. Several studies indicate that the RIDD mechanism contributes to ER stress-induced cell death, notably by degrading

several miRNAs involved in the repression of caspase-2 mRNA expression [75,76]. However, other studies propose that RIDD activity, by targeting mRNAs specifically translated at the endoplasmic reticulum, reduces the influx of newlysynthesized proteins, and thus participates in the adaptive survival process [82]. Moreover, the IRE1α-mediated targeting through RIDD of the mRNA coding for the death receptor 5 protein, a cell surface transducer of apoptotic signals could also limit ER stress-induced cell death [83]. The IRE1α activation level, stability, conformation, and oligomerization status appear to be also regulated by the interaction with many different protein partners such as for example HSP47 which facilitates the dissociation of BiP from its luminal domain thus helping in activation of IRE1α signaling under low stress conditions [10,84,85]. IRE1α associates also with additional partners through its cytosolic domain to induce different signaling pathways. TRAF2 (TNF receptor-associated factor), an adaptor protein,associates with IRE1α's kinase domain. The IRE1α/TRAF2 complex was found to interact with ASK1 (apoptosis signal-regulating kinase 1) to activate the c-Jun N-terminal kinase (JNK) and induce apoptosis [62,86]. Thus, the JNK arm of IRE1α pathway was initially thought to promote cell death. However, the function of this pathway in vivo is still controversial and has been described in some cases as pro-death and in other cases as pro-survival [62,86]. The nature and the intensity of the stimulus may account for these results. As in the case of ATF6α, IRE1α behaves as a sensor of the general cellular state through its multiple interactions with cofactors, regulators, and other members of the UPR signaling cascades and centralizes a set of signals in order to balance between anti- and proapoptotic signals.

Recent work has demonstrated that the activity of PERK and ATF6α can also be regulated by specific interacting proteins (reviewed in [10]). These results indicate that theactivity of the three UPR effectors is extremely finely tuned. In addition, these effectors can establish crosstalk between each other during the UPR response and therefore more detailed analyses of these proteins and their identified partners remain necessary to betterunderstand how they contribute on their own and altogether to the overall cell's response during UPR activation.

3. Hematopoiesis and Leukemias

3.1. Hematopoiesis

Hematopoiesis is the physiological process that is responsible for the production of the mature pools of blood cells from undifferentiated precursors, the stem cells. Hematopoiesis, which takes place mainly in the bone marrow of long and flat bones, is a crucial process as it allows the maintenance of blood cell homeostasis, producing approximately 10^{12} bloodcells daily in a healthy adult. The hematopoietic system functions as a pyramid-like hierarchy organized from a hematopoietic stem cell (HSC) at the top, able to self-renew ordifferentiate to produce all the cells of the hematopoietic system (Figure 2). In the bone marrow, long-term hematopoietic stem cells (LT-HSC) are quiescent, in the G0 phase of the cell cycle with a very low mitochondrial activity, but a high self-renewal potential [87].These cells are maintained throughout life. In classical hematopoiesis, LT-HSC division leads to the generation of new LT-HSC or ST-HSC, for Short-Term Hematopoietic Stem Cell, which are able to produce all mature hematopoietic lineages [88,89]. These cells then differentiate into multipotent progenitors (MPPs) and then either in common lymphoid progenitors (CLPs) or in common myeloid progenitors (CMPs) [90]. CLPs produce B andT lymphocytes and natural killer cells, while CMPs generate granulocyte-macrophage progenitors (GMPs), which, as their name implies, are then differentiated into granulocytes and macrophages, and megakaryocyte–erythrocyte progenitors (MEPs) which themselves differentiate into red blood cells and platelets [91,92] (Figure 2). Hematopoiesis is a tightly regulated mechanism, and therefore impaired hematopoiesis can be the cause of leukemias, malignant disorders resulting from defects of the stem cells at different stages of maturation, with subsequent clonal expansion [92]. Leukemias include acute and chronic leukemia and are also classified into lymphoblastic and myeloblastic leukemias according to the cell type affected. Acute leukemias are characterized by the proliferation of immature, unfunctional white blood cells called "blasts", decreasing normal hematopoietic cells in the bone marrow while chronic leukemias are characterized by theexpansion of differentiated cells in the blood [93]. Acute leukemias are divided into acute myeloid leukemias (AML) or acute lymphoblastic leukemias (ALL) and chronic leukemiainto chronic myeloid leukemias (CML) or chronic lymphoblastic leukemias (CLL).

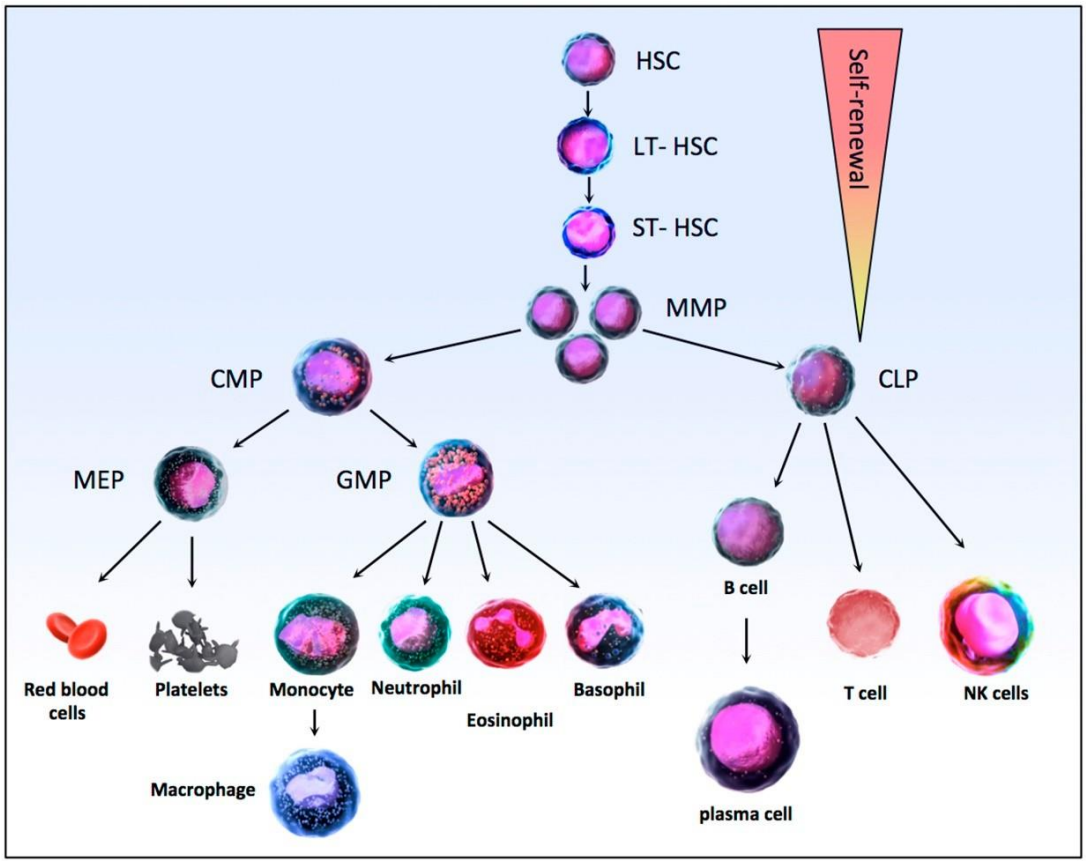


Figure 2. Schematic of the HSC differentiation hierarchy in normal hematopoiesis. HSC, Hematopoietic stem cells; LT-HSCs (Long-Term Hematopoietic Stem Cell) are able to generate new LT-HSC or to differentiate into ST-HSC (Short-Term Hematopoietic Stem Cell) then into MPPs (MultiPotent Progenitors) with reduced self-renewal capacity. Downstream of MPPs, a strict separation takes place between the myeloid (CMP, Common Myeloid Progenitors) and lymphoid (CLP, Common Lymphoid Progenitors) lineages. CMP then produce MEPs (Megakaryocyte–Erythrocyte Progenitors), which differentiate into platelets and erythrocytes, and GMPs (Granulocyte-Macrophage Progenitors) produce granulocytes (neutrophils, eosinophils, and basophils) and macrophages. In the lymphoid lineage, the CLPs then produce T and B lymphocytes and natural killer cells. The whole hematopoietic differentiation process is tightly regulated by a number of intrinsic and extrinsic factors, like cytokines and transcription factors.

3.2. Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) is a group of phenotypically and genetically het- erogeneous diseases, which is among the most common adult leukemia (it accounts for about 80% of leukemias in adults), with an average age of first diagnosis over 60 years [94]. This is a complex pathology triggered by the accumulation of chromosomal translocations and/or multiple mutations and resulting in the transformation and clonal expansion of hematopoietic progenitors. AML is thought to initially develop from at least two types of somatically acquired genetic alterations: mutations that confer advantages in terms of proliferation and survival and mutations that interfere with cell differentiation and apoptosis mechanisms [95]. Recent advances in sequencing methodologies have shown that AML represents a dynamic disorder in which multiple sub-clones compete and coexist, not only during the normal progression of the disease but also under pressure generated by anticancer agents [96]. While the majority of patients are in complete remission after the initial chemotherapy, AML has been associated with a poor prognosis because most patients tend to relapse due to the emergence of therapy-resistant clones [97]. Identifyingthe genetic alterations associated with resistance to chemotherapy is essential for risk stratification and to predict response to treatment of each AML patient. Three main classesof genetic aberrations have been described in AML: non-random chromosomal alterations, multiple gene mutations, and epigenetic alterations [98,99]. The most common of these chromosomal alterations include rearrangements leading to the formation of genes coding for chimeric proteins and upregulation of gene expression by juxtaposition with strong promoters. Among these rearrangements, we find translocations t(8;21) AML1-ETO or RUNX1, t(15;17) PML-RARA, inv(16) CBFB-MYH11, and t(9;11) MLL-AF9, which are associated with a better prognosis, whereas translocations t(11;19) MLL-ENL, t(6;11) MLL-AF6, t(10;11) MLL-AF10, or complex karyotypes are associated with a worse prognosis [100]. One of these translocations, t(15;17) (q22;q12), is peculiar because it is characteristic of a subtype of acute myeloid leukemia

named acute promyelocytic leukemia (APL) [101]. This specific chromosomal translocation leads to the expression of the PML-RAR α fusion pro-tein. APL is unique among all leukemias because of its high level of sensitivity to all-trans retinoic acid (ATRA), the vitamin A acid form [102]. The prognosis of this pathology is very good in general [103,104]. Among the genes that have been found mutated in AML we can mention retinoic acid receptor- α (RAR- α), core binding factor (CBF), HOX gene family or MLL. Mutations in oncogenes such as FLT3, KIT, N-RAS, GATA-1, JUN B, MYC,p53, PU.1, RB, FES, FOS, MPL, WT1, WNT, CEBPA, and NPM1 or mutations affecting epigenetic modifiers such as DNMT3A, ASXL1, TET2, IDH1, and IDH2 have also been characterized [105–107].

3.3. Acute Lymphoblastic Leukemia (ALL)

Acute lymphoblastic leukemia (ALL), also called acute lymphocytic leukemia, is a rare genetically heterogeneous clonal malignant disorder of the bone marrow character- ized by immature lymphoid precursors proliferation leading to the crowd out of normal hematopoietic cells [108]. ALL, which can occur at virtually any age, is more frequently seen in children and adolescents. This pathology results from clonal proliferation of ab- normal B cell progenitors (B-ALL) accounting for approximately 85% of ALL or T cell progenitors (T-ALL) accounting for roughly 15% of ALL. Most of the genetic alterations leading to leukemogenesis, including chromosomal translocations, somatic mutations, aneuploidy, and gene copy number alterations have been characterized in both T-ALL and B-ALL. Like in AML, these genetic alterations are important prognostic factors for disease-risk stratification and treatment [109,110]. Among the genetic alterations found inB-ALL, TCF3–PBX1 t(1;19), ETV6–RUNX1 t(12;21), and hyperdiploidy are associated with a favorable outcome while MLL rearrangements, TCF3–HLF t(1;19) and rearrangements of CRLF2, JAK2A, or BL-class tyrosine kinase genes are of poor prognosis [111,112]. Alter- ations involving the KRAS, NRAS, FTL3, PTPN11, and epigenetic modifiers like CREBBPor WHSC1 are frequent genetic events [111,113]. The genetics of T-ALL is extremely heterogeneous, with chromosomal abnormalitiesin nearly all patients. Mutations in the NOTCH1 gene leading to constitutive activation ofNOTCH signaling is the main oncogenic pathway found in the majority of patients. Thesealterations are generally associated with loss of p16 (INK4A) and p14 (ARF) suppressor genes at the CDKN2A locus. In addition, in 50% of patients with T-ALL, chromosomal translocations affect genes encoding oncogenic transcription factors like TAL1, TAL2, MYC, MYB, LYL1, TLX1 (HOX11), TLX3 (HOX11L2), or HOXA genes, placing these genes under the control of powerful T cell specific activators [114]. As for the AML example, it is not possible to exhaustively list all the genetic alterations and different combinations encountered, so we refer the reader to references dealing morespecifically with this pathology [115–117].

3.4. Chronic Myeloid Leukemia (CML)

Chronic myeloid leukemia (CML) is a slow-growing myeloproliferative neoplasm characterized in more than 95% of cases by the t(9;22) (q34.1;q11.2) chromosomal translo- cation leading to the formation of the Philadelphia chromosome (Ph*), resulting in the BCR-ABL1 gene fusion. The subsequent BCR/ABL1 chimeric protein is a constitutively active tyrosine kinases oncoprotein which activates transduction pathways involved in cell growth and differentiation such as RAS, MYC, STAT, AKT RAF, or JUN, and is therefore capable of transforming hematopoietic stem cell into neoplastic one [118,119]. Before targeted therapies became available, the main treatment options for CML included allogeneic stem cell transplantation and chemotherapy. However, the prognosisfor CML improved considerably since the use of tyrosine kinase inhibitors (TKIs), most notably imatinib in the early 2000s, which inhibit the BCR-ABL1 fusion protein by blocking its kinase domain [120]. Several generations of TKI have been developed since, but the appearance of TKI resistances remains a major issue [121]. It is therefore also crucial for this pathology to identify new therapeutic approaches in order to better stop its progression and avoid evolution to advanced disease states which may account for as much as 15% ofall CML deaths [121].

3.5. Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is characterized by a clonal proliferation and accumulation of mature but defective lymphocytes in the blood, bone marrow, lymph nodes, and spleen. CLL is the most common form of leukemia in Western countries. It is highly heterogeneous in its evolution, with some patients needing chemotherapy early after diagnosis and others never requiring specific treatment and having a survival rate similar to the general population. More than 95% of people with CLL develop the B cell type [122]. CLL is a heterogeneous disease, which divides into an aggressive form that expresses a wild type immunoglobulin heavy-chain variable region (IGVH) gene, and an indolent form that expresses a mutated IGVH, reflecting the stage of normal B cell differentiation [123,124]. Chronic lymphocytic leukemia cells exhibit many complex genetic alterations, which have been used by clinicians as prognostic biomarkers in order to predict

survival and disease progression and guide treatment decisions [124]. Many recurrent cytogenetic abnormalities are encountered in CLL. The main ones are (i) deletion of the long arm of chromosome 13 (del(13q)), leading to the loss of the DLEU2/MIR15A/MIR16-1 genes, which is found in more than 50% of CLL cases and is of good prognosis when isolated; (ii) trisomy 12, associated with an intermediate prognosis with median overall survival; (iii) deletion of the long arm of chromosome 11 (del(11q)) that leads a more aggressive disease due to the loss of the ATM gene (for Ataxia Telangiectasia Mutated) which is essential for the regulation of the cell cycle; and (iv) deletion of the short arm of chromosome 17 (del(17p)) resulting in the loss of the TP53 gene which is of poor prognosis [125–127]. At least one of these abnormalities can be found in approximately 80% of patients [122,128,129]. Translocations are reported in approximately 20% of CLL [130]. These translocations predominantly involve the immunoglobulin genes, mainly IGH, andthe 13q14 locus. Common partners are CCND1, BCL2, and BCL3 [130]. In addition to chromosomal rearrangements, sequencing studies have also revealed numerous recurrentmutations in CLL mostly in the P53, ATM, NOTCH1, SF3B1 (Splicing Factor 3B subunit 1), and BIRC3 genes [131].

A variety of targeted drugs including BCR signaling pathway inhibitors, anti-CD20 antibodies and BCL-2 inhibitors have been used in therapeutics and have significantly improved the management of this disease [132,133]. However, despite the increasing number of available therapeutic alternatives, chemotherapy does not currently provide adefinitive cure and additional strategies are still required.

4. Endoplasmic Reticulum Stress Induction in Hematopoietic and Leukemic Cells

4.1. ER Stress Activation in HSCs

Hematopoietic stem cells (HSCs) are sitting at the apex of the hematopoietic hierarchy. They are the most immature cells and are capable of replenishing all hematopoietic cell types [134,135]. As long-life cells, HSCs require a highly regulated protein quality controlin order to avoid the accumulation of damages that could ultimately affect their DNA integrity and promote tumorigenesis. At steady state, HSCs are quiescent and display lower protein synthesis rates in vivo and in vitro compared to their progeny [136]. Furthermore, HSCs have been associated with low protein folding capacity that can be explained by a lower expression of chaperones proteins compared to hematopoietic progenitors [137]. Moreover, protein synthesis deregulation has a great impact on HSCs' viability and self- renewal capacities and can lead to HSCs loss [136,138]. Investigation of ER stress role in regulating hematopoietic stem cells fate, revealed a high expression of PERK and a low expression of eIF2α in HSCs when compared to progenitor cells [139,140]. PERK upregulation in HSCs appears to increase their sensitivity to ER stress, compared to more committed progenitor cells, through activation of the PERK-peIF2α-ATF4/CHOP arm that can trigger apoptosis. It has been suggested that this sensitivity to ER stress could prevent accumulation of damaged cells in the HSCs compartment and potential subsequent malignant transformation [139]. In agreement with this hypothesis, Miharada et al. showed that reducing ER stress levels in vitro in HSCs through the overexpression of the RNA binding protein Dppa5 (Developmental pluripotency-associated 5) improved their self- renewal activity by protecting them from apoptosis [141]. However, the IRE1α-XBP1 UPR branch can also be activated in HSCs and in this case plays a significant cytoprotective role. For example, estrogen treatment of HSCs activates the IRE1α-XBP1 branch and increases repopulation capacities of HSCs upon transplantation [142]. In a mouse model system, Liu et al. also showed that IRE1α-XBP1 activation in HSCs in vivo prevents ER stress-induced apoptosis, preserves HSC clonogenicity and improves reconstitution capacity [143]. Xie et al. also demonstrated that increased cytoprotective ER stress (induced by the pharmacological inhibition of the sphingolipid enzyme DEGS1) participates together with autophagy in the setting up of a prosurvival response aimed to maintain stemness properties [144].

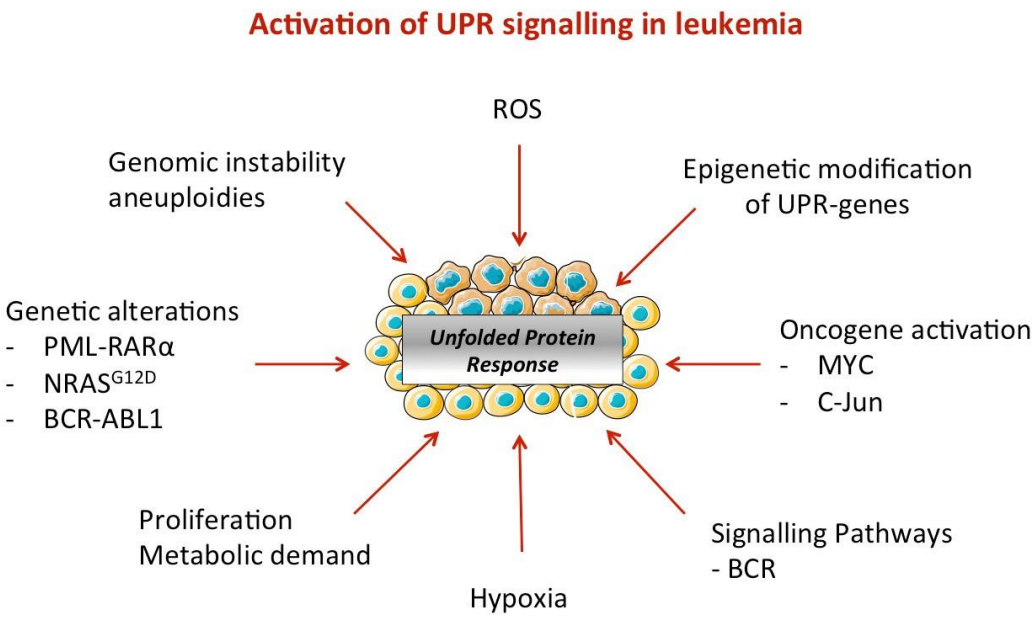
Increased ERAD has also been recently reported to actively participate in the main- tenance of proteins homeostasis in HSCs and appeared to be essential for stem cell pool maintenance [145]. In addition to low protein synthesis rates and low folding capacity, it has been reported that protein quality control by ERAD maintains HSCs pool. Altogethercurrently known data indicate that increased basal UPR induced at least in part by unfa- vorable growing conditions in the bone marrow environment, such as, e.g., hypoxia [146],helps in maintaining HSC integrity as well as clearing damaged HSCs and therefore playcritical functions at the early steps of hematopoiesis [147]. Of note, in our article we refer to "basal UPR" as the activation status of the different signaling pathways of the UPR in cells growing either in vitro or in vivo without any treatment by chemotherapeutic drugsor chemical compounds.

4.2. ER Stress Activation in Leukemic Cells

Recent lines of evidence link activation of the three UPR branches to most hallmarksof cancer and especially those aimed to protect the cells against the numerous aggressionsthey undergo during their growth inside

tumors [20]. This is especially true for solid tumors, which develop in a highly adverse environment but also for leukemic cells. Indeed, hematopoietic cells, either normal or leukemic, are exposed in the bone marrow to an adverse environment caused by hypoxia, high levels of reactive oxygen species (ROS), and nutrient deprivation, often resulting in ER stress activation [13,16,23,148]. Thus, many studies have reported the activation, to variable extents, of each of the three UPR branches(IRE1 α , PERK, and ATF6 α) in a wide range of hematopoietic tumors (leukemia, lymphoma,and myeloma) [137,149–151]. As in solid cancers, UPR plays a fundamental role in the adaptation of leukemic cells to cellular stress by inducing different mechanisms, which attempt to reestablish ER homeostasis in order to restore its proper functions.

In AML patients, increased expression of XBP1, BiP, and Calreticulin has been detected in 17.4% of cases [152]. Schardt et al. demonstrated a correlation between a high expression of XBP1s and complex karyotype in AML [152]. Another clinical study from Tanimura et al. reported activation of the IRE1 α -XBP1 pathway in AML patients; however, no significant correlation between ER stress activation and genetic features could be revealed [153]. Interestingly, UPR activation in some hematological malignancies is not always the consequence of stress integration but can also be induced through aberrant pathway activation. For example, in chronic lymphoid leukemia (CLL), UPR activation is observed in response to surface immunoglobulin M stimulation and activation of the kinases BTK and SYK [154]. In pre-B-ALL, Xbp1 expression is activated by various oncogenic tyrosine kinases via STAT5 signaling [155]. Moreover, the transcription factor c-Jun, overexpressedin AML and CML, promotes the transcription of general UPR target genes such as Xbp1 and Atf4 by a direct mechanism [156]. The modulation of expression of some UPR effectors inleukemia has been shown to involve epigenetic modifications in their promoters [157,158].In addition, mutations in epigenetic splicing factors, which are considered as first hit mutations, have pleiotropic effects that might be linked to ER stress activation (Figure 3).The comparison between healthy donor and AML patient samples revealed hypomethyla- tion of Xbp1′s promoter that has been suggested to lead to



overexpression of XBP1. On the contrary, in large diffuse B cells lymphoma, IRE1 α expression is reduced through a mechanism involving the histone methyltransferase, EZH2 (Enhancer of Zeste Homolog 2).

Figure 3. Activation of UPR signaling in leukemia. Different mechanisms of ER stress activation have been reported in leukemia, which include (epi)genetic modifications and genomic instability (e.g., mutations, translocations, hypomethylation), oncogenic signaling, and metabolism rewiring due to a high proliferation in blasts. Microenvironment is also a well-known source of ER stress (e.g.,hypoxia) that contributes to UPR activation.

Furthermore, transcription of ER stress-related proteins by oncogenic pathways alsoparticipates in the UPR activation in leukemia. For instance, the MAPK pathway promotes the transcription of Xbp1 through STAT5 activation. In pre-B acute lymphoblastic leukemia (ALL), BCR-ABL1 or NRASG12D signals through MAPK-STAT5-XBP1 [155]. Indirectly, inlymphomagenesis the transcription factor MYC, by promoting a rapid cell proliferation, increases the rate of misfolded proteins in ER that triggers the UPR [159].

Compared to HSC, in which both cytoprotective (IRE1 α -XBP1) and cell death-promoting (PERK) UPR pathways can be activated in the basal state, the activation of an adaptive UPR (mainly via IRE1 α signaling) appears to be preferred in leukemic cells. However, theincrease in basal UPR could sensitize these cells to additional stress

induced, for example, by chemotherapy treatment (see next chapter). By analogy with what is observed in HSCs under normal growth conditions, the UPR response may represent a real checkpoint influencing cell fate of leukemic cells experiencing chemotherapy: either the stress can be resolved via an adaptive phase and cancer progresses or the damage accumulates and becomes unrecoverable. In this latter case, excessive or prolonged stress triggers proapoptotic signaling through a terminal process [137,150,151,160]. This important issue is discussed below.

4.3. UPR Modulation: A Double-Edged Sword to Fight Against Leukemia

Despite the numerous pieces of evidence of reticulum stress activation in multiple cancers, the question of whether UPR reduces or promotes tumor growth in patients is still the subject of intense debate [149]. Two therapeutic strategies exploiting ER stress and UPR could be possible in order to induce leukemic cell death: either inhibition of the adaptiveUPR response (cytoprotective) or activation of the terminal UPR response (cytotoxic). Thechoice between these two strategies may be difficult as their relative efficacy may be highly dependent on the cellular deregulation that led to the disease.

As mentioned above, various studies have shown that leukemic cells often possess basal UPR activity with a cytoprotective function, which favors tumor progression and additionally may increase chemoresistance of the cells to various drugs. For example, theER stress sensor BiP was found to be highly expressed in B-ALL and its pharmacological inhibition by epigallocatechin gallate (a polyphenolic compound purified from green tea) sensitized cells to the anti-leukemic drug vincristine [161]. In the same pathology, the increase in expression and activity of BiP and the IRE1 α /XBP1 pathway were found to be essential for cell survival and pharmacological inhibition of IRE1 α RNAse domain bythe drug STF-083010 reduced the proliferation and survival of patient-derived pre-B ALLcells [155]. Of note, increased Xbp1 mRNA levels at diagnosis appear of poor prognosis for patients with the disease [155]. In CML, activation of the PERK-eIF2 α pathway has a cytoprotective effect and increases their resistance to imatinib, a tyrosine kinase inhibitor widely used in cancer chemotherapy [162]. Resistance to imatinib in CML was also shownto result from the activation of ATF6 α, which appears mediated by the protein disulfide isomerase 5 (PDIA5) upon ER stress and a PDIA5 inhibitor, 16F16, increased cells' sensitivity to treatment with imatinib [163]. Moreover, a pharmacological inhibitor of IRE1 α, B109, was reported to suppress CLL tumor cell progression in a murine model and to sensitize human CLL cells to the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib [164]. A pro- survival role for IRE1 α was also reported in AML and the pharmacological inhibition of IRE1 α by 2-hydroxy-1-naphthaldehyde (HNA) switched the cells towards apoptosis and in addition synergized with treatments with bortezomib and arsenic trioxide, two widely used anticancer drugs [157]. Moreover, analysis of Philadelphia chromosome (Ph)-positive AML patient samples revealed increased expression of the BiP, CHOP, and Xbp1s mRNAs and the authors demonstrated that inhibition of the IRE1 α and ATF6 α pathways sensitized cells expressing the Bcr-Abl fusion protein to imatinib- and etoposide-induced apoptosis [165].More recently it was shown that Jun itself induces the expression of several UPR effectors thereby enhancing UPR induction and this appeared essential to AML cell proliferation and survival, thus demonstrating that Jun could contribute to induce an adaptive UPR insome AML subtypes [156].

Altogether, the data presented above have largely validated the inhibition of adaptive UPR as an effective means of fighting leukemia and a significant number of pharma- cological inhibitors of central UPR effectors are currently under preclinical studies or clinical trials [137,150].

However, these promising results should not lead us to neglect the other strategy aimed at inducing a cytotoxic response in the cell through terminal UPR induction, the "second edge of the sword". Indeed, leukemic cells, which usually experience unfavorablegrowth conditions and maintain increased levels of ER stress and basal UPR, may show an increased susceptibility to enter terminal UPR in response to different treatments. Indeed,artificially increasing the unfolded protein load can lead to a cytotoxic cellular response in some leukemic models. Thus, in ALL treatment with the drug pevonedistat, which inhibits the NEDD8 conjugation pathway and impairs degradation of misfolded proteins by the proteasome, induces a reorientation of UPR towards apoptosis [166]. Interestingly, inhibiting the ER-associated degradation (ERAD) pathway of proteins by knockdown of one of its components, UFD1, also results in the induction of a terminal UPR process in T-ALL cells in response to the accumulation of unfolded proteins [167]. In mast cell leukemia, it was demonstrated that moderate pharmacological inhibition of IRE1 α could stop leukemic cell proliferation by impairing adaptive UPR but with non-significantly in-duced cell death. Interestingly, stronger inhibition of IRE1 α induced a switch from adaptive to terminal UPR. Enhancing ER stress by pharmacological inhibition of proteasome activity with bortezomib also induced terminal UPR in this model [168]. In Philadelphia-positive ALL, pharmacological inhibition of IRE1 α with MKC-8866 also appeared able to reorient the initial cytoprotective UPR program towards cell death induction when combined with the inhibition of BCR-ABL1 with nilotinib [169].

Few studies describing the induction of cell death in leukemic cells by a strategy delib- erately aimed at redirecting

the cell response towards terminal UPR has yet been described. However, the analysis of data published over the last two decades and describing the use of antileukemic drugs shows, strikingly, that for many of them (see Table 1) their mode of action involves the induction of a terminal UPR pathway or related UPR-induced cell death processes. Indeed, although adaptive UPR was found to contribute to chemoresistance in10 out of the 91 chemical compounds tested against leukemic cells and listed in Table 1, for the remaining compounds (i.e., 89% of the whole) the induction of UPR signaling pathways was associated with cytotoxicity. There appears to be no apparent correlation between the type of leukemia and the final response, cytoprotective or cytotoxic, to UPR induction. This also seems to be the case if we consider the mode of action of the drugs used. Similarly, no strict correlation can be found between the UPR pathways activated in response to the drugs and the final response of the cell (pro-survival or pro-death)and all UPR pathway have been reported to be induced whatever the final outcome on leukemic cell's viability. It can be noted, however, that the CHOP pathway is very fre- quently activated when UPR induction results in cell death. This appears not surprising asthe PERK-peIF2α/ATF4/CHOP signaling pathway plays a crucial function in inducing cell apoptosis in the cell [46] and was reported to be a major cell death-inducing UPR pathwayin hematopoietic stem cells, as described above (see Chapter 4.1). However, induction of the PERK-peIF2α/ATF4/CHOP signaling pathway can also be detected in leukemic cellsresponding to treatment by an adaptive UPR. As in other pathological models, leukemic cell response to UPR induction is a complex process, which may rely on a subtle balance between the activation levels of the different branches of the UPR. Anyway, it appears that for a large number of chemotherapeutic agents or candidate compound, this process is critical for the final death/survival outcome of leukemic cells.

Therefore, it seems important to further investigate terminal UPR induction, on its own as well as in combination with other pharmacological treatments, for the improvement of therapeutic strategies in leukemia

5. Conclusions

We have reviewed the currently available data in the literature dealing with the various roles played by UPR in leukemia. We also presented some of the UPR-mediated molecular processes that can induce cytoprotection of leukemic cells or direct them towards cell death through apoptosis induction. In the light of all of the currently reported data from studies carried out to dissect the role of UPR in the progression of leukemia, it is clear that the cytoprotective/cytotoxic balance regulation is a complex, highly dynamic machinery, still poorly understood and that a wide and integrative approach is needed to discover the genuine mechanisms underlying this crucial process. The specific networks that regulate ER stress-induced cytoprotection or apoptosis may be dependent on the nature, the intensity and the length of the stimuli. It is probable, even if contradictory results have sometimes been published, that it also depends on the cell type being stressed.A better understanding of the UPR mechanisms acting in response to chemotherapy appears also essential to provide new therapeutic pathways aimed to eradicate neoplastic cells either by inhibiting the adaptive UPR, or by activating UPR-mediated cell death pathways [265].

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Table 1. Consequences of UPR induction by drugs in leukemic cells. For each drug, the chemical nature, the pubchem compound ID, and the molecular target are provided when available. The type of leukemia on which the work was carried out was provided as well as the inferred or demonstrated role of UPR activation. The involved effectors are also indicated, when they have been identified (n.d.: not determined).

Molecule	Chemical Nature	Pubchem Compound CID	Target	Type of Leukemia	Proposed Role of ER Stress/UPR Activation	Mainly Implicated Pathways/Effectors	Ref.
BIX-01294		25150857	Histone methyltransferase G9A	AML	Adaptive	PERK (prosurvival, via NRF2)	[170]
SCH727965 (Dinaciclib)		46926350	CDKs 1,2,5 and 9	AML, CML, T-ALL	Adaptive	XBP1s	[171]
Ski, ROMe		16760659	spingosine kinases 1 and 2	T-ALL	Adaptive	unclear	[172]
MDA-7/IL-24)	cytokine	-	n.d.	AML, APL	Adaptive	GRP78/Bip, IRE1α, GADD34	[173]
sorafenib	multikinase inhibitor	216239	MEK/ERK pathway	U937 cell line	Adaptive	PERK	[174]
Digoxine	cardiac glycoside	2724385	Na+/K+ ATPase	K562 (erythroleukemic) and THP-1 (acute monocytic leukemia) cell lines	Adaptive	PERK, IRE1α	[175]
Shikonin	naphthoquinone	479503	pyruvate kinase-M2 (PKM2), proteasome inhibition, NFkB, Thioredoxin reductase (TrxR1)	HL60	Adaptive	ERP57 and Calreticulin	[176]
3-deazaneplanocin A (DZNeP)	cyclopentenyl analog of 3-deazaadenosine	73087	histone methyltransferase	MV4-11, MOLM-14, Mono-Mac-1, THP-1, HL60 and KG-1 AML cell lines	Adaptive	GRP78, GRP94, PERK, PDIA isoform 3, 4, and 5	[177]
wolfberry phytochemicals	n.d.	-	n.d.	Jurkat cell line	Adaptive	all UPR pathways	[178]
Imatinib		5291	tyrosine kinases inhibitor	CML/LAMA-84 CML cell line and murine myeloid progenitor primary cells	The constitutive activation of PERK in CML cells protects from imatinib treatment	PERK	[162]
Metformin		4091	multiple, see PMID: 28776086	T-ALL, B-ALL	Switch form adaptive to terminal	IRE1α, CHOP	[179]
Nilotinib + MKC8866		644241	Tyrosine kinases (Nilotinib); IRE1 (MKC8866)	ALL (Ph+)	Switch form adaptive to terminal	IRE1α (cytoprotective); PERK and ATF6α (cytotoxic)	[169]
2-deoxy-D-glucose	glucose analog	108223	n.d.	ALL Cell Lines	Switch form adaptive to terminal	GRP78/Bip, CHOP	[180,181]
Selenite	sodium selenite	24934	n.d.	NB4 cell line (APL)	Switch form adaptive to terminal	PERK/eIF2α/ATF4	[182]

Table 1. *Cont.*

Molecule	Chemical Nature	Pubchem Compound CID	Target	Type of Leukemia	Proposed Role of ER Stress/UPR Activation	Mainly Implicated Pathways/Effectors	Ref.
Asperuloside	iridoid glycoside	84298	n.d.	cell lines HL60 and U937, primary leukemic cells	Terminal: apoptosis induction	all UPR pathways	[183]
JA3 & JA7	Aldehyde biphenyl chalcones	134820953	n.d.	(AML); T-ALL; CML	Terminal: immunogenic apoptosis-like cell death	CHOP; PERK	[184]
Oprozomib	tripeptide analog of carfilzomib	25067547	immunoproteasome subunit β5i/LMP7 (ubiquitin–proteasome pathway)	CML	Terminal: apoptosis induction	PERK, IRE1α (via ASK/JNK/Bim)	[185]
VAS3947	given in [186] fig 1a	7471335	NADPH oxidases	AML	Terminal: apoptosis induction	IRE1α, PERK	[186]
Arsenic trioxide + Gilteritinib		14888/49803313	FLT3 (for Gilteritinib)	AML (FLT3-ITD)	Terminal: apoptosis induction	IRE1α	[187]
GSK-J4		71729975	H3K27me3 demethylase	AML	Terminal: apoptosis induction	PKCα; Bcl2 phosphorylation	[188]
CXL146	4H-chromene derivative	-		AML (or APL): HL60; CML	Terminal: apoptosis induction	PERK, IRE1α, ATF6α	[189]
FF-10501	given in paper	124343	inosine monophosphate deshydrogenase	AML	Terminal: necrotic cell death	CHOP	[190]
Retinoic acid+Tunicamycin+ arsenic trioxide		444795/11104835	n.d.	AML	Terminal: Cytotoxic UPR	CHOP, XBP1s	[191]
[Retinoic acid or arsenic trioxide] + tunicamycin		444795/11104835	n.d.	APL	Terminal: Cytotoxic UPR		[192]
MIM1 and UMI-77		135691163/992586	n.d.	AML; T-ALL	Terminal: Cytotoxic UPR	NOXA	[193]
PFR	peptide	-	n.d.	AML	Terminal: necroptosis		[193]
Genistein	Isoflavone	5280961	n.d.	AML or APL (HL60 cell line)	Terminal: Cytotoxic UPR		[194]
Camalexin	Phytoalexin (structure given in paper)	636970	n.d.	AML	Terminal: apoptosis induction	PERK, CHOP	[195]
Ibrutinib (PCI-32765)		24821094	Bruton's tyrosine kinase	B-ALL	Terminal: apoptosis induction	ATF4; CHOP	[196]
OT-55	bis-coumarine derivative	-	n.d.	CML	Terminal: Immunogenic cell death induction; apoptosis induction	not well documented	[197]

Table 1. *Cont.*

Molecule	Chemical Nature	Pubchem Compound CID	Target	Type of Leukemia	Proposed Role of ER Stress/UPR Activation	Mainly Implicated Pathways/Effectors	Ref.
RS-F3	fistularin-3 stereoisomer	-		AML	Terminal: Cytotoxic UPR (assumed)	PERK; XBP1s; CHOP	[198]
Bardoxolone methyl (CDDO-Me)	triterpenoid	400769	Nrf2 and NF-κB	Chronic myeloid leukemia, K562 cell line	Terminal: apoptosis induction	PERK, IRE1α, CHOP	[199]
3-O-trans-p-coumaroyl-alphitolic acid (3OTPCA)	triterpenoid	-	n.d.	U937, Molt-4 and Jurkat cell lines.	Terminal: apoptosis induction	XBP-1 and CHOP	[200]
Nelfinavir		64143	HIV protease inhibitors	T-ALL, B-ALL, and AML; CLL primary leukemic cells	Terminal: apoptosis induction; In CLL, contributes to the induction of cell death in choroquine treated cells	CHOP	[201,202]
CB-5083	1-[4-(benzylamino)-5H,7H,8H-pyrano[4,3-d]pyrimidin-2-yl]-2-methyl-1H-indole-4-carboxamide	439268	Valosin-Containing Protein/p97	B-ALL Cell Lines (BALL1, REH, NALM6, OP1, ALL-PO, 697, RS4;11, BV173, SEM, and SUPB15)	Terminal: apoptosis induction	all UPR pathways	[203]
Tunicamycin ± Quizartinib (AC220)	AC220	11104835 + 24889392	FLT3	AML (FLT3-ITD)	Terminal: apoptosis induction	PERK, CHOP	[204]
Cryptotanshinone	lipophilic diterpene quinone	160254	n.d.	CCRF-CEM cell line (ALL)	Terminal: apoptosis induction	IRE1α-XBP1, PERK-eIF2α-ATF4	[205]
Oxalicumone A	dihydrothiophene-condensed sulfur chromone	90676613	n.d.	KG-1a, HL60, U937, and K562 cell lines (AML)	Terminal: apoptosis induction	IRE1α-XBP1, PERK–CHOP	[206]
Pevonedistat (MLN4924)	adenosine sulfamate analog	16720766	NEDD8-activating enzyme	T-ALL (CCRF-CEM, Jurkat) and B-ALL (REH, NALM6, SupB15) cell lines	Terminal: apoptosis induction	all UPR pathways	[166]
Carfilzomib (PR-171)	tetrapeptide epoxyketone	11556711	ubiquitin–proteasome pathway	CLL MEC1 and MEC2 cell lines and primary leukemic cells	Terminal: apoptosis induction	ATF4, CHOP	[207]
Miltirone	abietane-type norditerpenoid quinone	160142	n.d.	Jurkat, U937, AML and ALL primary leukemic cells	Terminal: apoptosis induction	PERK	[208]
Arsenic trioxide		14888	n.d.	NB4 cell line (AML)/CML	Terminal: apoptosis induction	IRE1α-XBP1/GRP78/Bip, CHOP, Xbp1 (unspliced …)	[209,210]

Table 1. *Cont.*

Molecule	Chemical Nature	Pubchem Compound CID	Target	Type of Leukemia	Proposed Role of ER Stress/UPR Activation	Mainly Implicated Pathways/Effectors	Ref.
JPH203	O-[(5-Amino-2-phenyl-7-benzoxazolyl)methyl]-3,5-dichloro-L-tyrosine dihydrochloride	24853505	LAT1 (L-type amino-acid transporter 1)	Ke37, DND41, Sil-ALL, Peer, Molt-16, Jurkat and SupT1 T-ALL cell lines	Terminal: apoptosis induction	CHOP	[211]
Wogonin	5,7-dihydroxy-8-methoxyflavone	5281703	n.d.	HL-60 cell line.	Terminal: apoptosis induction	all UPR pathways-CHOP	[212]
Farnesol	acyclic sesquiterpene alcohol	445070	n.d.	Molt4 T-ALL cell line	Terminal: apoptosis induction	PERK-eIF2α-ATF3/4	[213]
APO866	(E)-N-[4-(1-benzoylpiperidin-4-yl)butyl]-3-pyridin-3-ylprop-2-enamide	6914657	nicotinamide phosphoribosyltransferase (NAMPT)	OCI/AML2, OCI/AML3, HL-60, HEL, KG1a, SET1, MV4-11, MEC.1, MEC.2, LAMA-84 cell lines and B-CLL and AML primary leukemic cells	Terminal: apoptosis induction	IRE1α-CHOP	[214]
Bortezomib	boronic acid	387447	proteasome (26S)	NB4 cell line (APL)	Terminal: apoptosis induction	Nd	[215]
CX-4945	5-(3-chloroanilino)benzo[c][2,6]naphthyridine-8-carboxylic acid	24748573	casein kinase 2	T-ALL cell lines and primary cells	Terminal: apoptosis induction	GRP78/BIP-IRE1α-CHOP	[216]
compound 3 (Pyrimidine analogue)	1-(5,5,5-trichloro-2-methoxy-4-oxopenten-2-yl)-4-trichloromethyl-pyrimidin-2(1H)-one	-	n.d.	L1210, CEM, JURKAT cell line (ALL)	Terminal: apoptosis induction	CHOP and caspase-12	[217]
R7, R13	Naphtylchalcones		n.d.	murine lymphoblastic leukemia	Terminal: apoptosis induction (assumed)	CHOP	[218]
S1 (BH mimetic)				APL	Terminal: cytotoxic through NOXA induction	PERK; XBP1s, NOXA	[219]
Gossypol (BH3 mimetic)	polyphenol	3503	phospholipase A2	AML, APL	Terminal: cytotoxic through NOXA induction	PERK; NOXA	[220]
Cariporide		151172	Na + H+ exchanger 1 (NHE1)	CML, APL, T-ALL	Terminal: sensitizes to extrinsic, TRAIL-induced, apoptosis	CHOP	[221]

Table 1. *Cont.*

Molecule	Chemical Nature	Pubchem Compound CID	Target	Type of Leukemia	Proposed Role of ER Stress/UPR Activation	Mainly Implicated Pathways/Effectors	Ref.
Auranofin		6333901	thioredoxin reductase	CLL	Terminal: contributes to the induction of cell death in treated cells	PERK; XBP1s, CHOP	[222]
[Cu(thp)4][PF6]	phosphine copper(I) complex		n.d.	B-ALL	Terminal: apoptosis induction	Xbp1s, CHOP	[223]
Z-Leu-Leu-Nle-CHO	leupeptin analog	-	γ-Secretase	CLL primary leukemic cells	Terminal: apoptosis induction	IRE1α, CHOP	[224]
curcumin	Diferuloylmethane	969516	n.d.	WEHI-3 myelomonocytic leukemia cell line/NB4 and UF-1 APL cell lines/HL60 cell line	Terminal: apoptosis induction	IRE1α, ATF6α, CHOP/PERK, CHOP, ASK	[225–227]
LQB 118	pterocarpanquinone	46233300	n.d.	K562 and Jurkat cell lines	Terminal: apoptosis induction (assumed)	caspase 12	[228]
Flavopiridol	Flavonoid alkaloid	5287969	CDKs inhibitor	CLL primary leukemic cells	Terminal: contributes to the induction of cell death in choroquine treated cells	IRE1α/XBP1 and CHOP	[229]
Safrole		5144		AML (HL-60)	Terminal: apoptosis induction (assumed)	ATF6α, CHOP	[230]
Clofibrate		2796	peroxisome proliferator-activated receptor (PPAR) alpha	T-ALL	Terminal: apoptosis induction (assumed)	ASAPK/JNK	[231]
Abnobaviscum F ®	Mitletoe aqueous extract	135343633	n.d.	CML	Terminal: contribution to the induction of cell death in treated cells (assumed)	GRP78/Bip, CHOP	[232]
MJ-29	Quinazolinone	-	n.d.	murine myelomonocytic leukemia	Terminal: apoptosis induction	GRP78/Bip, CHOP, PERK	[233]
Imatinib (STI571)		5291	BCR-ABL tyrosine kinase		Terminal: apoptosis induction	not well documented	[234]
glycyrrhizic acid		14982	n.d.	murine myelomonocytic leukemia	Terminal: contributes to the induction of cell death in treated cells	GRP78/Bip, CHOP	[235]
Gypenosides		-	n.d	HL-60 AML cell line	Terminal: apoptosis induction	ATF6α and ATF4	[236]

Table 1. *Cont.*

Molecule	Chemical Nature	Pubchem Compound CID	Target	Type of Leukemia	Proposed Role of ER Stress/UPR Activation	Mainly Implicated Pathways/Effectors	Ref.
AICAr (+ methotrexate)	5-aminoimidazole-4-carboxamide (AICA) riboside	266934	n.d	Nalm6 and CCRF-CEM cell lines (ALL)	Terminal: apoptosis induction	CHOP (C/EPB homologous protein)	[237]
Emodin	6-methyl-1,3,8-trihydroxyanthraquinone	3220	n.d.	WEHI-3 murine myelomonocytic leukemia cell line	Terminal: apoptosis induction (assumed)	n.d.	[238]
Syrbactin	azamacrocyclic product	-	proteasome (26S)	REH ALL cell line	Terminal: apoptosis induction	CHOP (C/EPB homologous protein)	[239]
ABT-737 and GX15-070	BH3 mimetics	11228183/46930997	BCL2 family proteins	Jurkat, NB4 and K562 cell lines	Terminal: Cytotoxic UPR	ATF4, ATF3, CHOP and NOXA,	[240]
NPB001-05	n.d.	-	BCR-ABL	K562 cell line	Terminal: apoptosis induction (assumed)	not well documented	[241]
Ras inhibitor farnesylthiosalicylic acid (FTS, Salirasib)	2-[[(2E,6E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl]thio]-benzamide	5469318	RAS	K562 cell line	Terminal: Cytotoxic UPR	not well documented	[242]
PYZD-4409	3,5-dioxopyrazolidine compound, 1-(3-chloro-4-fluorophenyl)-4-[(5-nitro-2-furyl)methylene]-3,5-pyrazolidinedione	60111983	ubiquitin-activating enzyme UBA1	K562, NB4, THP1, and U937 cell lines and AML primary leukemic cells	Terminal: apoptosis induction	PERK, CHOP, ATF4	[243]
Korbazol	n.d.	-	n.d.	CLL primary leukemic cells	Terminal: apoptosis induction (assumed)	n.d.	[244]
Polymethoxyflavone tangeretin (TAN)	Flavonoids	-	n.d.	K562 cell line	Terminal: apoptosis induction	IRE1α, PERK, CHOP	[245]
Shiga toxine type 1 (Stx1)	n.d.	-	ribosomes (protein synthesis)	THP-1 cell line	Terminal: apoptosis induction	CHOP, TNF-related apoptosis-inducing ligand (TRAIL), DR5 and calpain	[246]
Eicosapentaenoic acid		446284	n.d.	HL60 (AML or APL)	Terminal: apoptosis induction (assumed)	PERK	[247]
Xanthohumol	prenylated chalcone	639665	n.d.	CLL (patient samples)	Terminal: apoptosis induction	PERK, CHOP	[248]

Table 1. *Cont.*

Molecule	Chemical Nature	Pubchem Compound CID	Target	Type of Leukemia	Proposed Role of ER Stress/UPR Activation	Mainly Implicated Pathways/Effectors	Ref.
Tunicamycin (UPR inducer)		11104835	N-acetylglucosamine phophotransferase	AML (U937 and HL60)	Terminal: cytotoxic through induction of lysosomal apoptotic pathway	GRP78/Bip, CHOP	[249]
arsenic sulfide	[As4S4 (AS)]	61569	n.d.	BCR/ABL-positive K562 cell line	Terminal: apoptosis induction	not well documented	[250]
Fenretinide	synthetic retinoid derivative (related to vitamin A)	5288209	n.d.	NB4, U937 and HL60 cell lines	Terminal: apoptosis induction	PERK/eIF2α-CHOP (C/EPB homologous protein)	[251,252]
PABA/NO	O2-[2,4-dinitro-5-(N-methyl-N-4-carboxyphenylamino)phenyl] 1-(N,N-dimethylamino)diazen-1-ium-1,2-diolate	-	PDI	HL60 cell line	Terminal: Cytotoxic UPR	CHOP (C/EPB homologous protein)	[253]
alkyl gallate and gallamide derivatives		-	n.d.	HL60 cell line	Terminal: apoptosis induction	not well documented	[254]
Trichosanthin	type I ribosome-inactivating protein	596174	Ribosomes	HL60 cell line	Terminal: apoptosis induction	CHOP (C/EPB homologous protein)	[255]
auraptene	monoterpene coumarin ether	1550607	n.d.	Jurkat cell line	Terminal: apoptosis induction	Caspase 8	[256]
4-hydroxybenzylretinone	fenretinide analogue/synthetic retinoid derivative (related to vitamin A)	-	n.d.	HL60 cell line	Terminal: Cytotoxic UPR	CHOP (C/EPB homologous protein)	[257]
tipifarnib combined with bortezomib	quinolone and boronic acid	159324/387447	Farnesyltransferase Inhibiteur and 26 s proteasome inhibitor	KG-1, and U937 cell lines	Terminal: Cytotoxic UPR	not well documented	[258]
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride	186136	serine protease inhibitor	NB4 cel line	Terminal: Cytotoxic UPR	not well documented	[259]
Thapsigargin (UPR inducer)	sesquiterpene lactone	446378	sarco/endoplasmic reticulum Ca++ ATPase	K562 cell line	Terminal: apoptosis induction	not well documented	[260,261]
arsenic trioxide (ATO) + kinase inhibitor imatinib mesylate (STI571)		14888/5291	BCR-ABL tyrosine kinase	K562 cell line and CML primary leukemic cells	Terminal: apoptosis induction	not well documented	[262]
Tetrocarcin-A		54681516	n.d.	CLL/T-ALL	Terminal: apoptosis induction	not well documented	[263,264]

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