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Pyrimidine and NAD salvage pathways: unexpected allies in the maintenance of genome stability

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Pyrimidine and NAD salvage pathways: unexpected allies in the maintenance of genome stability

Voies de sauvetage du pool des pyrimidines et du NAD: des alliés inattendus dans le maintien de la stabilité du génome

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Abbreviations

53BP1	p53-binding protein 1	**BS**	Bloom syndrome
ACMS	A-amino-β-carboxymuconate-ε-semialdehyde	**cADPR**	Cyclic-ADP-ribose
AD	Alzheimer's disease	**CAP**	Cdh1-anaphase promoting
ADP	Adenosine Diphosphate	**CDA**	Cytidine deaminase
ADPr	ADP-ribose	**Cdc2 kinase**	cell division cycle protein 2 homolog kinase
AID	Activated cytidine deaminase	**CDH4**	Chromodomain Helicase DNA Binding Protein 4
AIF	Apoptosis-induced factor	**Cenp-A/B/C**	Centromere protein A/B/C
AKT	Protein kinase B	**CHFR**	Checkpoint with Forkhead And Ring Finger Domains
ALC 1	Amplified in liver cancer 1	**Chk1**	Checkpoint kinase 1
ALI	Acute lung injury	**CLL**	Chronic lymphocytic leukemia
ALS	Amyotrophic lateral sclerosis	**CNS**	Central nervous system
AML	Acute myeloid leukemia	**CNT**	Concentrative nucleoside transporters
AMPK	AMP-activated protein kinase	**CoaSt6**	Collaborator of signal transducer and activator of transcription 6
APH	Aphidicolin	**CO_2**	Carbon dioxide
APLF	Aprataxin And PNKP Like Factor	**COPD**	Chronic obstructive pumunary disease
Ara-C	cytarabine	**CREB**	c-AMP response element binding protein
ARH 3	ADP-ribosylhydrolase 3	**CS**	Cockayne syndrome
AT	Ataxia Telangiectasia	**CSR**	Class-switch recombination
ATF4	Activacting transcription factor 4	**CtIP**	CtBP-interacting protein
ATM	Ataxia Telangiectasia mutated	**DAG**	Diacylglycerol
ATP	Adenosine triphosphate	**DAPI**	4',6'-diamino-2-phenylindole
ATR	Ataxia telangiectasia and Rad3-related	**dCK**	Deoxycytidine kinase
ATRIP	ATR-interacting protein	**DDR**	DNA damage response
Aza-C	Aza-cytidine	**dGK**	Deoxyguanosine kinase
BAL	B-aggressive lymphoma	**DLB-CL**	Diffuse large B-cell lymphoma
BAT	Brown adipose tissue	**DNA**	Deoxyribonucleic acid
BBAP	B-lymphoma and BAL-associated protein	**Dna2**	DNA Replication Helicase/Nuclease 2
BER	Base excision repair	**DNA-PK**	DNA-dependent protein kinase
BLM	Bloom Syndrome RecQ Like Helicase	**dNDP**	Deoxynucleotide diphosphate
BRCA 1/2	Breast cancer type ½ susceptibility protein	**dNTP**	Deoxynucleotide triphosphate
BRCT	BRCA1 C-terminus domain	**DSB**	Double-strand break
BrdU	5' –bromodeoxyuridine	**EME1**	Essential Meiotic Structure-Specific Endonuclease 1

EMMPRIN	Extracellular matrix metalloproteinase inducer	**IRAP**	Insulin-responsive aminopeptidase
ENT	Equilibrative nucleoside transporters	**IRE1α**	Inositol requiring enzyme 1 alpha
ER	Endoplasmic reticulum	**IRS-1/2**	Insulin receptor substrate -1/2
ERα	Estrogen receptor alpha	**ISG**	Interferon-induced gene
ERCC1	Excision Repair Cross-Complementation Group 1	**JNK**	c-Jun N-terminal kinases
ETC	Electron transport chain	**LCCoA**	Long chain coenzyme A
EXO1	Exonuclease 1	**MacroD1/2**	MACRO Domain Containing 1/2
FA	Fanconi anemia	**MAPT**	Microtubule-associated protein tau
FANCD2	Fanconi anemia group D2 protein	**MAR**	Mono-ADP-ribose
FANCI	Fanconi anemia, complementation group I	**MMP-9**	Matrix metallopeptidase 9
FFA	Free fatty acid	**MPK-1**	MAPK phosphatase -1
FOXO	Forkhead box	**MnSOD**	Manganese superoxide dismutase
5-FU	5-fluorouracil	**MRE11**	Meiotic recombination 11
FZEB	5-fluorozebularine	**mTOR**	Mammalian target of rapamycin
G1 phase	Gap 1 phase	**MTorc2**	mTOR Complex 2
G2 phase	Gap2 phase	**MUS81**	Crossover Junction Endonuclease MUS81
G6PD	Glucose-6-phosphate dehydrogenase	**NA**	Nicotinic acid
GLUT 1/4	Glucose transporter 1/4	**NAAD**	Nicotinic acid adenine dinucleotide
GKRP	Glucokinase regulatory protein	**NAD**	Nicotinamide adenine diphosphate
GSK3	Glycogen synthase kinase 3	**NAFLD**	Non-alcoholic fatty liver disease
GSIS	Glucose-stimulated insulin secretion	**NAM**	Nicotinamide
GSSH / GSH	Oxidized /reduced glutathione disulphide	**NAMN**	Nicotinic acid mononucleotide
H_2O_2	Hydrogen peroxide	**NAMPT**	Nicotinamide phosphoribosyltransferase
HD	Huntington's disease	**NAPRT**	Nicotinic acid phosphoribosyltransferase
HFD	High-fat diet	**NAR**	Nicotinic acid ribose
HI	Hypoxia ischemia	**NBS11**	Nijmegen Breakage Syndrome 1
HIF-1α	Hypoxia-inducible factor 1-alpha	**NDPK**	Nucleoside diphosphate kinase
HJ	Holliday junctions	**Ndufs4**	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4
HR	Homologous recombination	**NEMO**	NF-kappa-B essential modulator
HSF-1	Heat shock transcription 1	**NES**	Nuclear export signal
HSP90	Heat shock protein 90	**NF-κB**	Nuclear factor kappa-light-chain-enhancer of activated B cells
HU	Hydroxyurea	**NHEJ**	Non-homologous end-joining
ICAM-1	Intercellular adhesion molecule 1	**NKR**	Nicotinamide ribose kinase
IOP	Intraocular pressure	**NLS**	Nuclear localization signal
IR	Insulin receptor	**NMN**	Nicotinamide mononucleotide

NMNAT	Nicotinamide mononucleotide adenyltransferase
NMPK	Nucleoside monophosphate kinase
NNT	Nicotinamide nucleotide transhydrogenase
NOS	Nitric oxide synthetase
NSCLC	Non-small cell lung cancer
OS	Oxidative stress
OSCC	Oral squamous cell carcinoma
OXPHOS	Oxidative phosphorylation
PAI-1	Plasminogen activator inhibitor-1
PAR	Poly-ADP-ribose
PARG	Poly(ADP-ribose) glycohydrolase
PARP	Poly (ADP-ribose) polymerase
PBEF	Pre-B-cell colony-enhancing factor
PBL	Peripheral blood lymphocytes
PBR	PAR-binding regulatory motif
PBZ	PAR-binding zinc finger
PCNA	Proliferating cell nuclear antigen
PD	Parkinson's disease
PDC	Pyruvate dehydrogenase complex
PGAM	Phosphoglycerate mutase
PGC-1α	PPARγ co-activator 1 alpha
PI3-kinase	Phosphatidyl inositol 3 Kinase
PICH	PLK1-interaction checkpoint helicase protein
PKC	Protein kinase C
PML	Promyoletic leukemia protein
PNS	Peripheral nervous system
PPARγ	Peroxisome proliferator-activator gamma
POL δ/ε	DNA polymerase δ/ε
POT1	Protection of telomeres homologue 1
PRPP	phosphoribosylpyrophosphate
QA	Quinolinic acid
QAPRT	Quinolinic acid phosphoribosyltransferase
RAD51	Radiation sensitive 51
RIF1	Replication Timing Regulatory Factor 1
RMI 1/2	RecQ-mediated genome instability protein 1 /2
RNR	Ribonucleotide reductase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPA	Replication Protein A
SARM1	Sterile alpha and toll/interleukin receptor motif-containing protein
SCE	Sister-chromatid exchange
SIRT	Sirtuins
SMARCA 5	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5
SMC	Smooth muscle cells
SOD	Superoxide dismutase
S phase	Synthesis phase
SSA	Single-strand annealing
SSB	Single-strand break
ssDNA	Single-strand DNA
STAT 3/6	Signal transducer and activator of transcription 3/6
TANK	Tankyrase
TCA	Tricarcarboxylic acid
TCCD	2,3,7,8- tetraclorodizenbo-p-dioxin
TD2M	Type 2 diabete mellitus
TEP 1	Telomerase-associated protein 1
TFAM	Mitochondrial transcription factor A
TGF-β1	Transforming growth factor beta 1
THU	Tetrahydrouridine
TK	Thymidine kinase
TNF-1α	Tumor necrosis factor receptor 1
Topo	Topoisomerase
TRF 1/2	Telomere repeat 1/2
Trp	tryptophan
TS	Thymidylate synthetase
UA	Uric acid
UFB	Ultra-fine anaphase bridge
UV	Ultra-violet
VCAM-1	Vascular cell adhesion protein 1

V(D)J recombination	Variable and joining recombination
VEEV	Venezuelan equine encephalitis virus
WAT	White adipose tissue
WNT	Wingless-type MMTV-integration site family member
WRN	Werner Syndrome RecQ Like Helicase
XRCC1/4	X-repair cross-complementing 1/4
XLF	XRCC4-like factor
XP	Xeroderma pigmentosum

Abstract

Bloom syndrome (BS) is a rare human autosomal recessive disorder resulting from mutations of both copies of the *BLM* gene encoding BLM, a 3'-5' RecQ DNA helicase. BS cells display strong genetic instability, and BS predisposes patients to a wide range of cancers common in the general population. BLM depletion leads to the downregulation of cytidine deaminase (CDA), an enzyme of the pyrimidine salvage pathway that catalyzes the hydrolytic deamination of cytidine (C) and deoxycytidine (dC) to uridine (U) and deoxyuridine (dU), respectively. CDA defects lead to an excess of cellular dC and deoxycytidine triphosphate (dCTP) in BS cells or BLM-expressing cells, jeopardizing genome stability. Indeed, this nucleotide pool disequilibrium is responsible for some of the cellular abnormalities associated with the BS phenotype, including the high frequency of sister chromatid exchange (25-40%), slower replication fork speed and high frequency of ultrafine anaphase bridges (UFBs). CDA deficiency and the resulting pyrimidine pool imbalance also result in significantly lower levels of basal poly (ADP-ribose) polymerase 1 (PARP-1) activity. These low levels of PARP-1 activity disturb Chk1 activation and decrease the efficiency of downstream checkpoints, leading to the accumulation, during mitosis, of unreplicated DNA at some "difficult-to-replicate" loci in the genome, such as centromeres and fragile sites, leading to excess UFB formation. The objective of my PhD project was to decipher the mechanism underlying the decrease in basal PARP-1 activity in the absence of CDA. We performed a metabolomic study in two independent pairs of isogenic cell models of CDA deficiency and their CDA-expressing counterparts. PARP-1 is involved principally in the synthesis and transfer onto target proteins a polymer of multiple ADP-ribose residues (poly-ADP-ribosylation or PARylation) resulting from the cleavage of NAD^+ into nicotinamide (NAM) and ADP-ribose. We therefore focused, in particular, on NAD metabolism pathways, investigating possible alterations to these pathways in CDA-deficient cells. Our metabolomic study revealed higher levels of nicotinamide (NAM) levels, the substrate of nicotinamide phosphoribosyltransferase (NAMPT), and lower levels of nicotinamide mononucleotide (NMN), the product of NAMPT, in CDA-deficient cells. We confirmed the lower levels of nuclear NAMPT activity in CDA-deficient cells. We found that siRNA-mediated NAMPT knockdown or chemical NAMPT inhibition reproduced the decrease in basal PARP-1 activity in CDA-proficient cells, and that this decrease in PARP-1 activity was

independent of NAD^+ levels. Moreover, the expression of exogenous wild-type NAMPT, but not of the NAMPT catalytic mutant, fully rescued basal PARP1 activity, abolishing the increase in UFB frequency in CDA-deficient cells. These results indicate that the low levels of basal PARP-1 activity in CDA-deficient cells result from a decrease in NAMPT activity. We propose a model in which the intracellular accumulation of dC/dCTP resulting from CDA deficiency impairs nuclear NAMPT activity, resulting in an intracellular accumulation of NAM, a known natural inhibitor of PARP-1, and a decrease in PARP-1 activity. Our results provide the first evidence of a link between pyrimidine pool and NAD salvage pathways.

Résumé

Le syndrome de Bloom (SB) est une maladie humaine autosomique récessive rare résultant de la mutation des deux copies du gène *BLM*, qui code pour la protéine BLM, une 3'-5' ADN hélicase de la sous-famille RecQ. Les cellules SB présentent une forte instabilité génétique et les patients atteints du SB sont prédisposés au développement de tous les types de cancers affectant la population générale. La déplétion en BLM conduit à une chute drastique de l'expression de la cytidine désaminase (CDA), une enzyme de la voie de sauvetage des pyrimidines qui catalyse la désamination hydrolytique de la cytidine (C) et de la désoxycytidine (dC) en uridine (U) et désoxyuridine (dU). La déficience en CDA conduit à un excès de dC et de désoxycytidine triphosphate (dCTP) dans les cellules SB, mais également dans les cellules qui expriment BLM, ce qui entraîne une instabilité génétique. Ainsi, ce déséquilibre du pool de pyrimidines résultant de la déficience en CDA est responsable de plusieurs anomalies cellulaires associées au phénotype SB : il contribue à l'augmentation de la fréquence des échanges entre chromatide-sœurs (25-40%) et est pleinement responsable du ralentissement de la vitesse de progression des fourches de réplication et de l'augmentation de la fréquence des ponts anaphasiques ultrafins (UFBs). Ce déséquilibre du pool de pyrimidines conduit également à une diminution de l'activité basale de la poly (ADP-ribose) polymérase 1 (PARP-1) qui entraîne une réduction de l'activation de la kinase Chk1, ce qui affaiblit l'efficacité des points de contrôle du cycle cellulaire en aval, favorisant l'accumulation en mitose de séquences d'ADN non répliquées qui conduisent à une formation excessive d'UFBs. Ces séquences correspondent essentiellement des régions du génome « difficiles à répliquer », comme les centromères et les sites fragiles. L'objectif de mon projet thèse était de décrypter le mécanisme conduisant à la réduction de l'activité basale de PARP-1 dans les cellules déficientes en CDA. Nous avons effectué une étude comparative des métabolomes de deux couples de lignées isogéniques exprimant ou non CDA. L'activité principale de PARP-1 étant la synthèse et le transfert sur des protéines cibles de polymères de multiples résidus ADP-ribose (Poly-ADP-ribosylation ou PARylation) issus du clivage du NAD^+ en nicotinamide (NAM) et ADP-ribose, nous avons porté une attention particulière aux voies du métabolisme du NAD afin de déterminer si elles pouvaient être altérées dans les cellules déficientes en CDA. Notre étude métabolomique a ainsi révélé une augmentation du niveau de NAM, substrat de la

nicotinamide phosphoribosyltransférase (NAMPT), et une diminution du niveau de nicotinamide mononucléotide (NMN), produit de la NAMPT, dans les cellules déficientes en CDA. Nous avons confirmé la réduction de l'activité de la NAMPT nucléaire dans les cellules déficientes en CDA. Nous avons montré que la déplétion en NAMPT par ARN interférence ou l'inhibition chimique de l'activité de la NAMPT reproduit la réduction de l'activité basale de PARP-1 dans les cellules exprimant CDA, et que cette diminution de l'activité de PARP-1 était indépendante du niveau cellulaire de NAD^+. De plus, l'expression exogène de la NAMPT sauvage, mais pas celle de la NAMPT mutée dans son site catalytique, restaure complètement l'activité basale de PARP-1 dans les cellules déficientes en CDA, entraînant de fait une normalisation de la fréquence des UFBs dans ces cellules. Ces résultats indiquent que la réduction de l'activité basale de PARP-1 dans les cellules déficientes en CDA résulte de la diminution de l'activité de la NAMPT. Nous proposons un modèle dans lequel l'accumulation intracellulaire de dC /dCTP résultant de la déficience en CDA pourrait entraver l'activité de la NAMPT nucléaire, provoquant une accumulation intracellulaire de NAM, un inhibiteur naturel connu de PARP-1, qui par conséquent réduirait l'activité basale de PARP-1. Nos résultats révèlent pour la première fois, un lien entre les voies de sauvetage du pool des pyrimidines et du NAD.

Introduction

Bloom syndrome and the BLM protein

1. Bloom syndrome

Bloom syndrome (BS) was first described in 1954 by the dermatologist Dr. David Bloom as a "congenital telangiectatic erythema resembling lupus erythematosus in dwarfs" (Bloom 1954). This syndrome is a rare autosomal recessive disorder, resulting from mutations in both copies of the *BLM* gene encoding the BLM protein which displays a 3'-5' helicase activity and belongs to the DExH box-containing RecQ DNA helicase subfamily (Ellis, Groden et al. 1995). A register program, named Bloom Syndrome Registry was created in 1960 with the main objective of surveilling the risk of cancer development in the affected families. This registry comprised 168 individuals (93 males and 75 females) until the beginning of 1991, when this program was discontinued (German 1969, German, Bloom et al. 1977, German, Bloom et al. 1979, German, Bloom et al. 1984, German and Passarge 1990, German 1995). BS frequency is uncommon in the general population, being is 1 in 10.836.000 in Japan, 1 in 6.331.000 in the United States, 1 in 5.590.000 in West Germany and 1 in 2.395.000 in Netherlands (James and Hiraku 1989) but relatively frequent in the Ashkenazi Jewish population (1 in 48.000) (German 1969, Shahrabani-Gargir, Shomrat et al. 1998). The frequency of BS in the Jewish population is due to the founder effect, where 1% of the Ashkenazi Jewish is heterozygous carrier for the *blm*^{Ash} mutation (Shahrabani-Gargir, Shomrat et al. 1998).

1.1. Clinical features

BS patients present some persistent clinical phenotypes called the "three cardinal features" (Bloom 1966): a) a proportional pre-and post-natal growth retardation that follows through child and adulthood. The mean height for men is 147,5 cm (it ranges from 130 to 162 cm) and the mean height for women is around 138,6 cm (range from 122 to 151 cm), b) the presence of a telangiectatic erythema in the face, affecting mainly the butterfly area of the face. This erythema can also affect the lips, cheeks, nose, forehead and sometimes the forearms and the dorsa of the hands (German 1969, German 1993), and, c) the sun-sensitivity of the patients, that develops during the first or second summer-life and its responsible for the appearing of skin lesions (Bloom 1966, German 1969, German 1993). Other clinical phenotypes can be present:

- Dolicephaly, in which is associated to a "bird-like" narrow face, to nasal prominence and mandibular hypoplasia (German 1969, German 1993, German 1997).

- Hypo- and hyperpigmentation of the skin that resembles “café au lait” spots (German 1993).
- High-pitched and strident voice (“mickey mouse” voice) (German 1993).

In addition to the physical features, BS patients can suffer from moderate to severe immunodeficiency that appears during infancy. This immunodeficiency is manifested by life-threatening respiratory and gastrointestinal infections. This pathology disappears with the advance of the age but, for now, its origin is unknown. Moreover, in the Bloom syndrome register was mentioned that certain BS patients (20 patients from 168) can developed insulin resistance and non-insulin dependent type diabetes (at a mean age of 24.9 years old). Infertility in males is due to defective spermatogenesis and reduced fertility in women is a consequence of early menopause (German 1993, German 1997). Intelligence is normal, but some cases of mental retardation were reported (German 1969, German 1995).

1.2. Cancer predisposition

The most striking feature of BS is the cancer predisposition of the patients to all types of cancer that commonly affect the general population, at an early age. Two hundred and twelve cancers were reported from 136 patients of the Bloom syndrome registry (Table 1). This registry demonstrated that the BS patients had a similar distribution to the general population but cancer development occurred at a premature age: the mean age was 27 years old (Cunniff, Bassetti et al. 2017). Interestingly, no melanoma was found in BS patients, though this syndrome leads to the development of skin lesions and other skin cancers (Cunniff, Bassetti et al. 2017).

Cancer site/type		Number of cancers	Mean age at diagnosis
Epithelial (carcinoma)	Small and large intestine	31	35
	Skin	27	32
	Upper gastroint/respiratory tract	22	38
	Genitalia & urinary tract	19	17
	Breast	17	35
	Lower respiratory tract	9	33
	Liver	1	15
Hematologic	Lymphoma	35	22
	Acute lymphoblastic leukemia	13	20
	Acute myelogenous leukemia	27	18
Other	Sarcoma	4	16
	Germ-cell	2	24
	Medulloblastoma	1	3
	Retinoblastoma	1	1
	Metastatic – primary unknown	3	34
All		212	27

Table 1. The 212 cancers from the 136 patients of the Bloom Syndrome Registry (adapted from Cunniff *et al*., 2017).

Although BS was described in 1954, its proneness for cancer development was only described in 1965 (German 1993). On the contrary of the others genetic instability-associated syndromes, in which, it leads to the development of specific tumors, BS is the only syndrome that is associated to the predisposition to all types of cancer (German, Bloom et al. 1977, Chu and Hickson 2009). It is suggested that one or several main events that lead to carcinogenesis may be already initiated in BS patients, which could explain the appearance of cancer at a very early age. In fact, it is proposed that the cancers that arise in these patients have several steps in common with tumor progression event. BS presents all the features that lead to genomic instability and consequently to neoplasia transformation and tumor progression (German 1993). Therefore, this syndrome is one of the best model to understand the carcinogenesis process in the general population.

1.3. Bloom syndrome cellular phenotypes

BS is a rare disease that belongs to the group of “Chromosomal breakage syndromes”, that also includes the Ataxia Telangiectasia (AT), Fanconi anemia (FA), Xeroderma Pigmentosum (XP), among others (Duker 2002). All these syndromes are characterized by genomic instability, the presence of induced and/or spontaneous chromosomes breaks and are associated to cancer predisposition.

1.3.1. Cytogenetic features

As mentioned earlier, BS cells presents several cytogenetic abnormalities. This was highlighted through karyotype analyses of BS cells, where it was observed an increase in chromosomes breaks and some chromosomal rearrangements. Moreover, 1 to 5% of metaphase BS cells presented a quadriradial configuration (German 1964, German, Archibald et al. 1965) due to non-sister chromatids exchanges of homologous chromosomes. This study was also demonstrating for the first time, the crossing-over process in somatic cells (Chaganti, Schonberg et al. 1974).

Nevertheless, one important feature of BS cells is the increase frequency in sister-chromatid exchanges (SCEs), which was demonstrated to be a product of RAD51- mediated Homologous Recombination (HR) during DNA replication. BS cells present a 10-fold-increase frequency in SCEs in compared with normal cells (Chaganti, Schonberg et al. 1974). SCEs are observed by the incorporation of DNA base analog, 5’-bromodeoxyuridine (BrdU) during two rounds of replication, in combination with Hoechst dye 33258 staining and finally by using a DNA dye, Giemsa (Latt 1973, Perry and Wolff 1974). This technique allows chromosomes to have an appearance of “Arlequin” pattern (Figure 1), where the sister chromatid that incorporated BrdU in only one strand (mono-substituted) is stained and visualized as black, whereas the bi-substituted strand is less stained and visualized as slight grey (Wilson and Thompson 2007).

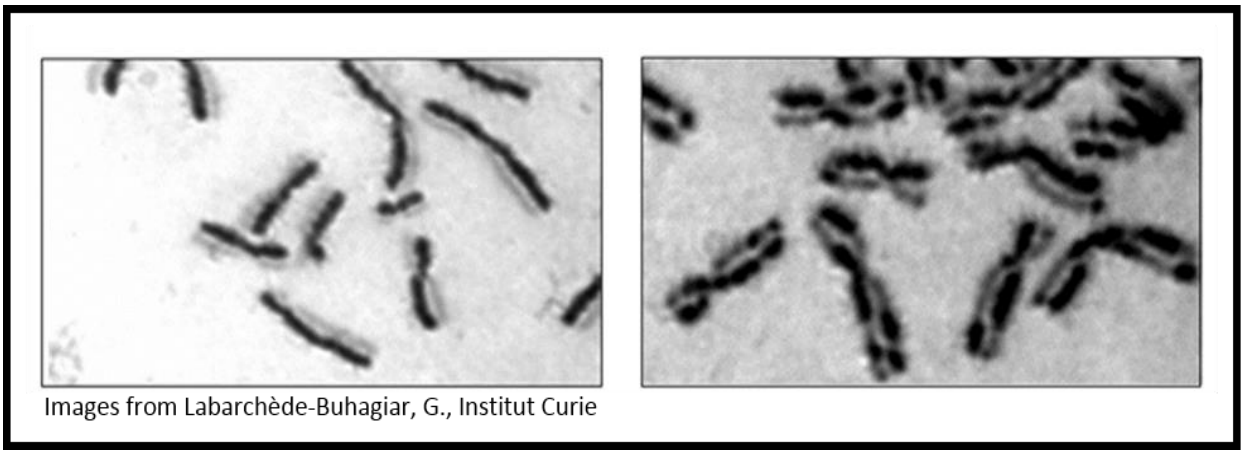


Figure 1. Example of SCE from BS cell expressing BLM (left) and BS cells (right).

Until recently, the increase in SCE frequency was the only objective criteria for BS diagnosis, but, in nowadays, the identification of *BLM* gene mutations by DNA sequencing is also performed (Amor-Guéret, Dubois-d'Enghien et al. 2008).

1.3.2. DNA Replication abnormalities

Even before the discovery of the *BLM* gene, it was already known that BS presented several abnormalities in DNA replication. By performing a technique of DNA fiber autoradiography with a radioactive pulse, Hand and German (1975) demonstrated, for the first time, that BS cell lines shown a slowing-down of the replication forks speed (Hand and German 1975, Giannelli, Benson et al. 1977, Hand and German 1977). This work was later confirmed through DNA molecular combing, a new and more specific technique for determining DNA replication speed (Rao, Conti et al. 2007). They also found an increase in the frequency of asymmetric replication forks, reflecting an accumulation of stalled replication forks. Moreover, Rao *et al.* (2007) reported a decrease in inter-origins distance and an increase in replication origins activation. To compensate a defective DNA replication, sleeping origins of replication are activated in BS cells to fulfill the replication of their genome (Rao, Conti et al. 2007).

1.3.3. Mitotic abnormalities

From cells derivate from BS patients, it was observed an increase in anaphase bridges frequency, the presence of late chromosomes associated to loss of chromosomal fragments and defective segregation due to non-disjunction of chromosomes (Figure 2) (German 1969).

Such mitotic abnormalities lead to aneuploid cells, binuclei cells and/or cells with micronuclei (German 1969, Rosin and German 1985).

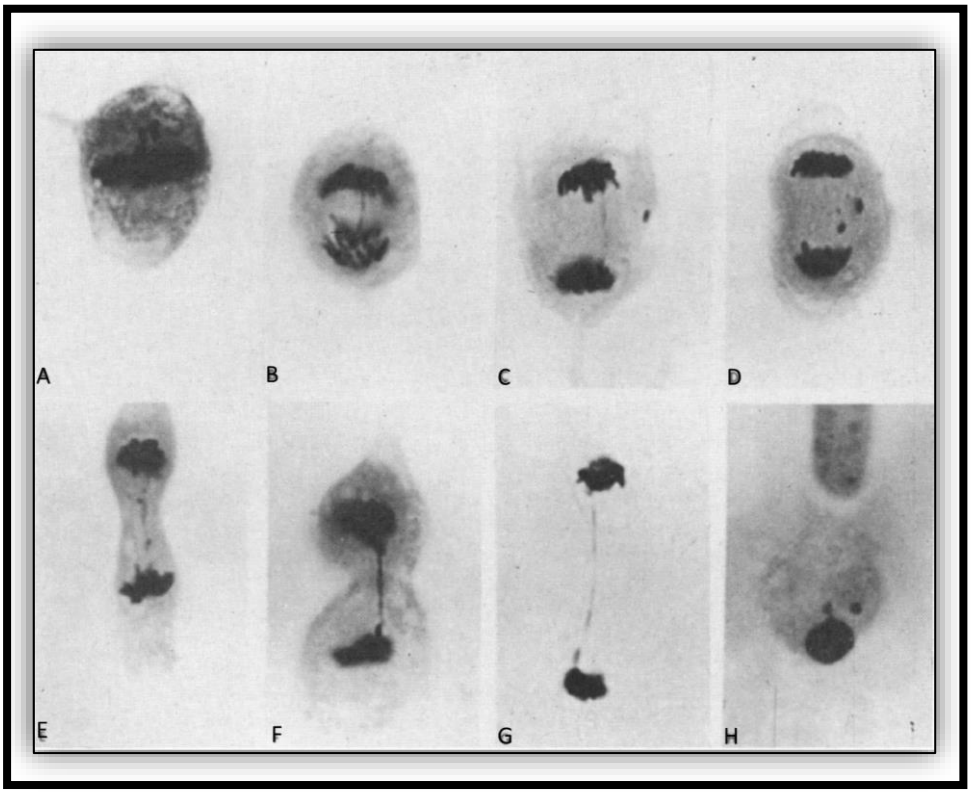


Figure 2. Mitotic abnormalities presented in BS cells. A) Late chromosomes, B-G) anaphase bridges and H) micronuclei (from German, 1969).

Remarkably, anaphase bridges are a well-known marker for genomic instability and may contribute to tumor progression (Chan, North et al. 2007). These anaphase bridges are DNA structures that bound both poles of the cell during anaphase and there are two types of anaphase bridges: chromatin bridges and ultra-fine anaphase bridges (UFBs). Chromatin bridges (Figure 3) are pathological DNA structures resulting from a defect in chromosome segregation during anaphase (Acilan, Potter et al. 2007, Fenech, Kirsch-Volders et al. 2011, Bizard and Hickson 2018). These bridges are constituted of histones and can be visualized by classical DNA intercalants like 4’,6’-diamidino-2-phenylindole (DAPI) (Chan, North et al. 2007).

Micronuclei result from the breakage of chromatin bridges that have not been resolved during anaphase (Hoffelder, Luo et al. 2004).

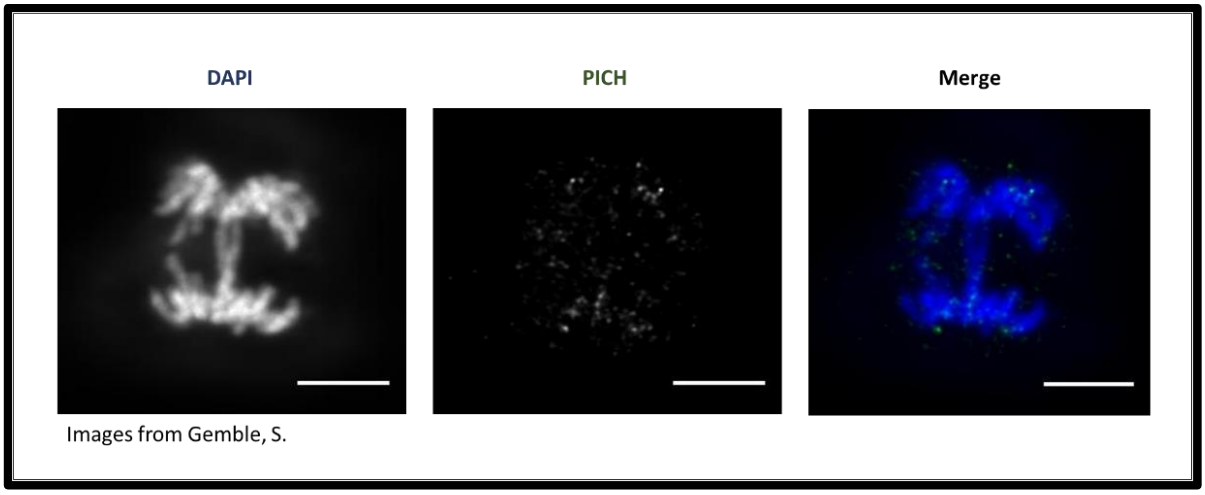
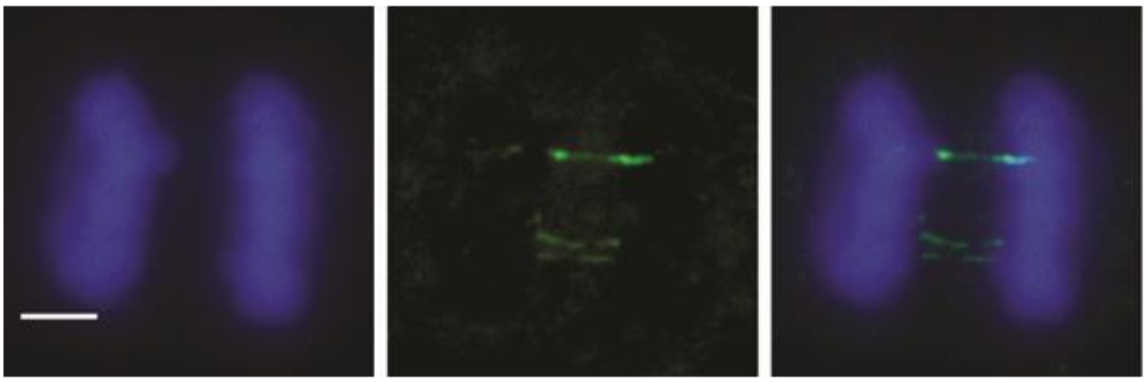


Figure 3. Example of a chromatin bridge.

UFBs (Figure 4) are also DNA structures, since they are sensitive to DNAse treatment. They are physiological structures that are thought to contain either catenated DNA or unreplicated DNA that persists in mitosis (Chan, North et al. 2007, Gemble, Ahuja et al. 2015). UFBs cannot be stained by conventional dyes like DAPI nor by antibodies against histones; they are visualized by immunofluorescence using specific antibody against helicase-like protein called PLK1-interaction checkpoint helicase protein (PICH) and/or antibody against BLM (Baumann, Körner et al. 2007, Chan and Hickson 2009, Chan and Hickson 2011, Liu, Nielsen et al. 2014). UFBs, mostly of centromeric origin, are found in all cultured cells tested and are probably physiological structures whose function is still unknown (Chan and Hickson 2009, Liu, Nielsen et al. 2014). Also, treatment of cells with aphidicolin (APH), a DNA polymerase α inhibitor, leads to the increase in the frequency of common fragile site (CFS) UFBs (CFS-UFBs) (Chan, Palmai-Pallag et al. 2009). A small proportion of UFBs extend from telomeres (T-UFBs) (Chan and Hickson 2009, Liu, Nielsen et al. 2014), or from ribosomal DNA loci (R-UFBs) in chicken (Nielsen and Hickson 2016) and in human cells (Bou Samra, Buhagiar-Labarchède et al. 2017).



Images from Cunha Silveira, S.

Figure 4. Example of an ultra-fine anaphase bridge (UFB).

1.3.4. Oxidative stress

Oxidative stress (OS) is described as an imbalance between the production of reactive oxygen species (ROS)/ reactive nitrogen species (RNS) and the levels of antioxidant molecules that can scavenge them. It is well known that OS can damage important cellular macromolecules such as DNA (Pisoschi and Pop 2015, Van Houten, Santa-Gonzalez et al. 2018).

BS is characterized by a genomic instability and it has been proposed that oxidative stress might contribute to this phenotype (Nicotera 1991). Several studies revealed abnormalities in the redox balance in BS cells. Nicotera and colleagues (1989) reported that addition of α-tocopherol, an antioxidant compound, led to a moderate decrease in SCE frequency (10 to 15%) (Nicotera, Notaro et al. 1989). Another study showed that BS cells (both fibroblasts and lymphocytes) exhibit an increase in superoxide dismutase (SOD) activity (for about 200%) as a reflection of the intracellular accumulation of superoxide anion ($O_2^{\cdot -}$). In addition, this work highlighted a correlation between ROS concentration, and more important the intracellular accumulation of $O_2^{\cdot -}$, and the increase in SCE frequency. When BS cells are treated with antioxidant agents, such as α-tocopherol, a decrease in SCEs rate (17 to 23%) is observed. A few years later, Nicotera and colleagues (1993) confirmed that BS cells present an increase (for about 3 times) in ROS intermediate species in compared to normal cells (Nicotera, Thusu et al. 1993).

Finally, the presence of redox imbalance is BS cells was confirmed through a clinical study (where blood and urine were used as samples). It was observed that leukocyte levels of 8-hydroxy-2'-deoxyguanosine (8-OHDG), a biomarker for DNA damage, were increased in four

patients versus 40 controls. In addition, plasma levels uric acid (UA), one important antioxidant present in the blood, was found increased, suggesting a compensatory mechanism to the elevated oxidative stress present in BS. In addition, the oxidized and reduced forms of glutathione disulfide ratio (GSSH/GSH ratio), which is primordial for the maintenance of redox balance, was decreased in BS patients (Zatterale, Kelly et al. 2007).

All these data support that BS cells presents a chronic oxidative stress state that might explain some aspects of the BS phenotype such as growth retardation and insulin resistance. Furthermore, a better understating of the pro- and antioxidant homeostasis in BS could be a promising information for new therapeutic strategies (Maciejczyk, Mikoluc et al. 2017).

2. The BLM protein

2.1. BLM localization

The *BLM* gene was mapped at the chromosome 15 at the band 15q26.1, which encodes a protein with the same name. BLM is a nuclear protein and in normal conditions can be localized in two types of structures: in large and diffuse patches, called microspeckles and in bright foci, where the number of which is dependent on the cell cycle: the maximum number and intensity of these foci are found in late S phase and in the beginning of G2 phase (Yankiwski, Marciniak et al. 2000, Bischof, Kim et al. 2001). The most part of these foci are found in promyoletic leukemia protein (PML) bodies (Bischof, Kim et al. 2001). The BLM protein is also detected in the nucleolus in the beginning of the S phase (Sanz, Proytcheva et al. 2000, Yankiwski, Marciniak et al. 2000, Cunniff, Bassetti et al. 2017).

During mitosis, BLM is phosphorylated and excluded from an insoluble fraction containing mainly chromatin and cytoskeleton (Dutertre, Sekhri et al. 2002). Part of the hyperphosphorylated mitotic BLM is associated with the mitotic spindle during pre-metaphase (Nousiainen, Silljé et al. 2006). Also, in cells treated with DNA damage agents, especially agents that affect DNA replication, BLM is found at stalled or damage replication forks (Cunniff, Bassetti et al. 2017). Finally, the lab demonstrated that BLM localized at centromeres during G2 phase until mitosis. This leads to the recruitment of topoisomerase IIα

(Topo IIα), facilitating decatenation of centromeres at the metaphase-anaphase transition (Rouzeau, Cordelières et al. 2012).

2.2. BLM expression

BLM protein is expressed in all normal and tumor tissues and in proliferative cells, but not in quiescent, serum-deprived cells or unstimulated lymphocytes (Kawabe, Tsuyama et al. 2000). BLM expression is regulated during the cell cycle: the BLM protein accumulates in S phase, persists in G2/M and sharply declines in G1, strongly suggesting its degradation during mitosis (Dutertre, Ababou et al. 2000). Finally, BLM expression increased in cells treated with DNA damage agents (ionizing radiations, bleomycin) or with DNA replication inhibitors (hydroxyurea (HU), aphidicolin) (Ababou, Dutertre et al. 2000, Bischof, Kim et al. 2001).

2.3. Functions of BLM

BLM, a 3'-5' DNA helicase belonging to the RecQ helicase subfamily, is known as genome caretaker, due to its important role in maintaining genomic stability (Bachrati and Hickson 2008, Chu and Hickson 2009). It was demonstrated that BLM is involved in DNA repair and in the restart of blocked replication fork (Ralf, Hickson et al. 2006).

2.3.1. BLM and DNA replication

As mentioned earlier, BLM is implicated in the restart of the replication forks and in preventing replication forks blockage by secondary structures. Indeed, during DNA replication, some secondary structures like G-quadruplexes can lead to the stalling of replication forks (León-Ortiz, Svendsen et al. 2014, Magdalou, Lopez et al. 2014).

BLM acts as a "roadblock" remover during DNA replication by disrupting complex structures such as G-quadruplexes or DNA hairpins (Huber, Duquette et al. 2006, Wu, Hou et al. 2015). It has also been suggested that, thanks to its reverse branch migration activity, BLM plays a key role in restarting replication after the stalling of the fork (Ralf, Hickson et al. 2006). This hypothesis is supported by Rao *et al.* (2007), which, as already mentioned, reported an increase in asymmetric replication forks when BLM is absent (Rao, Conti et al. 2007).

2.3.2. BLM and homologous recombination

HR is a pathway that functions during the S and G2 phase of the cell cycle that allow the repair of double-strand DNA breaks (DSBs). HR is initiated by 5' to 3' resection of the ends of the broken DNA mediated by the Mre11-RAD50-Nbs1 (MRN) complex in association with CtIP (C-terminal-binding protein) that catalyzes a limited resection (Symington 2014). A second long resection is performed by DNA exonuclease 1 (Exo1) or by the helicases WRN/BLM-Dna2 complex, producing a 3' overhang single strand DNA (ssDNA) (Nimonkar, Genschel et al. 2011, Sturzenegger, Burdova et al. 2014). Then, this 3' overhang ssDNA is coated first by the ssDNA-binding protein replication protein A (RPA), which in turn, is replaced by the recombinase RAD51 via its mediator BRCA2 (Liu, Doty et al. 2010, Ma, Gibb et al. 2017). The RAD51 nucleoprotein filament starts the homology search for the strand exchange with the homologous sequence, creating a D-loop structure (Renkawitz, Lademann et al. 2014). After, the 3' end of the invading strand is extended by DNA synthesis by polymerases. In the context of a DSB, the second end of the break is capture by annealing to the D-loop, leading to the formation of a double Holliday junctions (HJ), that are resolved by GEN1 giving rise to both crossover or non-crossover products (Ip, Rass et al. 2008) or by BLM - Topo IIIα - RMI1 complex giving rise to non-crossing recombinant products (Wu and Hickson 2003, Bussen, Raynard et al. 2007) (Figure 5).

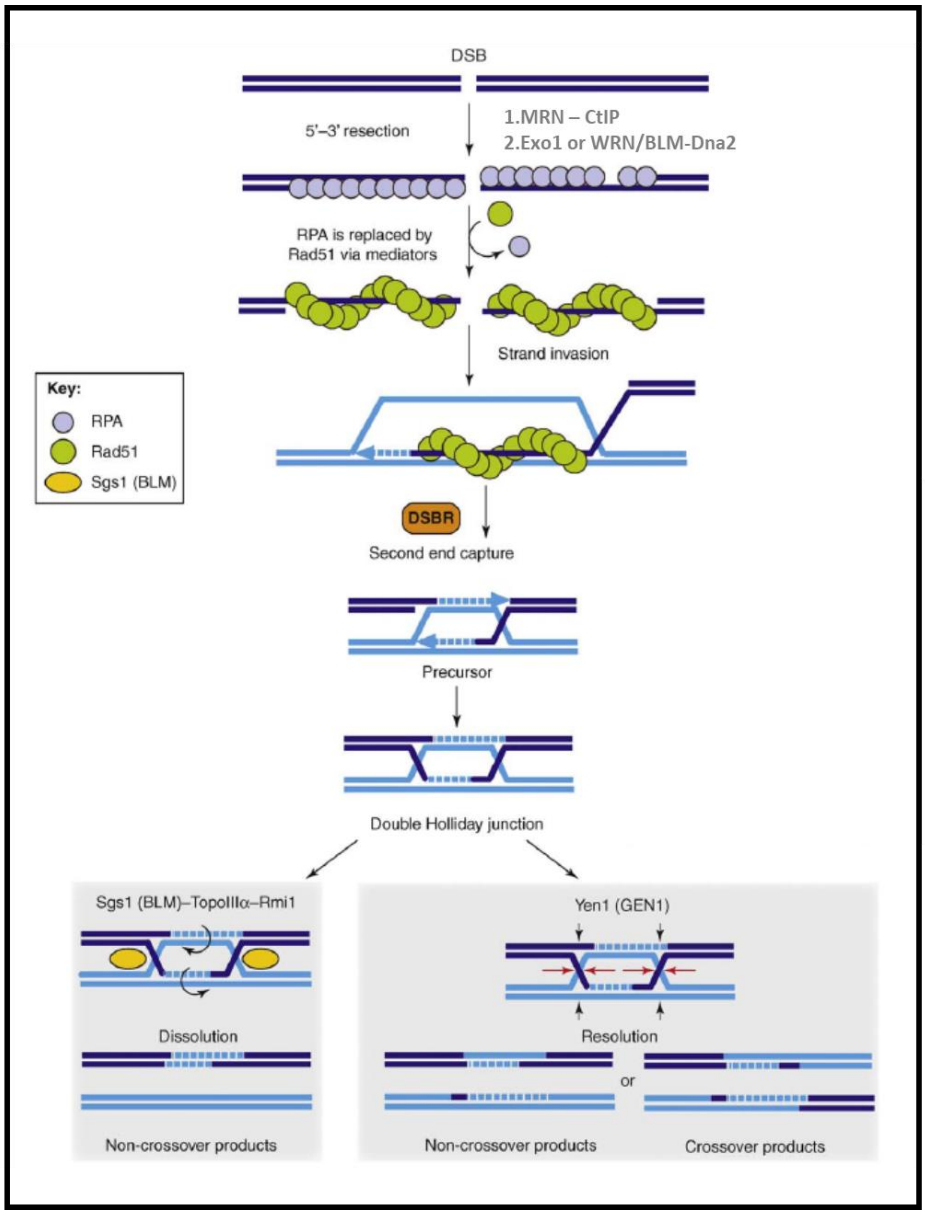


Figure 5. Homologous recombination pathway. In the context of a DSB repair, HR is started by the 5' to 3' end resection of DSB mediated by the MRN complex in association with CtIP. A second resection (long resection) is performed by Exo1 or BLM-Dna2 complex producing a 3' overhang ssDNA Then, the produced 3' overhang ssDNA is coated, first, by RPA (light purple dots), which in turn is replaced by the recombinase RAD51 (green dots) via its mediator BRCA2 (not depicted). The RAD51 nucleoprotein filament starts the homology search for the strand exchange, creating a D-loop structure. After, the 3' end of the invading strand is extended by DNA synthesis by polymerases (light blue discontinuing line). Then, the second end of the break is capture by annealing to the D-loop, leading to the formation of a double HJ, that can be resolved by GEN1 (Yen1 in Saccharomyces cerevisiae) giving rise to both crossover or non- crossover products or by BLM (Sgs1 in Saccharomyces cerevisiae) - Topo IIIα - RMI1 complex giving rise to non-crossing recombinant products (adapted from Mimitou and Symington 2009).

BLM can be important in the early steps by stimulating resection as well as in late steps of HR with the function of resolving HJ (Chu, Hanada et al. 2010) (Figure 6). In the early steps of HR, BLM acts as both an anti- and pro-recombination factor leading to the ① disruption of the RAD51-ssDNA filament, an important process in the early steps of HR, and ultimately inhibiting the DNA strand exchange and HR (Bugreev, Yu et al. 2007). Conversely, the same group reported that BLM can also ② stimulate RAD51 DNA strand exchange activity (Bugreev, Mazina et al. 2009). The interaction of BLM with RAD51 is dependent of the conformational state (active or inactive) of the RAD51-ssDNA filament: if RAD51 is present in an ADP-bound form (inactive), BLM leads to the disruption of RAD51-ssDNA filament (Bugreev, Yu et al. 2007) and if RAD51 is in its active form, then, BLM promotes RAD51 and DNA strand exchange (Bugreev, Mazina et al. 2009). ③ Furthermore, BLM also disrupts D-loops which are formed by the RAD51 strand exchange activity, allowing the repair of DSBs by other mechanism and suppressing HR (van Brabant, Ye et al. 2000). Finally, the balance between the anti-and pro-recombinant function is regulated by BLM SUMOylation. In fact, BLM SUMOylation promotes RAD51 function for homology search and DNA strand exchange activity whereas absence of SUMOylation of BLM impairs RAD51 activity (Ouyang, Woo et al. 2009, Ouyang, Matunis et al. 2013).

④ BLM also participates in the late steps of HR by resolving HJ that are formed during this pathway and gives rise to non-crossover products. BLM interacts with Topo IIIα and RMI1-RMI2 to dissolve these structures, by promoting the branch migration of the two HJ toward each other (Karow, Constantinou et al. 2000, Wu and Hickson 2003).

⑤ In addition to its role during DSB repair by HR, BLM is also implicated in the replication fork restart. When the progression of the replication fork is impaired by DNA lesions, this could lead to replication fork collapse and DSBs formation. BLM can promote replication fork restart via the promotion of the replication fork regression, leading to the formation of particular structures named “chicken foot”. The formation of these structures facilitates the stabilization of the replication fork for the repair of DNA lesion. However, DNA lesion can be bypass by extension of the leading strand that uses the lagging strand as template (Ralf, Hickson et al. 2006, Atkinson and McGlynn 2009) Finally, due to its activity of inverse branch migration, BLM

promotes the restart of the replication fork (Karow, Constantinou et al. 2000, Wu and Hickson 2003, Amor-Guéret 2006).

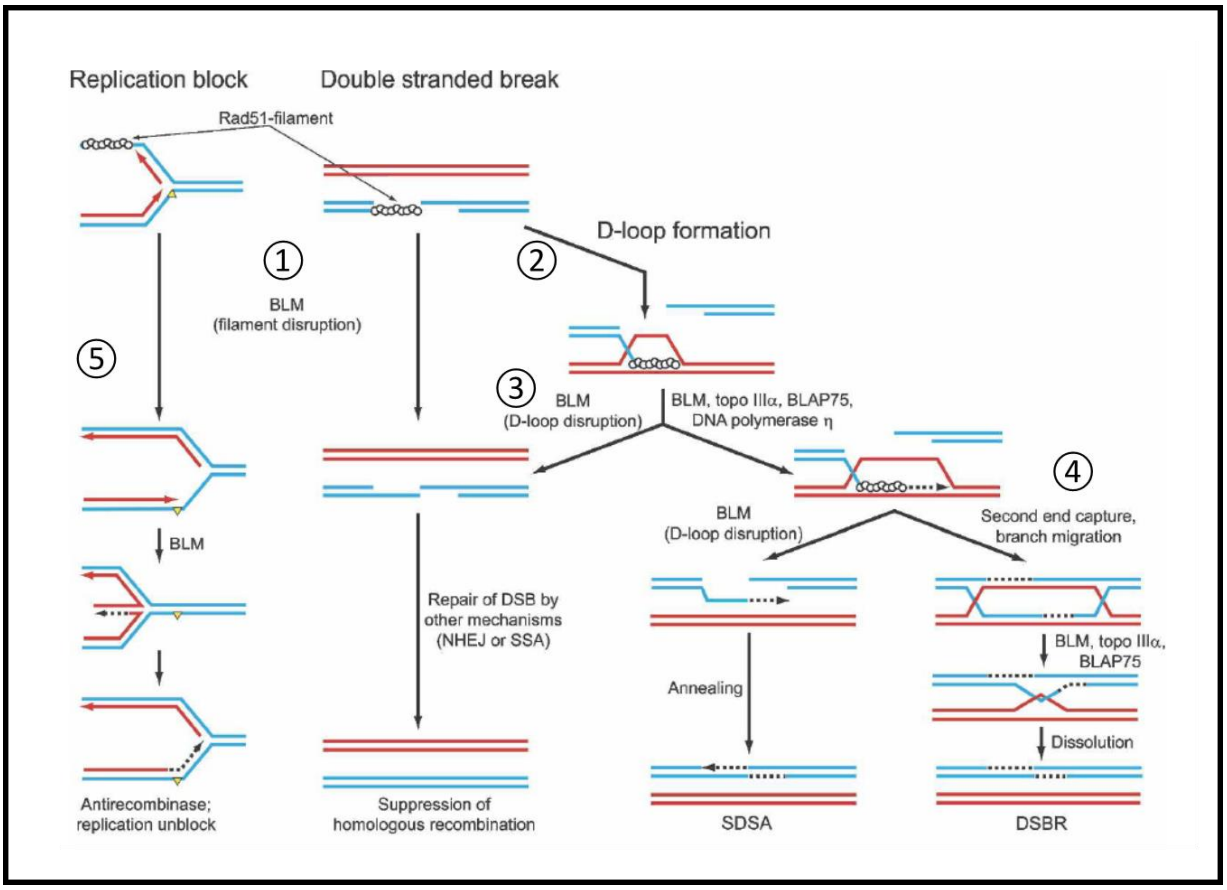


Figure 6. The functions of BLM in the HR. BLM is implicated in both the disruption and stimulation of RAD51 depending of its conformational state. BLM can also suppress HR by resolving D-loops that are formed by the DNA strand invasion. BLM is also important for HR since it is responsible for the dissolution of HJ structures. In addition to its role in HR, BLM is also implicated in the restart of stalled replication forks (adapted from Bugreev *et aL,* 2007).

2.3.3. BLM and non-homologous end joining

Non-homologous end joining (NHEJ) consist of another mechanism for DSBs repair that allows the re-ligation of extremities without homologues sequences. This mechanism is performed by two pathways: the canonic or classical (C-NHEJ) pathway (Figure 7A) and alternative pathway (Alt-NHEJ) (Figure 7B), source of chromosomal rearrangements. In contrast to HR that can take place only during S and G2 phases of the cell cycle, Alt-NHEJ is active during all the cell cycle (Bétermier, Bertrand et al. 2014, Williams, Hammel et al. 2014).

The most predominant NHEJ pathway, C-NHEJ (Figure 7A), initiates by the loading of Ku70/Ku80 complex to the DNA ends, which in turn, recruits the DNA-PKcs catalytic subunit and forms the DNA-PK complex. The Ku70/Ku80 complex is also important for the recruitment of the XRCC4/DNA ligase IV complex to the DNA ends (Nick McElhinny, Snowden et al. 2000, Mari, Florea et al. 2006). The XRCC4 protein is going to interact with Ku70 whereas DNA ligase IV is going to interact with Ku70/Ku80 complex (Costantini, Woodbine et al. 2007, Hsu, Zhang et al. 2012). Then, the other factors such as XLF/Cernunos are recruited via interaction with the Ku70/Ku80 complex or via interaction with XRCC4 to promote DSBs repair (Mari, Florea et al. 2006).

If the C-NHEJ is compromised, cells are able to repair DSBs by activating the Alt-NHEJ (Figure 7B) that operates at slower kinetics. The first steps of Alt-NHEJ are common with HR and it consists of DNA resection by the complex CtIP/Mre11 (Rass, Grabarz et al. 2009, Lee-Theilen, Matthews et al. 2010). The ligation step is performed by DNA ligase III with the help of XRCC1 and/or ligase I and promoting DSBs repair (Della-Maria, Zhou et al. 2011, Lu, Duan et al. 2016). PARP-1 was also reported to be implicated in Alt-NHEJ by promoting the recruitment of Alt-NHEJ factors (Haince, McDonald et al. 2008).

BLM prevents Alt-NHEJ and large deletions (>200 nucleotides) that are mediated by the CtIP/MRE11 complex. BLM represses Alt-NHEJ through an epistatic regulation of 53BP1 and its partner, RIF1. In G1 phase, 53BP1 and RIF1 confine the resection activity of BLM. These data, leads to suggest that BLM, as in HR, has a double role in Alt-NHEJ: by protecting the extensive DNA resection by the CtIP/MRE11 complex and to promote DNA resection when 53BP1 or RIF1 are absent (Grabarz, Guirouilh-Barbat et al. 2013).

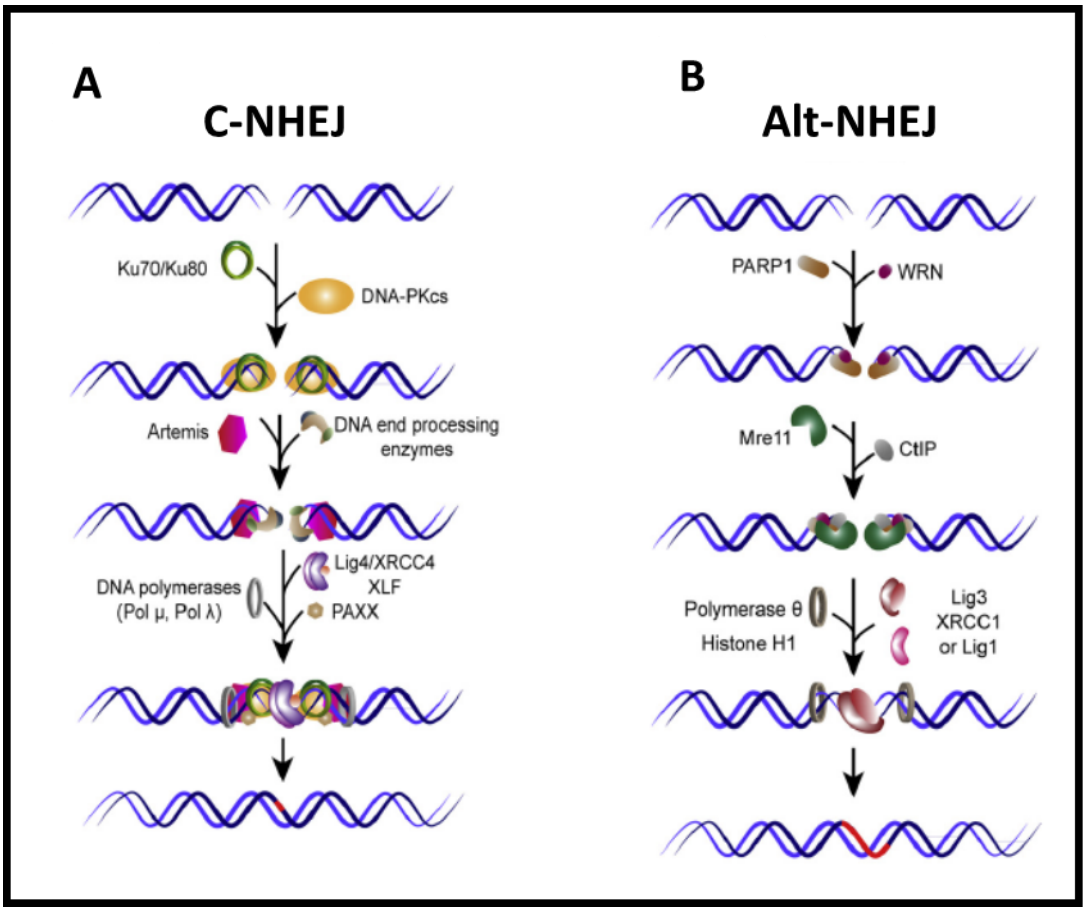


Figure 7. The canonical and alternative NHEJ. The most prominent NHEJ pathway is the C-NHEJ, however, if this pathway is compromised, cells can repair DSB by the Alt-NHEJ, which is a source of chromosomal rearrangements (Iliakis *et al.*, 2015).

2.3.4. BLM and telomeres

Telomeres are DNA-protein complexes located at the extremities of the chromosomes. They have an important role in the maintenance of genome stability, in cell survival and cell proliferation (O'Sullivan and Karlseder 2010). The telomeric sequences can form DNA secondary structures such as D-loop (or T-loop) or G-quadruplexes structures, that need to be resolved for a correct telomere replication (Maciejowski and de Lange 2017). G-quadruplexes are four-strand DNA structures that results from the folding of G-rich (TTAGGG) repeats strands that are present in telomeres 3' ends (Wright, Tesmer et al. 1997, Hänsel-Hertsch, Di Antonio et al. 2017).

BS cells present a slowdown of the replication fork speed at telomeres associated with an increase in G-quadruplexes frequency (Drosopoulos, Kosiyatrakul et al. 2015). BLM depletion also leads to the association of telomeres between the arms of homologous chromosomes

(Lillard-Wetherell, Machwe et al. 2004). Moreover, BLM is recruited at telomeres and interacts with telomeric proteins telomeres repeat 1 and 2 (TR1, TR2) and protection of telomeres homolog 1 (POT1) stimulating its T-loops dissociation activity (Opresko, von Kobbe et al. 2002, Lillard-Wetherell, Machwe et al. 2004, Opresko, Mason et al. 2005, Barefield and Karlseder 2012). These data suggest that BLM might facilitate the progression of replication forks at telomeres by resolving secondary structures upstream the forks (Opresko, Mason et al. 2005, Drosopoulos, Kosiyatrakul et al. 2015) .

2.3.5. BLM and mitosis

As mentioned in point 2.1, Amor-Guéret's team reported that: BLM is hyperphosphorylated in mitosis which leads to its exclusion from an insoluble fraction, containing mainly chromatin and nuclear matrix. This phosphorylation is mediated by cdc2 kinase. This was confirmed by treating cells with ionizing radiation, leading to inactivation of cdc2 kinase and consequently to the dephosphorylation of BLM and its possible recruitment to specific DNA damage sites (Dutertre, Sekhri et al. 2002).

During mitosis, BLM is also associated to certain mitotic abnormalities. It was reported that both BLM helicase and PICH can localize on some chromatin bridges (presented in point 1.3.3), as well as BLM protein complex topo IIIα and RMI1, suggesting a role in their resolution (Chan, North et al. 2007). Moreover, BLM and PICH are also localized in UFBs during mitosis. It was demonstrated that PICH is recruited into UFBs during metaphase whereas BLM is recruited to UFBs during anaphase (Baumann, Körner et al. 2007, Chan, North et al. 2007).

Until now, it was thought that absence of BLM in BS cells was the full responsible for the increase in chromatin bridges and UFB frequency. Nonetheless, Gemble, S. *et al.* (2015) demonstrated that supernumerary UFBs in BS cells are entirely due to the pyrimidine pool imbalance as a result of cytidine deaminase (CDA) deficiency and independently of BLM status (Gemble, Ahuja et al. 2015), whereas high chromatin bridge frequency results directly from BLM deficiency, and not from CDA deficiency[1].

[1]*The work about the consequences of nucleotide pool imbalance in Bloom syndrome will be further explain in point 3 of this chapter.*

Finally, BLM is recruited at centromeres from G2 phase to mitosis (Rouzeau, Cordelières et al. 2012). BLM cooperates with PICH at centromeres to recruit active Topo IIα to eliminate DNA catenation before the onset of anaphase. BLM-deficient prometaphase cells and PICH-deficient cells display changes in the structure of centromeric chromatin. They proposed a model in which the combined action of BLM and PICH promotes the organization of centromeric chromatin, thereby rendering some centromeric catenates accessible to Topo IIα. This mechanism might prevent centromeric non disjunction and the potential formation of supernumerary UFBs that might interfere with abscission (Rouzeau, Cordelières et al. 2012).

3. Bloom syndrome and the nucleotide pool imbalance

Nucleotide pool imbalance leads to genome instability, as well as, to mutagenesis in both bacteria and eukaryotic cells (Kunz 1982, Meuth 1984, Bester, Roniger et al. 2011). In mammalian cells, nucleotide pool disequilibrium is responsible for chromosomal aberrations and formation of SCEs (Popescu 1999).

Several years ago, it was suggested that some BS phenotypes could be associated to a nucleotide pool imbalance. Indeed, in 1986, Bamezai and Shiraishi, demonstrated that co-culturing BS lymphocytes with normal lymphocytes, was immediately leading to a decrease in SCE frequency in the BS lymphocytes, and after 8 days, to an increase in SCE frequency in normal lymphocytes. These observations suggested the existence of one or several transmissible factors (Bamezai and Shiraishi 1986). Three years later, the same research group, demonstrated that four BS lymphocytes cell lines presented a defective activity (50 to 60%) of thymidylate synthetase. This enzyme catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). Thus, these data suggested a decrease in thymidine pool in BS cells (Shiraishi, Taguchi et al. 1989).

3.1. Bloom syndrome and Cytidine deaminase

While studying the genes and pathways dysregulated in the absence of BLM, Amor-Guéret's team demonstrated that BLM deficiency was associated with a strong dowregulation of cytidine deaminase (CDA) expression in BS fibroblasts (Figure 8) (Chabosseau, Buhagiar-Labarchède et al. 2011). As presented in the next section, CDA is an enzyme of the pyrimidine salvage pathway that catalyzes the deamination of cytidine (C) and deoxycytidine (dC) to the corresponding uridine (U) and deoxyuridine (dU). CDA defects leads to a pyrimidine pool disequilibrium with an increase in C and dC and a decrease in U and dU. In BS cells, deoxycytidine triphosphate (dCTP) levels are twice those in the isogenic BS cells expressing BLM and thymidine triphosphate (dTTP) are slightly but significantly lower (Chabosseau, Buhagiar-Labarchède et al. 2011).

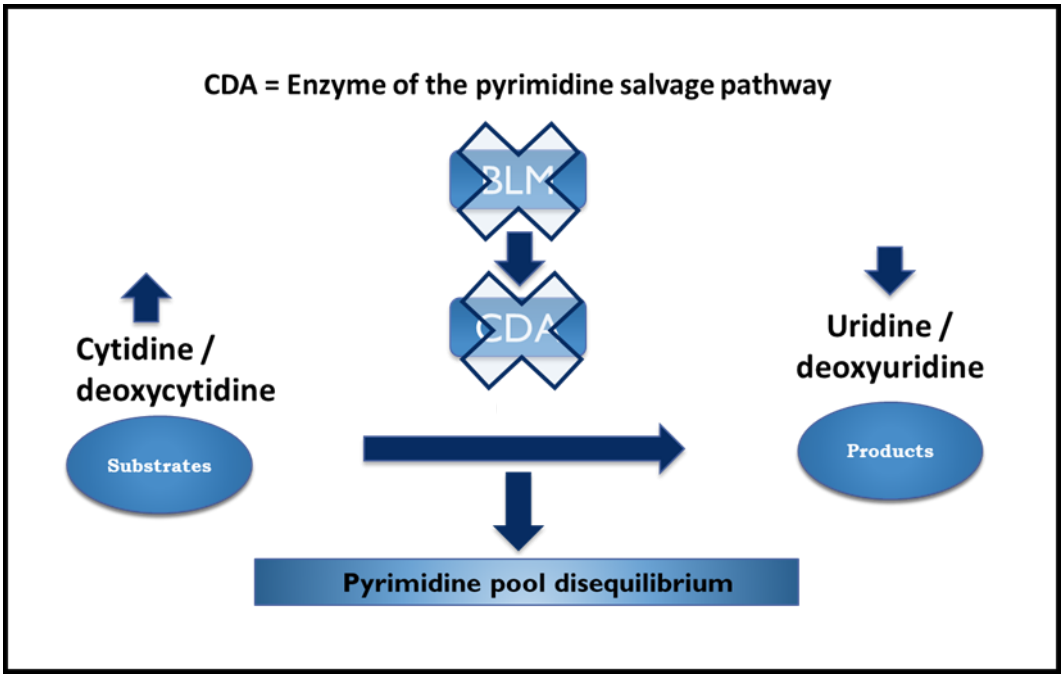


Figure 8. BLM deficiency is associated with CDA downregulation. CDA is an enzyme of the pyrimidine salvage pathway, where its defect leads to nucleotide pool imbalance.

The team demonstrated that the pyrimidine pool disequilibrium resulting from CDA deficiency was responsible for some aspects of the BS phenotype:

- It contributes to the increase in sister chromatid exchange frequency (25-40%).
- It is fully responsible for the slowing-down of replication fork speed.
- It is fully responsible for the increase in UFBs frequency.

Furthermore, the stable expression of CDA protein in BS cells (BS-CDA cell line) restored the dC/dCTP pool, leading to a decrease in SCEs frequency (25%) and restored the replication fork speed, confirming CDA was fully responsible for the pyrimidine pool imbalance and was contributing to the BS phenotypes.

Next, the team was interested in understanding the mechanism underlying the mitotic abnormalities in BLM-deficient cells. In particular, the increase in the frequency of anaphase bridges (chromatin bridges and UFBs). The team demonstrated that chromatin bridges formation results from BLM deficiency whereas supernumerary UFB formation was entirely due to CDA deficiency. They also demonstrated that CDA deficiency and the subsequent dC/dCTP accumulation was responsible for the decrease in basal poly(ADP-ribose) polymerase 1 (PARP-1) activity that, consequently leads to excess UFB formation[2] (Gemble, Ahuja et al. 2015).

[2] *How CDA deficiency leads to supernumerary UFB formation will be presented in chapter 2 (point 2.5 – CDA and genetic instability)*

Due to the importance of CDA expression and activity in BS phenotypes, the next chapter will be focused on CDA and genetic instability.

Nucleotide pool, cytidine deaminase and genetic instability

1. Nucleotide metabolism

Nucleotides are organic molecules essential for several biological processes. High rates of nucleotide synthesis are required for both DNA replication and RNA production in order to sustain protein synthesis during the cell cycle. Nucleotides are continuously synthesized in cells, which is an energy intensive process and, therefore, it is strongly regulated at multiple levels (Lane and Fan 2015).

1.1. Nitrogenous bases, nucleosides and nucleotides

Nitrogenous bases or nitrogen-containing bases are the base of the nucleotides and their biological function is to bound nucleic acids together. There are six major nitrogenous bases that are classified in two major groups: purine and pyrimidines. The purine bases are Adenine (A) Guanine (G) and Hypoxanthine and the pyrimidine bases are Cytosine (C), Thymine (T) and Uracil (U). When a nitrogenous base is covalently bound to a pentose sugar, it is called nucleoside. When the nitrogenous base is attached to a 2'-deoxyribose sugar, it is called deoxyribonucleoside and if it is bound to a ribose sugar is called ribonucleoside. The deoxyribonucleosides are deoxyadenosine (dA), deoxyguanosine (dG), deoxycytidine (dC), deoxyuridine (dU), thymidine (T or dT), deoxyinosine, and the ribonucleosides are the adenosine (A), guanosine (G), cytidine (C), uridine (C), ribothymidine (or thymine riboside), a rare nucleoside, and inosine (Figure 9).

Sugar \ Base	Pyrimidine			Purine		
	Cytosine	Thymine	Uracil	Adenine	Guanine	Hypoxanthine
Deoxyribose	Deoxycytidine	Thymidine	Deoxyuridine	Deoxyadenosine	Deoxyguanosine	Deoxyinosine
Ribose	Cytidine	Ribothymidine	Uridine	Adenosine	Guanosine	Inosine

Figure 9. Chemical structures of nitrogenous bases and respective nucleosides.

Nucleotide is a nucleoside that contain one (NMP), two (NDP) or three (NTP) phosphate groups on the 5’-OH group of the pentose sugar (Figure 10A). When nucleotides are incorporated in to DNA, the adjacent nucleotides are linked by a phosphodiester bond between the 5’ phosphate group of the nucleotide and the 3’-OH group of the other nucleotide (Figure 10B). Furthermore, deoxynucleotide triphosphates (dNTPs) are used as substrates for DNA replication by DNA polymerases (Burke and Lupták 2018).

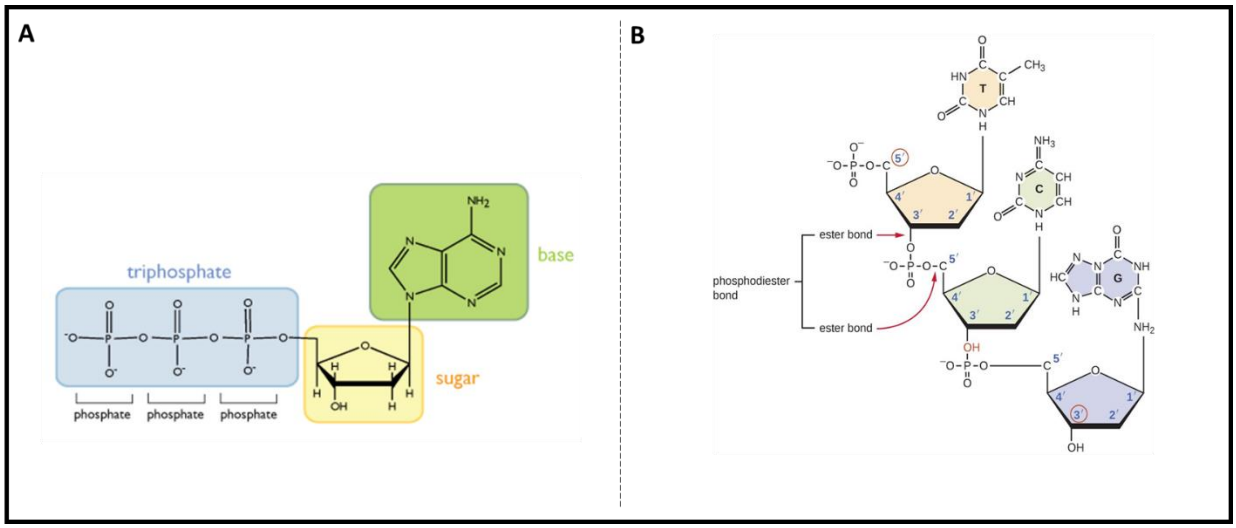


Figure 10. Chemical structure of a nucleotide and phosphodiester bond between nucleotides. A) Nucleotide structure. Nitrogenous base (in green) is bound to the pentose sugar (in yellow) and this one can present one to three phosphate groups (mono, di- or tri- phosphates) (in blue). **B)** Scheme of a phosphodiester bond between two nucleotides. Adjacent nucleotides are linked by a phosphodiester bond between the 5' phosphate group of the nucleotide and the 3'-OH group of the other nucleotide.

Nucleotides are the backbone of nucleic acids. They are synthetized from two major pathways: the *de novo* pathway, which purine and pyrimidine macromolecules are synthesized from simple molecules, and the salvage pathway, where nucleosides and nucleobases from cellular metabolism are converted into the corresponding nucleotide (Moffatt and Ashihara 2002) .

1.2. The *de novo* pathway

The *de novo* purine pathway is a highly conservative and energy-consuming process. It comprises ten initial steps, which starts by the synthesis of inosine monophosphate (IMP) from PRPP and glutamine (Figure 11A). Then, this pathway is divided in two branches, where IMP, initially leads to the formation of adenosine monophosphate (AMP) and guanosine monophosphate (GMP) and finally to the biosynthesis of dATP and dGTP, respectively (Adam 2005, Zrenner, Stitt et al. 2006, Pedley and Benkovic 2017). Like in the *de novo* purine pathway, the *de novo* pyrimidine biosynthesis is also an evolutionary conserved process. This pathway consists of six enzymatic steps that leads to the synthesis of uridine monophosphate (UMP) from glutamine (Figure 11B). This pathway is also called orotate pathway and one of

the enzymatic steps is only performed in the mitochondria (Huang and Graves 2003, Zrenner, Stitt et al. 2006).

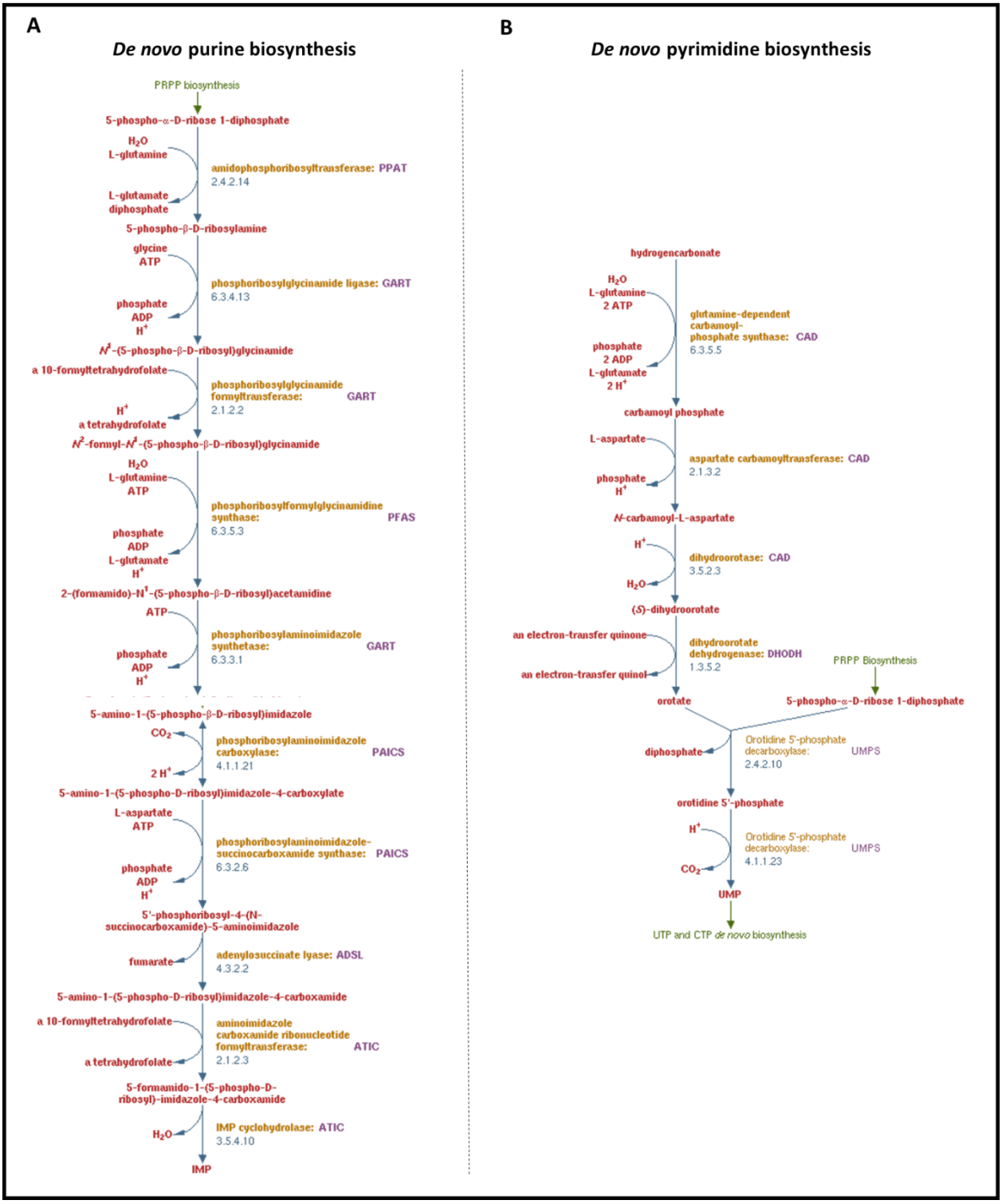


Figure 11. The de novo nucleotide biosynthesis pathway. **A**) In *the de novo* purine nucleotide biosynthesis pathway, IMP is synthesized from PRPP and glutamine after ten reactions steps**. B)** In the *de novo* pyrimidine biosynthesis pathway, UMP is produced from glutamine and hydrocarbonate after six reactions steps (from database Humancyc (www. humancyc.org).

Then, both metabolic pathways have common steps that are performed by two main enzymes that accept all the ribo- and deoxyribonucleotides. The end-product of these pathways are ribonucleotides, which are reduced by the ribonucleotide reductase (RNR) (❶) into deoxyribonucleotides diphosphate (dNDPs) and further phosphorylated into the corresponding deoxynucleotides triphosphates (dNTPs) by nucleoside diphosphate kinases (NDPKs) (❷). However, the synthesis of dTTP requires further metabolic steps. dTTP is synthetized from dUMP by the action of thymidylate synthetase (TS) (❸) into dTMP, then the latter is converted into dTTP by two sequential reactions performed by thymidine monophosphate (dTMP) kinase (❹) and by NDPK (❷), respectively (Figure 12) (Lane and Fan 2015, Wang 2016).

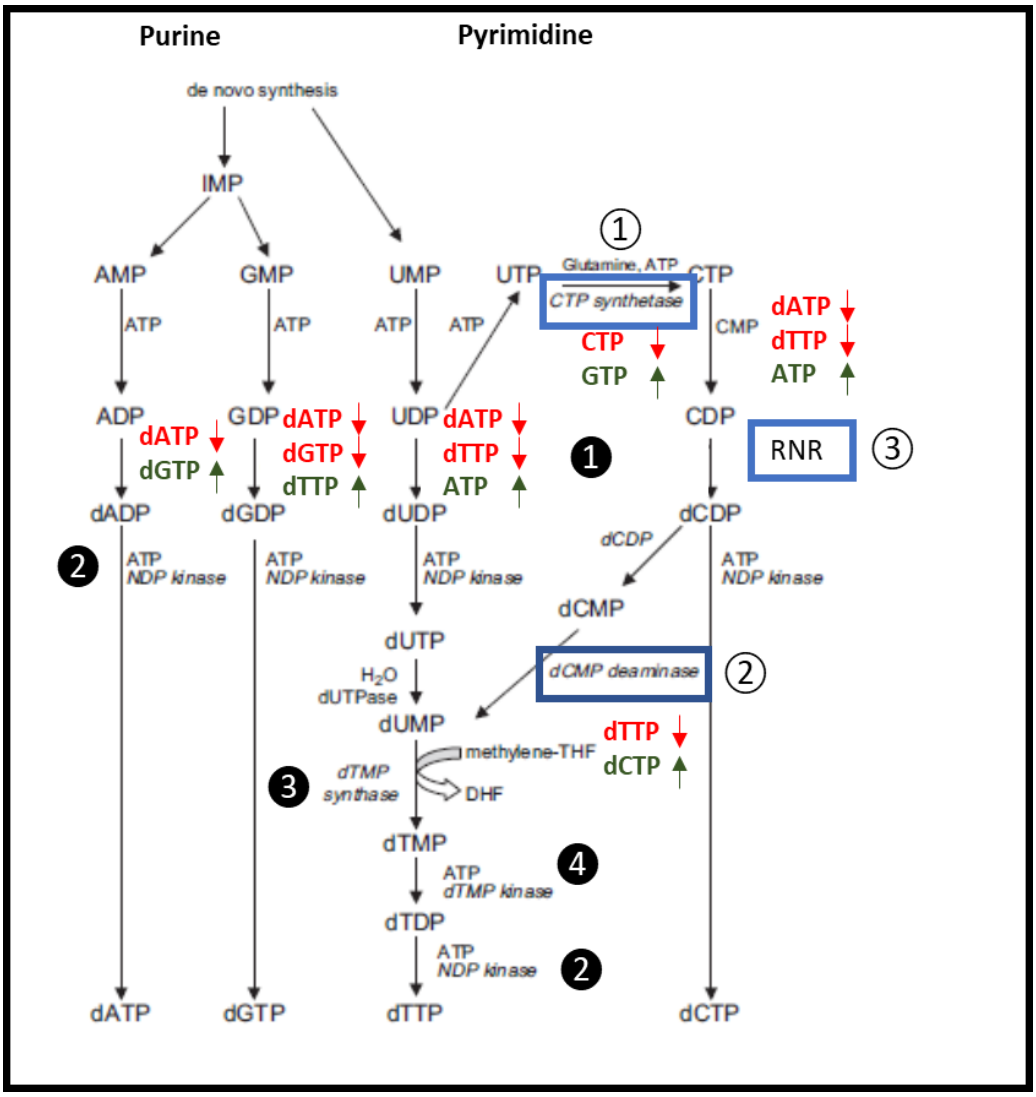


Figure 12. The common steps of nucleotide de novo biosynthesis (adapted from Kunz *et al.*, 1994 and Mathews, 2014).

The nucleotide synthesis is regulated by the concentration of these nucleotides. Moreover, three important enzymes in the *de novo* pathway are allosterically regulated*, which is a highly controlled mechanism in mammalian cells (Huang and Graves 2003). These enzymes are the CTP synthetase, (①) CMP deaminase (②) and RNR (③): their end-products act as activators (in green) or as inhibitors (in red) (Figure 12).

***Allosteric regulation:** Regulation of an enzyme by binding a substrate (effector) other than in the active site. This leads to a conformational change of the enzyme that leads to changes in the binding affinity.*

① **CTP synthetase** catalyzes the formation of CTP from UTP and glutamine deamination into glutamate in the *de novo* pyrimidine pathway. This enzyme is activated by GTP and inhibited in the presence of CTP. CTP synthetase is important for the synthesis of dCTP and has been considered as an attractive target for chemotherapy, because it is frequently overactivated in cancer. The deregulation of this enzyme leads to increased pools of CTP and dCTP, and consequently to an increase in the induction of spontaneous mutations (van Kuilenburg, Meinsma et al. 2000).

② **dCMP deaminase** catalyzes the deamination of dCMP into dUMP. This enzyme is allosterically regulated by dCTP and dTTP concentrations, where dCTP leads to the activation of dCMP deaminase and dTTP leads to its inhibition, but only at low levels. dCMP deaminase also contributes to the DNA synthesis by providing dUMP to the TS to produce dTTP, the unique DNA specific nucleotide. Moreover, a defective dCMP deaminase leads to the imbalance of the dCTP/dTTP ration, leading to mutagenesis (Reichard 1988, Maley and Maley 1990, Maley, Lobo et al. 1993).

③ **RNR** is the unique enzyme that catalyzes the reduction of all four essential ribonucleotides for DNA synthesis in both eukaryotic and prokaryotic cells (Reichard 1988). The activity of RNR and its regulation has an important role in maintaining the correct concentration and ratio of dNTPs, which are crucial factors for ensuring a high-fidelity DNA synthesis and consequently avoiding genome instability (Guarino, Salguero et al. 2014). RNR is composed of 2 homodimers (Uhlin and Eklund 1994): the large subunit (R1) and the small subunit (R2), except in

mammalian cells, where another R2 isoform is present, the p53R2 subunit which depends on p53 and is activated in response to DNA damage (Pai and Kearsey 2017). The association of these subunits is essential for RNR activity (Nordlund and Reichard 2006). Exactly like the former enzymes, RNR is allosterically regulated by ATP (allosteric activator) and by dATP (allosteric inhibitor). This allows the regulation of the two allosteric sites, the catalytic activity site and the substrate specificity site (Nordlund and Reichard 2006, Guarino, Salguero et al. 2014, Lane and Fan 2015). Moreover, RNR can also be regulated by the other dNTPs to promote specific substrates and provide the correct proportion of each dNTP for DNA synthesis (Reichard 2010, Lane and Fan 2015).

1.3. The salvage pathway

The salvage pathway recycles pre-existing nucleosides, deoxynucleosides, nucleobases and nucleotides released from products of cellular metabolism or from the catabolism of nucleic acids to synthetize the corresponding nucleotides (Moffatt and Ashihara 2002, Pedley and Benkovic 2017). This process is more energetically favorable for the cell since only one reaction uses ATP: the phosphorylation of nucleosides into nucleotides (Moffatt and Ashihara 2002, Pedley and Benkovic 2017). The salvage pathway is performed in both cytosolic and mitochondrial compartment. Briefly, this pathway starts, firstly, by the phosphorylation of deoxynucleosides by four deoxynucleoside kinases: the cytosolic thymidine kinase 1 (TK1) (①), deoxycytidine kinase (dCK) (②), mitochondrial thymidine kinase 2 (TK2) (③) and deoxyguanosine kinase (dGK) (④) into deoxynucleosides monophosphates (dNMPs) (Reichard 1988, Wang 2016). Then, dNMPs are further phosphorylated into dNTPS in two additional steps performed by nucleosides monophosphate kinases (NMPKs) (⑤) and NDPKs (⑥), respectively (Figure 13) (Wang 2016).

Among the enzymes that participate in the nucleotide salvage pathway, cytidine deaminase (CDA) (⑦), an enzyme involved in the pyrimidine salvage pathway, is the object of great attention in the team. CDA catalyzes the deamination of cytidine and deoxycytidine into uridine and deoxyuridine, respectively (Nygaard 1986). *Given that CDA deficiency plays an important role in my project, CDA will be further presented in the point 2 of this section.*

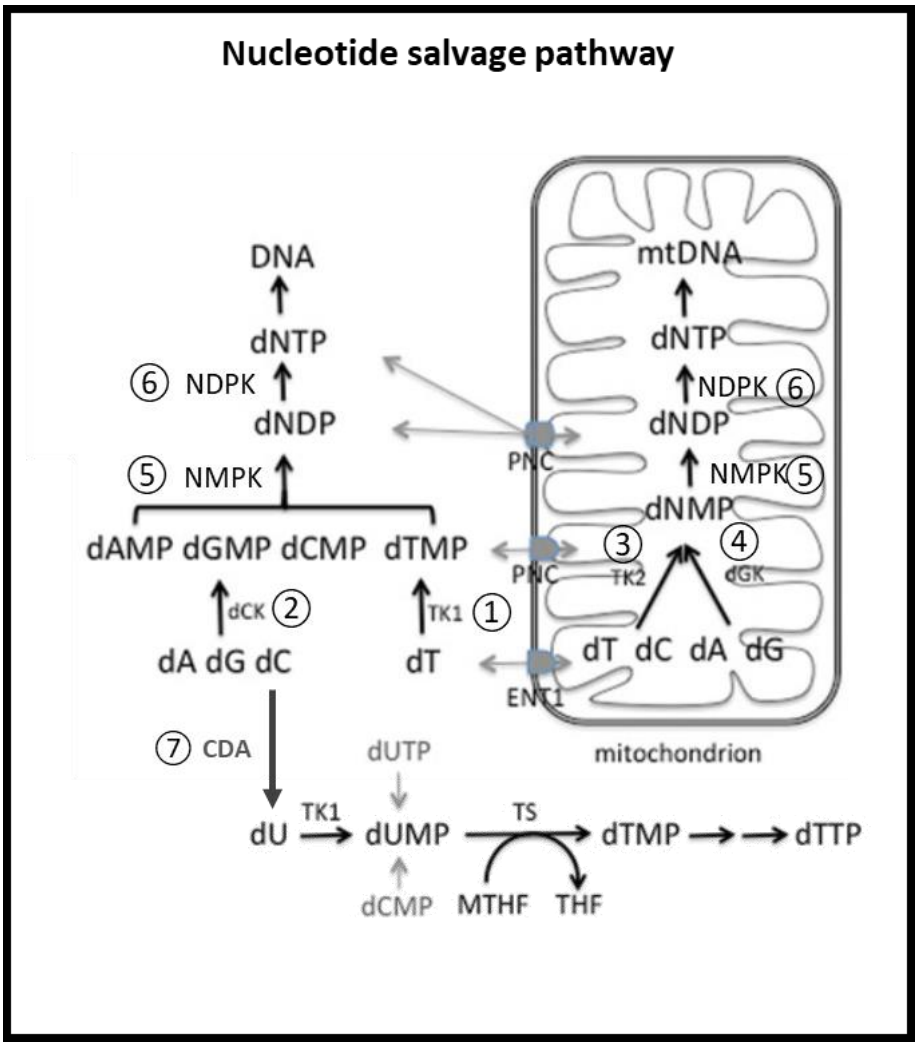


Figure 13. The nucleotide salvage pathway (adapted from Wang, 2016).

In the salvage pathway, nucleosides or bases present in the extracellular matrix can be passive [equilibrative nucleoside transporters (ENT)] or actively [concentrative nucleoside transporters (CNT)] transported into the cell to produce the corresponding nucleotides (Evans and Guy 2004, Pedley and Benkovic 2017). ENT are facilitated transporters proteins that mediate the diffusion of nucleosides in or out of the cell in accordance with the substrate concentration gradient (Wei, Karen et al. 2004). They belong to the SLC29 family and four members were reported: ENT 1, ENT 2, ENT 3 that transport both purine and pyrimidine nucleosides and ENT 2 that can also transport nucleobases and ENT 4 that transports only adenosine and monoamines. CNT are nicotinamide adenine dinucleotide (NAD^+)-dependent secondary active transporters that mediate the nucleosides transport into cells against their concentration gradient. They belong to the SLC28 family and three members were reported. They exhibit distinct substrate specificity, CNT1 transporting pyrimidine nucleosides, CNT2

purine nucleosides and CNT3 transporting both purine and pyrimidine nucleosides (Wei, Karen et al. 2004, Young, Yao et al. 2013, Rahman, Raj et al. 2018).

Nucleosides transporters also transport nucleoside analogs that are used in chemotherapy such as gemcitabine, clorofarabine, 5-fluorouracil (5-FU), 6-mercaptopurine and they contribute to the effectiveness and toxicities of these analogs. Also, nucleoside transporters can modulate several physiological processes by regulating the adenosine concentration available in cell surface receptors (Young, Yao et al. 2013).

1.4. Regulation of the nucleotide pool

1.4.1. Nucleotide pool size

The nucleotide pool is asymmetric because all the different dNTP pools have not the same concentration in cells. The size of a nucleotide pool depends on the rate of its synthesis, on its use in DNA replication, and on the rate of catabolism. This pool varies during the cell cycle, with the largest pool during S and G2 phases and the smallest pool during G1 phase. From all four dNTPs pool, dGTP is the smallest pool, making between 5 to 10% of the total pool (Bjursell and Skoog 1980, Reichard 1988, Martomo and Mathews 2002, Huang and Graves 2003). To have a little estimation of the concentration of dNTPs in the cells, the nucleotide pools were measured in S-phase synchronized HeLa cells and found to correspond to 60 µM dATP, 60 µM dTTP, 30 µM dCTP and 10 µM dGTP (Zhang and Mathews 1995, Martomo and Mathews 2002, Mathews 2006). Nonetheless, it was reported that cells that suffer oncogenic transformation present an elevated nucleotide concentration compared to normal cells, so this result needs to be interpreted cautiously (Kunz, Kohalmi et al. 1994, Martomo and Mathews 2002, Pai and Kearsey 2017). Indeed, the nucleotide pool is expanded for about 3-to 4-fold in tumor cells (Evans and Guy 2004). Moreover, size of the nucleotide pool depends of the organism and cell type (Martomo and Mathews 2002).

1.4.2. Cellular compartments of the nucleotide pool

Nucleotide pool compartments have been intensively studied and discussed and include several theories and contradictions. Firstly, in 1980, it was suggested the existence of aggregates within the cells that contain enzymes responsible for dNTP synthesis and DNA replication. Moreover, these aggregates were formed in the nucleus, and as cells progress into the S phase in the cell cycle, these aggregates could be channeling dNTPs to replication sites. This model was proposed by Prem Veer Reddy and Pardee (1980) as a "Replitase": a multiprotein complex located in the nucleus that contains the principal enzymes for dNTP and DNA synthesis when cells enter in the S phase (Prem veer Reddy and Pardee 1980). Unfortunately, this model was not yet confirmed and, four years later, Engström and colleagues (1984) by immunocytochemistry technique showed that the enzyme responsible for the reduction of ribonucleotides into deoxyribonucleosides, RNR, was strictly located in the cytoplasm (Engström, Rozell et al. 1984). Then, the deoxynucleosides were transported into the nucleus for DNA synthesis. This work was later confirmed (Pontarin, Fijolek et al. 2008). In fact, it was, also, suggested that dNTPs were produced near the outer surface of the nuclear and their uptake into the nucleus is somehow coordinated when DNA synthesis is initiated. However, this is merely speculative once this was not confirmed. Finally, a study performed in *S. cerevisiae* synchronized cells demonstrated that the dNTP synthesis and initiation of DNA replication are independent processes (Koç, Wheeler et al. 2003). However, based on results obtained by confocal microscopy, a new model for aggregates was proposed in 2008, in which only the enzymes necessary for the dNTPs synthesis form a complex. This new multiprotein complex was observed in the *de novo* purine synthesis pathway and is called "Purinosome"(An, Kumar et al. 2008). Purisonome is composed of six enzymes that participates in the first three steps of the *de novo* purine pathway. Moreover, it is suggested that the purisonome is formed as a response to depleted purine levels or high metabolic demands (Pedley and Benkovic 2017). The existence of purisonome is very criticized due to opposite results and that this aggregate can be as a result from the association of fluorescence proteins or due to conditions of stress (Zhao, Tsechansky et al. 2014). Further, it was suggested that dNTPs are compartmentalized in two places: in the cytosol and in the mitochondria and that the exchange of dNTPs between the two is due to specific nucleotide transporters (Kunz, Kohalmi et al. 1994).

1.4.3. Nucleotide pool regulation during cell cycle

dNTPs synthesis is important for supplementing the intracellular nucleotides pools for DNA replication and genome maintenance during all the cell cycle. The dNTP pools are regulated during the cell cycle, being the smallest at G1 phase and exponentially increasing during the G1/S transition (Koç, Wheeler et al. 2004). The dNTP pools are also upregulated in the S-phase through the induction of nucleotide synthetic enzymes such as RNR, TS and thymidylate kinase (TMPK), to supply the nucleotides necessary for DNA replication. After mitosis, these same enzymes are degraded, leading to a decrease in the nucleotide pools (Wang 2016). The modifications of the nucleotide pool size are due to transcriptional or post-transcriptional regulation of "key-enzymes" of the nucleotide biosynthesis pathways (Chabes, Pfleger et al. 2003). Indeed, the fluctuation of dNTPs concentration during the cell cycle is controlled by RNR activity, whose activity is highest during the S and G2 phase (Chabes, Pfleger et al. 2003, Chabes and Stillman 2007). As mentioned earlier, RNR is composed by two subunits: R1 and R2 subunit. Although the expression of R1 is constant during all the cell cycle, the expression of R2 increases during S phase and is degraded in late mitosis (Chabes, Björklund et al. 2004) The degradation of R2 is dependent of a so called "KEN box", present in the N-terminus of this subunit, that activates the cdh1-anaphase-promoting (CAP) complex and provides a signal for proteasomal degradation (Chabes and Stillman 2007, Pontarin, Ferraro et al. 2011). The increase in R2 levels in S phase leads to its association with R1 subunit and to the formation of an active RNR that leads to nucleotide synthesis. To prevent an excess of dNTPs synthesis, RNR is submitted to a feedback inhibition by dATP (Chabes and Stillman 2007).

1.4.4. DNA damage regulation of the nucleotide pool

As already explained in this chapter, RNR is responsible for the synthesis of dNTPs essential for DNA synthesis. But how the dNTP pool is regulated in response to DNA damage? A study in budding yeasts, showed that the dNTP pools increased via induction of RNR activity in response to DNA damage (Chabes, Georgieva et al. 2003). As mentioned earlier, mammalian RNR possesses a R1 subunit and two isoforms of the R2 subunit. The p53R2 subunit lacks the KEN box that promote protein degradation in late mitosis (Pontarin, Fijolek et al. 2008). This subunit is induced by tumor suppressor p53 (Håkansson, Hofer et al. 2006, Pontarin, Fijolek et

al. 2008, Pontarin, Ferraro et al. 2012) upon treatment with DNA damaging agents such γ-radiation, UVs and adriamycin (also named doxorubicin) (Tanaka, Arakawa et al. 2000). Regarding the results observed in yeast, showing that DNA damage leads to up-regulation of RNR, increasing the dNTP pools (Zhao, Chabes et al. 2001, Håkansson, Dahl et al. 2006), it was proposed that the induction of p53R2 subunit and its subsequent association with R1 could lead to the stimulation of dNTPs synthesis in response to DNA damage. However, only induction of p53R2 expression leading to a slight increase in the dNTP pool has been reported in mammalian cells (mouse and human fibroblasts cells) after DNA damage (for about 5-10% of the total size of dNTP pool in S-phase) (Håkansson, Hofer et al. 2006).

It has been reported that in mammalian cells, the pools of dNTPs are almost not expandable after DNA damage (by contrast to yeast) (Niida, Katsuno et al. 2010), suggesting that enzymes involved in the dNTPs metabolism may be recruited to the sites of damage, to coordinate dNTPs incorporation. This hypothesis is supported by the finding that TMPK and RNR are recruited to sites of DNA damage in tumor cells, to prevent dUTP incorporation during DNA DSB repair (Hu, Yeh et al. 2012), and that dCK regulates the G2/M checkpoint in response to DNA damage (Yang, Lee et al. 2012).

2. Cytidine deaminase and genetic instability

2.1. Principal characteristics of CDA protein

Cytidine deaminase (CDA) is encoded by the *CDA* gene, mapped at chromosome 1 (1p36.2-p.35) (Josephsen, Hammer-Jespersen et al. 1983, Micozzi, Carpi et al. 2014). CDA is an enzyme that participates in the pyrimidine salvage pathway, catalyzing the irreversible hydrolytic deamination of cytidine (C) and deoxycytidine (dC) to the corresponding uridine (U) and deoxyuridine (dU) (Nygaard 1986). This enzyme is also capable of deaminate cytosine analogues, such as cytarabine (ara-C), aza-cytidine (Aza-C) and gemcitabine, conventionally used in cancer treatment (Josephsen, Hammer-Jespersen et al. 1983, Kuhn, Bertling et al. 1993, Laliberté and Momparler 1994, Chung, Fromme et al. 2005, Micozzi, Carpi et al. 2014). CDA activity is inhibited by Tetrahydrouridine (THU) both *in vivo* and *in vitro* (Vincenzetti,

Cambi et al. 1996, Vincenzetti, Costanzi et al. 2004). Due to these properties, CDA as well as its inhibitors have a special interest for cancer therapy.

CDA is a tetrameric enzyme with four identical subunits (each have a mass of 15 kDa) containing each a zinc atom in the active site and that plays an important role in the catalytic process (Vincenzetti, Cambi et al. 1996, Chung, Fromme et al. 2005). It is an evolutionary conserved enzyme known to be highly polymorphic (Chung, Fromme et al. 2005, Fanciullino, Farnault et al. 2018). CDA presents three variants, CDA-1, CDA-2 and CDA-3 due to different nucleotide exchanges at different codon localization: CDA-1 and CDA-2 have both a polymorphism in codon 27 but one presents a glutamine and the second a lysine, respectively. CDA-3 presents a threonine in the codon 70 instead of an alanine. CDA-2 is the most frequent CDA variant found in the general population (approximately 70%), and the CDA-3 (4,3%) is the most uncommon, but very frequent in the Japanese population (Yue, Saikawa et al. 2003).

2.2. CDA expression and localization

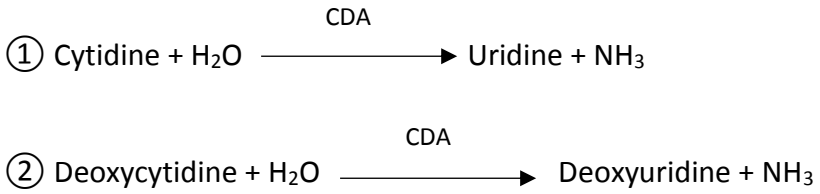
CDA is widely expressed in eukaryotic tissues but its expression levels vary in the different tissues and cells. At mRNA level, CDA is highly expressed in human tissues such liver, placenta, granulocytes and leukocytes and moderately to slightly expressed in kidney, lung, spleen and in human cells such fibroblasts, chondrocytes, monocytes, B-cells and T-cells. By contrast, no CDA expression was found in the brain, heart and muscle (Kuhn, Bertling et al. 1993, Laliberté and Momparler 1994, Watanabe and Uchida 1996). At the protein level, CDA is highly expressed in the liver, mature neutrophils and hepatic cells and slightly expressed in skeletal muscle, bone and cartilage (Micozzi, Carpi et al. 2014). Furthermore, vitamin D3 in the cell culture medium induced an increase in CDA expression in some cell lines (in four colorectal cell lines and two gastric carcinoma cell lines) (Watanabe and Uchida 1996). Also, genetic polymorphism in CDA promotor can be responsible for some changes in CDA expression level (Ge, Jensen et al. 2004). Finally, it was reported that, miR-484 negatively regulates CDA expression in breast cancer cell lines (Ye, Song et al. 2015).

As nucleotide synthetic enzymes were assumed to be mainly present in the cytoplasm, it was considered that CDA might had, also, a cytoplasmic localization. However, CDA presents a

classical nuclear localization signal in its N-terminal domain and has been reported to be in the nucleus in form of foci and present in the cytoplasm as diffuse form in several cellular models such HeLa (human cervix epithelial cancer cells), Cos-7 (monkey kidney cells), CCL-13 (human hepatocytes-like cells) and HepG2 (human liver cancer cells) cell lines (Somasekaram, Jarmuz et al. 1999). A Flag-tagged CDA overexpression confirmed that CDA was predominantly localized in the nucleus. Additionally, they observed that the import of CDA to the nucleus was due to the nuclear localization signal (NLS) sequence in the N-terminal domain. These results suggest that the salvage pathway could have some important enzymes located in both nuclear and cytoplasmic compartments (Somasekaram, Jarmuz et al. 1999). CDA activity was also found in the sera of nucleosides analogs-treated patients (DeAngelis, Kreis et al. 1992, Sugiyama, Kaniwa et al. 2006, Ciccolini, Dahan et al. 2010) as well in cell culture medium (Ge, Jensen et al. 2004), demonstrating an extracellular export of CDA. However, the export mechanism for CDA has not been yet elucidated.

2.3. CDA enzymatic activity

As already mentioned, CDA catalyzes the deamination of cytidine and deoxycytidine in to the corresponding uridine and deoxyuridine, respectively:



CDA is biologically active when in homotetrameric form, although the substrate binding to each subunit is independent (Figure 14). The tetrameric complex is stabilized by the tyrosine 33 (Y33) in each monomer, creating four active sites (Costanzi, Vincenzetti et al. 2004, S., B. et al. 2008, Micozzi, Pucciarelli et al. 2010). The residues phenylalanine 36 (F36) and F137 are important for the stabilization of the enzyme with the ligand, facilitating the catalytic activity of CDA (Vincenzetti, De Sanctis et al. 2003, Vincenzetti, Costanzi et al. 2004). Furthermore, CDA activity is inhibited by THU and 5-fluorozebularine (FZEB). These drugs inhibited CDA activity by dissociating the tetramer into the different subunits (Vincenzetti, De Sanctis et al. 2003).

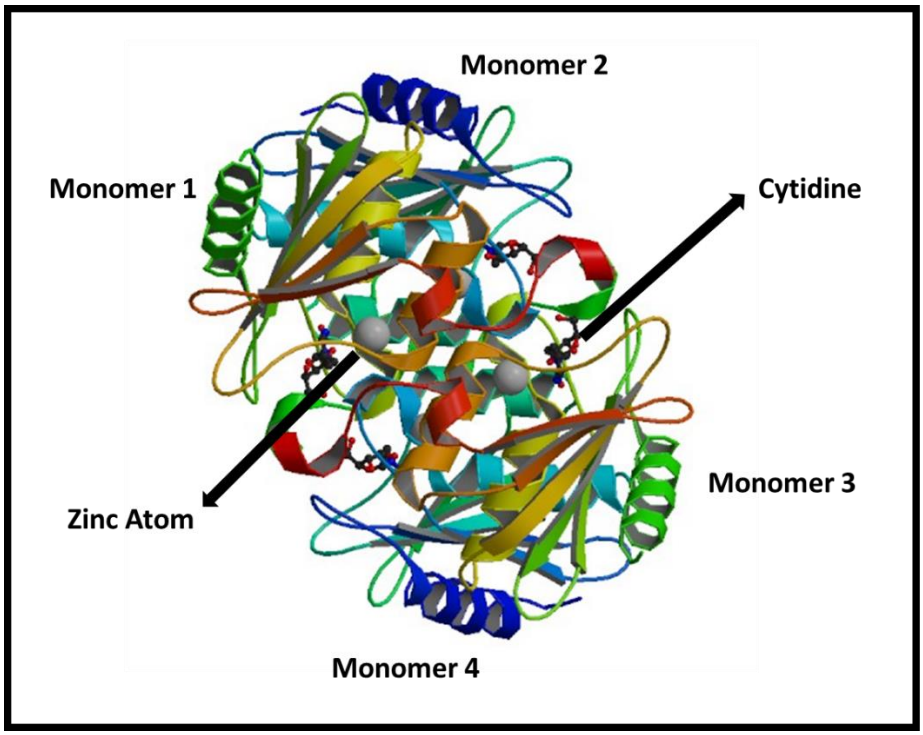


Figure 14. 3D structure of human cytidine deaminase (hCDA). From Protein Data Bank (PDB): 1MQ0 (https://www.rcsb.org/structure/1MQ0).

Finally, the genetic polymorphism mentioned earlier, leads to inter-individual variation of CDA activity, that can affect the deamination of the nucleoside analogs used in chemotherapy. This can, also, affect the concentration of the drug present in the blood and consequently influences the efficiency of cancer treatment (Micozzi, Carpi et al. 2014).

2.4. CDA and cancer

To my knowledge, only two independent groups reported results on CDA and cancer, both of which focused on CDA overexpression in cancers. One reported that miR-484 downregulates CDA expression by targeting its 3'-UTR, sensitizing breast cancer cells overexpressing CDA to gemcitabine (Ye, Song et al. 2015). The other identified CDA overexpression as a potential target for anticancer treatment, proposing the use of cytidine analogs that are selectively converted to their uracil counterparts and then incorporated into DNA in cancer cells overexpressing CDA, resulting in DNA damage and cell death (Zauri, Berridge et al. 2015). Amor-Guéret's team demonstrated for the first time that CDA expression is not only overexpressed in tumors, but is also downregulated in about 60% of tumors, mostly due to

DNA methylation (Mameri, Bièche et al. 2017). Indeed, immunohistochemistry assessments of CDA expression status in 300 microarrays (TMA) from six types of primary tumors revealed that 50% (endometrium) to 88% (triple-negative breast cancer, ovary or colon cancer) were expressing very low levels or not expressing CDA (Mameri, Bièche et al. 2017). They also showed that tumors from the same classically defined groups display differences in CDA expression status, resulting in contrasting cellular properties, such as SCE levels.

Finally, they showed that CDA gene expression in tumor cells is epigenetically regulated, and that epigenetic treatment of cancer cell lines with the DNA methyltransferase activity inhibitor 5-Aza-dC (5-Aza-2'-deoxycytidine), resulting in DNA demethylation, strongly induced the expression of a functional CDA in CDA-deficient tumor cells, with little or no effect on CDA expression in CDA-proficient cells. As proposed in Mameri *et al.* (2017), these findings suggest that DNA-demethylating agents could be assessed as a possible treatment for CDA-deficient tumors, to induce CDA overexpression and then sensitize these tumors to treatments with oxidized and epigenetically modified cytidine nucleosides, such as those reported in Zauri *et al.* (2015) (Zauri, Berridge et al. 2015). To determine whether CDA deficiency could be of clinical interest for cancer treatment, they performed an *in-silico* screening with the NCI CellMiner analysis tool and identified aminoflavone as a candidate for the targeting of CDA-deficient tumor cells. They confirmed that CDA-deficient tumor cells, but not CDA-proficient tumor cells, present a marked susceptibility to aminoflavone, indicating that CDA expression status could be used to guide anticancer therapy. These results constitute a proof-of-concept that CDA deficiency may turn out to be a new predictive marker of susceptibility to antitumor drugs (Mameri, Bièche et al. 2017) indicating that CDA deficiency might be of clinical interest for cancer treatment.

2.5. CDA and genetic instability

As already mention *(in chapter 1, point 3.1)*, the pyrimidine pool disequilibrium resulting from CDA deficiency contributes to the genetic instability associated with Bloom syndrome. Indeed, in BS cells, dCTP levels are twice those in normal cells: this pyrimidine pool imbalance due to a 2-fold increase in dCTP is fully responsible of the slowing down of the replication speed and contributes to the increase in sister chromatid exchange (SCE) frequency in these cells (Chabosseau, Buhagiar-Labarchède et al. 2011). Moreover, CDA downregulation on its own

leads to a 2-fold increase in dCTP in cells expressing BLM and reproduces several aspects of the genetic instability associated with BS, known to confer cancer predisposition, including the slowing down of replication speed and an increase in SCE frequency (Chabosseau, Buhagiar-Labarchède et al. 2011, Gemble, Ahuja et al. 2015, Gemble, Buhagiar-Labarchède et al. 2016). Indeed, Amor-Guéret's team reported that CDA deficiency leads to the intracellular accumulation of dC and dCTP, reducing the basal PARylation activity of poly(ADP-ribose) polymerase 1 (PARP-1), a multifunctional enzyme involved in many cellular processes, including the response to DNA damage (*see the following chapter*) (Gemble, Ahuja et al. 2015). They demonstrated that the resulting low levels of PARP-1 activity (i) disturb Chk1 activation and decrease the efficiency of downstream checkpoints, leading to the accumulation, during mitosis, of unreplicated DNA at some "difficult-to-replicate" loci in the genome, such as centromeres and fragile sites, leading to an excess UFB formation (Gemble, Buhagiar-Labarchède et al. 2016) (Figure 15), and (ii) lead to a defective sister chromatid disjunction (Gemble, Buhagiar-Labarchède et al. 2017).

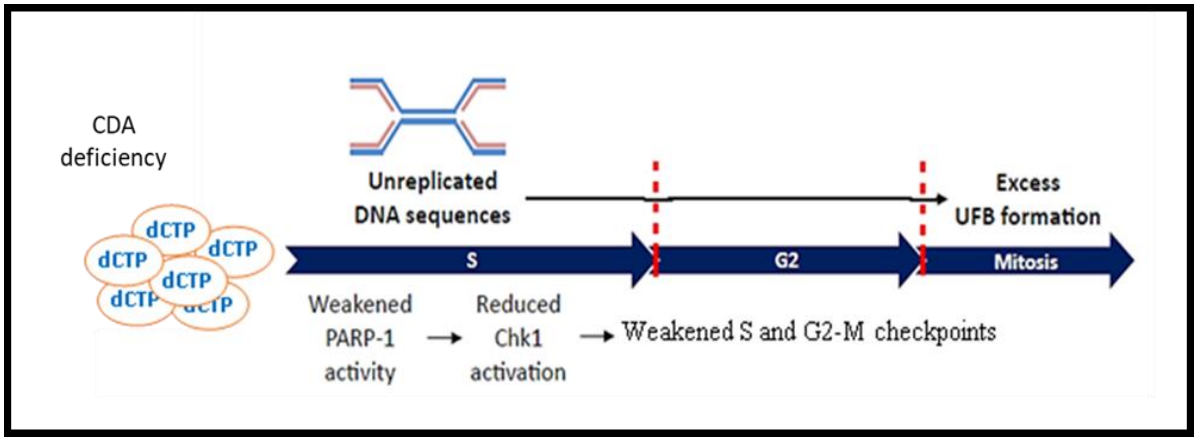


Figure 15. CDA deficiency leads to the reduction of PARP-1 reduced and consequently, to UFB formation. CDA deficiency leads to intracellular accumulation of dCTP, which in turn partially inhibits PARP-1 activity. This results in reduced chk1 activation and consequently to a weakened S and G2/M checkpoint, promoting the excess of unreplicated sequences during mitosis, thus, leading to supernumerary UFB (adapted from Gemble *et al.*, 2016).

Then came the question of how CDA-deficient cells survive with such genetic instability, tolerating DNA damage and replication stress. Bou Samra *et al.* (2017) performed a genome-wide RNAi-synthetic lethal screen and transcriptomic profiling to identify genes and pathways

enabling BLM- and/or CDA-deficient cells to survive despite constitutive DNA damage and replicative stress. They found a synthetic lethal interaction between CDA and microtubule-associated protein Tau (MAPT) deficiencies. Tau is overexpressed in CDA-deficient cells, and its depletion worsens genomic instability, compromising cell survival. They also reported new functions for Tau protein in the preservation of genome integrity. In brief, Tau binds to ribosomal DNA (rDNA) loci. Its depletion reduces the intracellular levels of ribonucleotides, downregulates rRNA synthesis, and impairs rDNA stability, resulting in the formation of ribosomal UFBs (r-UFBs), a new class of UFBs (Figure 16) (Bou Samra, Buhagiar-Labarchède et al. 2017).

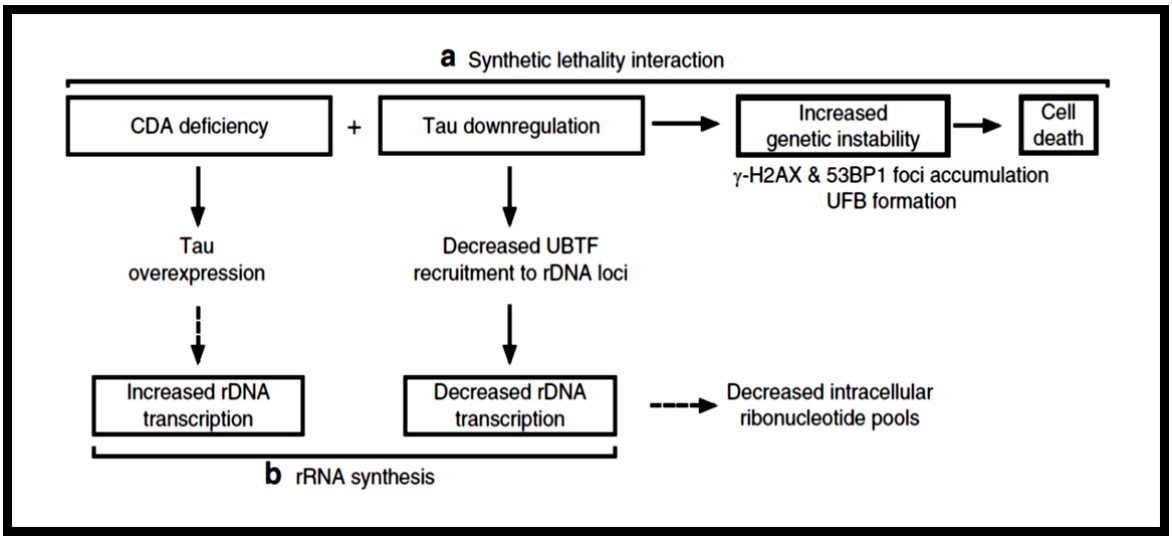


Figure 16. The interactions between CDA and Tau (from Bou Samra *et al*., 2017).

Moreover, Bou Samra *et al.* (2017), reported a negative correlation between CDA deficiency and Tau overexpression in several cancer such as breast cancer, papillary, cell kidney carcinoma and prostate adenocarcinoma (Bou Samra, Buhagiar-Labarchède et al. 2017).

Bou Samra *et al.* (2017) highlighted that Tau is important for maintaining survival of CDA-deficient cells as well as ribosomal DNA transcription and stability. Additionally, cancers that present the negative correlation between both CDA and Tau expression, might be a predictive marker for new cancer therapy strategies.

Amor-Guéret's team demonstrated, for the first time, the role of CDA in maintenance of genetic stability. CDA deficiency (and its subsequent pyrimidine pool imbalance) is responsible for the reduction of PARP-1, an important enzyme of the DNA repair machinery, leading to the formation of supernumerary UFBs, a hallmark of genetic instability.

Although Gemble et al. (2015) reported that intracellular accumulation of dC and dCTP resulting from CDA deficiency leads to a reduced PARP-1 activity, the mechanism by which this partial inhibition of PARP-1 activity was taking place was unknown. The aim of my project was to decipher how the intracellular accumulation of dC and dCTP impairs PARP-1 activity. Therefore, my next chapter focused on the PARP proteins and their functions.

PARP-1, a multifaceted protein

1. The PARPs superfamily

First described as “new DNA-dependent polyadenylic acid synthetizing nuclear enzymes”, the poly (ADP-ribose) polymerase (PARP) superfamily comprises 17 members. The homology sequence with the catalytic domain of PARP-1, the founding member, was the base to identified the proteins belonging to the PARP superfamily (Amé, Spenlehauer et al. 2004). The superfamily members are classified according to their functional domain or established functions:

- DNA-damage-dependent PARPs (PARP-1, PARP-2, PARP-3)
- Tankyrases (Tankyrase 1 [PARP-5a] and Tankyrase 2 [PARP-5b])
- CCCH-types zinc finger PARPs (PARP-12, PARP-13, tiPARP)
- Macro-PARPs (PARP-9/BAL1*, PARP-14/BAL2/CoaSt6*, PARP-15/BAL3*)
- Other PARPs (PARP-4/VPARP, PARP-6, PARP-8, PARP-10, PARP-11)

***BAL 1,2,3** – B-aggressive lymphoma 1, 2, 3; **CoaSt6** – collaborator of signal transducer and activator of transcription 6*

Each PARP is considered to exhibit either mono-(ADP-ribosyl)ation or MARylation, poly(ADP-ribosyl)ation or PARylation or no catalytic activity (Vyas, Chesarone-Cataldo et al. 2013).

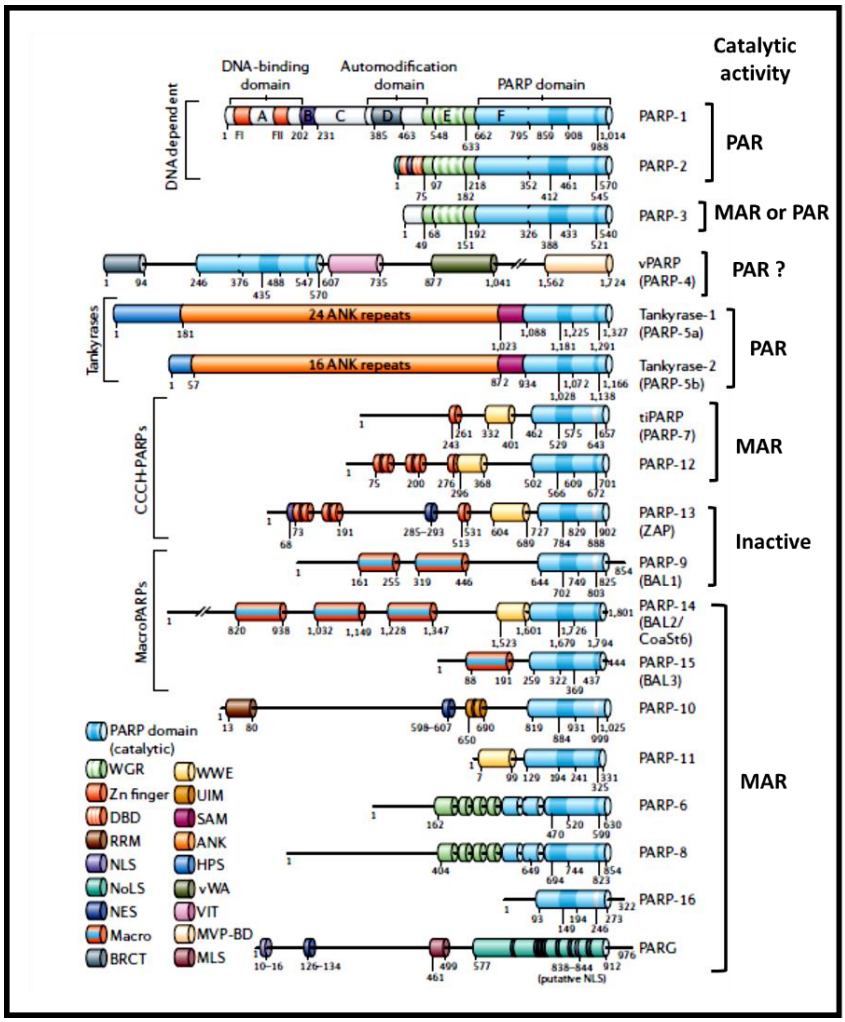


Figure 17. The PARP superfamily. The architecture of the domains of the 17 members of the PARP superfamily. All the functional domains of each protein are colored, and the corresponding legends are presented at the left of the figure. The catalytic PARP domain, which is conserved among all the members of PARP family is colored in blue (adapted from Schreiber *et al.*, 2006).

1.1. DNA damage- dependent PARPs

DNA-damage-dependent PARPs are known as the DNA damage sensors whose catalytic activity is stimulated by the presence of DNA breaks (Amé, Spenlehauer et al. 2004). PARP-1 (①), PARP-2 (②) and PARP-3 (③) belongs to this subfamily. In response to DNA lesions, these enzymes use NAD^+ as substrate to synthetize ADP-ribose (ADPr) polymers that are covalently attached to themselves and to target proteins. This allows the recruitment of repair proteins to DNA damage sites, increasing the DNA repair efficiency (Langelier, Riccio et al. 2014, Talhaoui, Lebedeva et al. 2016). These three proteins share a common conserved C-terminal region, which comprises the catalytic domain (CAT) and the WGR domain

(Tryptophan (W) – Glycine (G) – Arginine (R) residues). However, the N-terminal region, which is responsible for the DNA binding interface, is not conserved between these proteins (Figure 18). The different N-terminal regions might explain the different affinity of these proteins to DNA structures (Langelier, Riccio et al. 2014).

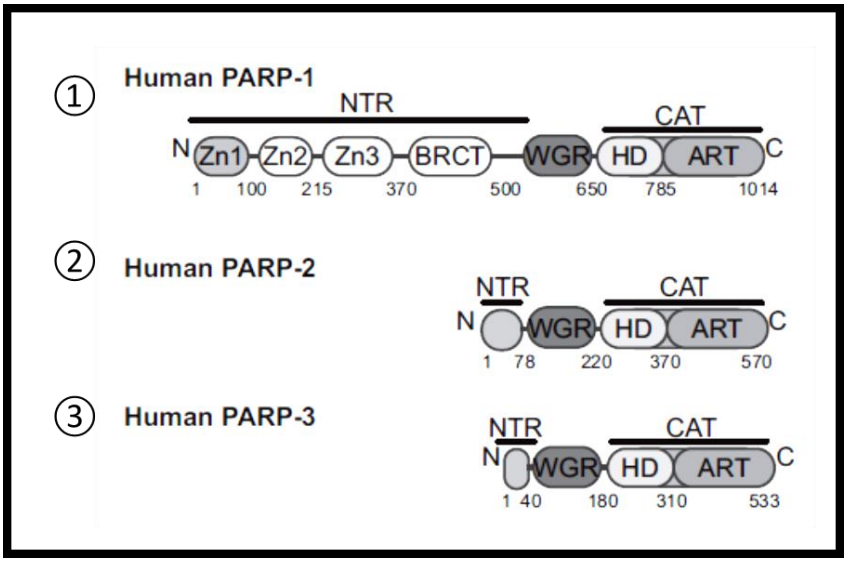


Figure 18. Domain architecture of DNA-damage-dependent PARPs. The WGR and CAT domains present in the C-terminal region are conserved whereas the N-terminus (NTR) differs between the three proteins (adapted from Langelier *et al.*, 2014).

① PARP-1, the founding member of this superfamily, is a 116 kDa protein that comprises six major domains. The N-terminus (NTR) (of ~ 500 residues) contains three zinc-finger (Zn) domains, Zn1, Zn2 and Zn3. The Zn1 and Zn2 recognize DNA breaks, allowing the binding of PARP-1 to DNA and stimulating its activity up to 500-fold (Langelier, Planck et al. 2011, Langelier and Pascal 2013) , whereas Zn3 mediates interdomain contacts (Langelier, Servent et al. 2008): through the extended loop of its zinc-ribbon fold, Zn3 contributes to interaction between the WGR domain and the CAT domain in the C-terminal region of PARP-1, possibly by modifying the orientation of these domains, leading to the activation of its catalytic function (Tao, Gao et al. 2008, Langelier, Ruhl et al. 2010, Langelier, Planck et al. 2012). PARP-1 also comprises a central region that includes an automodification domain (AD), containing a BRCA1 C-terminal (BRCT) motif and the C-terminus, where the WGR and CAT domains are present (Langelier, Servent et al. 2008, Langelier, Planck et al. 2012, Langelier and Pascal 2013).

② PARP-2 is a 65 kDa protein that was discovered as a result of the presence of residual PARylation activity in embryonic fibroblasts-derived from PARP-1-deficient mice (Shieh, Amé et al. 1998, Amé, Rolli et al. 1999). PARP-2 presents an NTR region smaller than the one of PARP-1 (only 78 residues in this region) (Langelier, Riccio et al. 2014). Although this region is important for DNA binding, DNA breaks recognition, PARP-2 recruitment to DNA damage sites and activation are mediated by the WGR and CAT domains (Langelier, Riccio et al. 2014, Ali, Khan et al. 2016, Obaji, Haikarainen et al. 2016). Recently, Fouquin *et al.* (2017) revealed that PARP-2 is involved in DSB repair independently of its poly ADP-ribose (PAR) synthesis activity. They demonstrated that PARP-2 orients DSB repair pathway choice, preventing the accumulation of 53BP1 at DNA damages sites and favorizing CtIP-mediated resection. Thus, PARP-2 enhances DSB repair by HR, single-strand annealing (SSA) or by Alt-NHEJ instead of C-NHEJ (Fouquin, Guirouilh-Barbat et al. 2017).

③ PARP-3 is a 63 kDa protein that was identified as component of the centrosome. Indeed, PARP-3 is associated to centrosomes and, more specifically, to the daughter centriole: PARP-3 might be responsible for the maturation of the daughter centriole within the centrosomes during G1/S phase (Augustin, Spenlehauer et al. 2003). With only 40 residues, PARP-3 displays the smallest NTR region from all DNA-damage-dependent PARPs (Amé, Spenlehauer et al. 2004, Langelier, Riccio et al. 2014). PARP-3 might also play an important role in some DNA repair pathways. It has been reported that PARP-3 participate in the C-NHEJ pathway by mono (ADP-ribosyl)ation (MARylation) the Ku70/Ku80 complex, interacting with this complex and the downstream proteins, DNA-PKcs, DNA ligase IV and Aprataxin And PNKP Like Factor (APFL). Thus, PARP-3 seems to facilitate the recruitment of DNA repair proteins to DSBs, stimulating both earlier and latter steps of C-NHEJ (Rouleau, McDonald et al. 2007, Rulten, Fisher et al. 2011, Beck, Boehler et al. 2014, Zarkovic, Belousova et al. 2018). More recently, it was reported that PARP-3 also negatively regulates the G4 quadruplexes formation, facilitating the DSBs repair by NHEJ or by HR (Day, Layer et al. 2017).

PARP-1, PARP-2 and PARP-3 are recruited to DNA damage sites but they are activated by different DNA lesions structures. PARP-1 is activated by both SSB and DSB and ssDNA (Talhaoui, Lebedeva et al. 2016), whereas PARP-2 is preferentially activated by SSB and by DNA intermediates such as gaps and flaps-containing DNA (Sukhanova, Abrakhi et al. 2016).

Finally, it was suggested both PARP-2 and PARP-3 are strongly activated by 5'-phosphorylated DNA breaks (Kutuzov, Khodyreva et al. 2013, Langelier, Riccio et al. 2014, Obaji, Haikarainen et al. 2016, Zarkovic, Belousova et al. 2018) .

Although these proteins are activated by different DNA damage structures (Mortusewicz, Amé et al. 2007, Boehler, Gauthier et al. 2011), they all have the same function: the synthesis of ADPr polymers mediated by the H-Y-E (Histidine-Tyrosine-Glutamate) amino acid residues in their C-terminus, which are necessary for PAR synthesis. The histidine (His) and tyrosine (Tyr) residues are required for the binding of the ribose groups of NAD^+, allowing its correct orientation in the catalytic site, initiating the ADP-ribosylation. Then, the glutamate (Glu) residue allows the transferring of successive ADPr, catalyzing the elongation of PAR chains (Vyas, Matic et al. 2014, Liu and Yu 2015). In mammalian cells, PARP-1 is responsible for 80 to 90% of total PAR synthesis in DNA damage sites and PARP-2 accounts for about 10 to 20% of this synthesis (Amé, Rolli et al. 1999). Although the function of PARP-3 has a PARylase (Boehler, Gauthier et al. 2011, Rulten, Fisher et al. 2011) was initially suggested, nowadays is accepted that PARP-3 presents a MARylase function (Loseva, Jemth et al. 2010, Grundy, Polo et al. 2016, Zarkovic, Belousova et al. 2018). Even if PARP-3 also presents the H-Y-E signature in its catalytic site, it is suggested that PARP-3 structurally donor distinct donor "D" loop might explain the absence of poly(ADP-ribosyl)ation activity (Gupte, Liu et al. 2017).

1.2. Tankyrases

The Tankyrase (TRF1-interacting, ankyrin-related ADP-ribose polymerases) protein subfamily comprises two major proteins, the ❶ Tankyrase 1 (TANK-1) or PARP-5a and ❷ Tankyrase 2 (TANK-2) or PARP-5b. These proteins comprise distinct domains: the ankyrin domain that contains ankyrin repeats arranged in five helices, the sterile alpha module (SAM) domain, and the PARP catalytic domain. The protein TANK1 also presents an additional domain, the HPS domain that is composed by H-P-S (Histidine-Proline-Serine) residues (Amé, Spenlehauer et al. 2004).

❶ TANK-1 was first discovered as a factor regulating the telomere homeostasis by PARylating the negative regulator of telomere length, TRF-1 (Smith, Giriat et al. 1998, Schreiber, Dantzer

et al. 2006). TANK-1 is involved in the telomere length, in the Wingless-type MMTV-integration site family (WNT) pathway, in the control of the mitotic checkpoint and mediates the insulin-stimulated glucose uptake (Riffell, Lord et al. 2012). Indeed, TANK-1 regulates the telomere length by inhibiting the negative action of TRF-1 in telomeres. TRF-1 impairs telomerase access to the telomeres. When TRF-1 is PARylated by TANK-1, telomerase is capable to bind to the end of the chromosome, leading to the elongation of the telomeres, facilitating, thus, telomere maintenance (Smith, Giriat et al. 1998, Seimiya, Muramatsu et al. 2005, Donigian and de Lange 2007, Riffell, Lord et al. 2012). TANK-1 also participates in the WNT signaling pathway by controlling β-catenin degradation. TANK-1 PARylates the proteins AXIN1 and AXIN2, preventing the degradation of β-catenin. This allows the latter to be translocated to the nucleus, inducing the transcription of target genes like c-Myc, an important onco-protein that is responsible for proliferation of tumoral cells (Huang, Mishina et al. 2009, Riffell, Lord et al. 2012, Mariotti, Pollock et al. 2017). Due to the participation of TANK-1 in this pathway, the Tankyrases could be interesting targets for cancer therapy. By inhibiting TANK-1, the WNT signaling pathway is consequently inhibited, leading to the decrease of tumoral cells proliferation (Seimiya 2006, Bao, Christova et al. 2012, Mariotti, Pollock et al. 2017). TANK-1 also controls the mitotic spindle by suppressing the cohesion of the sister chromatids or by PARylating proteins that are involved in the spindle assembly such as the nuclear mitotic apparatus protein complex (NUMA)(Riffell, Lord et al. 2012). Finally, both TANK-1 and TANK-2 have a role in the insulin-stimulated glucose uptake by binding to insulin-responsive aminopeptidase (IRAP), a transmembrane enzyme present in adipocytes and myocytes, that promotes the exocytosis of glucose transporter (GLUT) 4 store vesicles (GSVs) into the plasma membrane (Yeh, Sbodio et al. 2007, Riffell, Lord et al. 2012, Zhong, Ding et al. 2016).

❷ TANK-2 was discovered 3 years later through a double-hybrid screen to identify proteins interacting with TRF-1 (Kaminker, Kim et al. 2001). As already mentioned, TANK-2 is closely related to TANK-1 (Seimiya 2006), with the exception of its N-terminal region that lacks the HPS domain. TANK-2 functions are unknown. TANK-2 disruption in mice does not affect telomere length, but reduces the size of the mice, indicating a potential function of TANK-2 in cellular metabolism (Hsiao, Poitras et al. 2006, Schreiber, Dantzer et al. 2006).

Both proteins are ubiquitously expressed in several human tissues, in exception of TANK-1 that is not expressed in the placenta, whereas TANK-2 is highly expressed in this tissue (Riffell, Lord et al. 2012).

Finally, Tankyrases can also be inhibited by the same PARP-1 inhibitors conventionally used in cancer therapy (Lari, Nai-Wen et al. 2013, Teemu, Stefan et al. 2014). Since Tankyrases participate in the deregulation of WNT pathway, inhibiting Tankyrases could be a specific therapeutic target in cancer patients with up-regulated WNT pathway.

1.3. CCCH-type zinc-finger PARPs

The CCCH-type zinc-finger is a subfamily that comprises three members, the TCCD (as 2,3,7,8-tetraclorodibenzo-p-dioxin)-inducible PARP (tiPARP) or PARP-7 (❶), PARP-12 (❷) and PARP-13 (❸). They share a similar domain organization, the CCCH (Cys-Cys-Cys-His)-like zinc finger domain, mediating RNA binding (Hall 2005, Bock, Todorova et al. 2015), the WWE (Trp-Trp and Glu residues) domain, that is only present in some PARP proteins and ubiquitin ligases. WWE domain was reported to recognize iso-ADP-riboses, the smallest internal PAR structural unit containing the characteristic ribose-ribose glycosidic α(1”- 2’) bond that is formed during PARylation reaction, indicating that ADP-ribose can act as a ubiquitination signal (Wang, Michaud et al. 2012). CCCH-type PARPs also present the PARP catalytic domain (Schreiber, Dantzer et al. 2006). Both PARP-7 and PARP-12 present MARylases function, while PARP-13 lacks catalytic activity. X-ray crystallography analysis revealed that catalytic site of PARP-13 suffers a conformational change that inhibits NAD^+ binding to that site (Karlberg, Klepsch et al. 2015).

❶ Interestingly, tiPARP expression is induced by the aryl hydrocarbon receptor (AHR) in response to the exposure to dioxines such TCCD (Ma, Baldwin et al. 2001, Schreiber, Dantzer et al. 2006). Indeed, loss of tiPARP in mice increases the sensitivity to dioxin-induced toxicity, leading to liver steatosis and hepatotoxicity and subsequent death, indicating that tiPARP has a role in the resistance to dioxin-induce toxicity (Ahmed, Bott et al. 2015).

❷ PARP-12 participates in antiviral response by negatively regulating the expression of viral genes such as the Venezuelan equine encephalitis virus (VEEV) (Atasheva, Akhrymuk et al. 2012). Another study revealed PARP-12 as a interferon-induced gene (ISG) that might be implicated in cellular defenses against viral infections (Welsby, Hutin et al. 2014).

❸ Finally, PARP-13, also known Zinc-finger Antiviral Protein (ZAP) is mainly implicated in the anti-viral immunity. PARP-13 participates in the regulation of several viral genes by destabilizing their mRNA ensuring, thus, an antiviral role (Bick, Carroll et al. 2003, Guo, Ma et al. 2007, Bock, Todorova et al. 2015). Recently, a new role of PARP-13 has been reported in the pre-recruitment for PARP-1 to DNA damage sites by binding to heat shock transcription factor 1 (HSF-1), which is involved in the progression of neurodegenerative diseases and growth of malignant tumors. PARP-13 is present in both cytoplasm and nucleus. When this protein is in the nucleus, it acts as a scaffold protein, regulating the binding of PARP-1 to HSF-1 sites that are present in the genome. During DNA damage, PARP-1 auto-parylates, dissociating from the complex HSF-1-PARP-13-PARP-1 and is redistributed to DNA lesions and DNA damage-inducible gene foci. Finally, the HSF-1-PARP-13-PARP-1 complex mediated DDR machinery was also observed to protect tumor cells from DNA damage, mainly in BRCA-1-null mammary cancers that are sensitive to PARP-1 inhibitors (Fujimoto, Takii et al. 2017).

1.4. Macro-PARPs

Macro-PARPs subfamily is composed of three members that presents 1 to 3 macro domains in their N-terminal region. The macro-PARPs are PARP-9/BAL1[3] (❶), PARP-14/BAL2/CoaSt6[3] (❷) and PARP-15/BAL3[3] (❸). They are located within ~200 kbp of the same chromosome on the 3q21 chromosome region, which is involved in multiple hematological malignances (Aguiar, Yakushijin et al. 2000, Aguiar, Takeyama et al. 2005). In this subfamily, PARP-9 lacks catalytic activity *in vitro*, whereas PARP-14 and PARP-15 functions as MARylases (Vyas, Matic et al. 2014). The macro-PARPs are preferentially expressed in lymphoid tissues, indicating that these proteins may function in the immune system (Antoinette, Aline et al. 2008).

❶ PARP-9 have a role as transcription repressor when binding to the TK promotor (Aguiar, Takeyama et al. 2005). PARP-9 also plays a role in DNA damage response via its partner BBAP,

which is an E3 ubiquitin ligase. Indeed, in the DNA damage sites, PARP-1 PARylates PARP-9 , leading to PARP-9 and its partner BBAP recruitment to these sites, inducing local ubiquitination and promoting the recruitment of 53BP1 and BRCA1, facilitating the repair of DSBs (Yan, Xu et al. 2013). Finally, PARP-9 is overexpressed in aggressive diffuse large B-cell lymphoma (DLB-CL), indicating that it might promote the progression and dissemination of malignant B cells (Aguiar, Takeyama et al. 2005, Juszczynski, Kutok et al. 2006, Schreiber, Dantzer et al. 2006).

❷ PARP-14 activates the STAT6-mediated transcription in interleukin (IL)-4 -stimulated cells (Goenka and Boothby 2006, Schreiber, Dantzer et al. 2006, Mehrotra, Riley et al. 2011). PARP-14 is also important in DNA damage repair regulating HR. Indeed, PARP-14 knockdown results in reduced HR pathway, hypersensivity to DNA damaging agents and accumulation of DNA lesions, indicating that PARP-14 is important for HR machinery. Also, PARP-14 interacts with PCNA, promoting the replication of DNA breaks and common fragile sites. This study highlights a new role of PARP-14 in preventing replication stress and genome instability (Nicolae, Aho et al. 2015).

❸ Until now, no functions were reported for PARP-15.

[3] *I will mention the macro-PARPs as simply PARP-9, PARP-14 and PARP-15.*

1.5. Non-classified PARPs

A subgroup of PARPs that do not present the distinct characteristics mentioned in the other subfamilies are named as non-classified PARPs. This group comprises the PARP-4 (❶), PARP-6 (❷), PARP-8 (❸), PARP-10 (❹), PARP-11 (❺) and PARP-16 (❻).

❶ PARP-4 or VPARP is the largest protein of the superfamily (193 kDa) that was discovered associated to vault particles in a ribonucleoprotein complex. This complex is mainly present in the cytoplasm and is associated with two conserved proteins, the major vault protein (MVP) and the telomerase-associated protein (TEP1) (Amé, Spenlehauer et al. 2004). Moreover, PARP-4 can be in the nucleus and in the mitotic spindle (Zheng, Sumizawa et al. 2004). It has

been suggested that PARP-4 was involved in the cellular transport and in the multidrug resistance (Kickhoefer, Siva et al. 1999). I*n vitro*, PARP-4 can only synthetize mono (ADP-ribose) polymers but when associated with vault particles, it can synthetize PAR polymers (Kickhoefer, Siva et al. 1999, Vyas, Matic et al. 2014). PARP-4 knock-out mice are viable, fertile and do not present any phenotypes that are associated to the vault-related or telomerase-related phenotype (Liu, Snow et al. 2004). Nevertheless, these mice present an increased incidence for colon cancer when treated with 1,2-dimethylhydrazine, a chemical knowing to induce colon cancer (Gurley, Moser et al. 2015), indicating a probable role of PARP-4 in chemical-inducing cancers (Raval-Fernandes, Kickhoefer et al. 2005).

❷ PARP-6 functions as tumor suppressor by inhibiting the progression of the cell cycle, leading to the accumulation of PARP-6 expressing cells in S-phase (Tuncel, Tanaka et al. 2012). PARP-6 also induces apoptosis *in vitro* and *in vivo* in colorectal cancer cells (Qi, Kudo et al. 2016).

❸ To my knowledge, PARP-8 functions are unknown.

❹ PARP-10 possesses an ubiquitin interaction domain that contributes to the regulation of transcription factors by the NF-κB pathway (Verheugd, Forst et al. 2013). PARP-10 is a partner of the onco-protein c-Myc, a transcriptional regulator of cell proliferation. PARP-10 inhibits the transcription of c-Myc, independently of its MARylase activity, thus being a potent inhibitor of cell proliferation (Yu, Schreek et al. 2005). PARP-10 is implicated in the DNA damage response (DDR) by interacting with PCNA protein, facilitating the translational DNA synthesis (Nicolae, Aho et al. 2014).

❺ PARP-11 activity seems to be implicated in the spermatozoid differentiation in mice (Meyer-Ficca, Ihara et al. 2015) but this protein was not yet well studied.

❻ PARP-16 is a protein that seems to locate in the membrane of endoplasmic reticulum (ER). In fact, PARP-16 can MARylate protein kinase R like-endoplasmic reticulum kinase (PERK) and inositol requiring enzyme 1 alpha (IRE1α), both kinases that work as stress sensors in the ER,

by stimulating their activity when a defective folding of the protein occurs in this organelle (Jwa and Chang 2012).

2. Poly (ADP-ribosyl)ation or PARylation

Poly (ADP-ribosyl)ation or PARylation was first described 55 years ago by Chambon *et al.* (1963). PARylation is implicated in several molecular and cellular processes such as DNA repair, chromatin modification, transcription regulation, cell death and energy metabolism. All these processes are critical for physiological and pathophysiological processes, including maintenance of genomic stability, aging, neuronal function and carcinogenesis (d'Amours, Desnoyers et al. 1999, Kim, Zhang et al. 2005, Altmeyer, Messner et al. 2009). After PAR were found, for the first time, they were described as the “third type of nucleic acid” due to its chemical composition (Chambon, Weill et al. 1963).

2.1. Poly (ADP-ribose) polymers biosynthesis reaction

The PARylation reaction consists in the synthesis of poly(ADP-ribose) polymers (PAR) which is performed by the PARP proteins. The reaction starts in the catalytic site (CAT) in the C-terminus present in all PARP proteins. It comprises three main reactions (Figure 19): the initiation reaction (①), the elongation reaction (②) and the branching reaction (③) (d'Amours, Desnoyers et al. 1999, Altmeyer, Messner et al. 2009). Nevertheless, ADPr synthesis reaction is initiated by the hydrolysis of NAD^+ molecule that produces a nicotinamide (NAM) and an ADPr molecule as end products. For each molecule of NAD^+ catalyzed, one molecule of NAM and one molecule of ADPr are produced (d'Amours, Desnoyers et al. 1999). Noteworthy, in this reaction, a conformational change in the PARP catalytic site occurs to expose the NAD^+ binding site. Then, the His and Tyr residues present in this domain interact with the ribose group of the NAD^+ molecule. The third residue Glu is important for the synthesis and attachment of the second PAR to the acceptor protein. These three residues form the conserved triad H-Y-E, which is known as the PARP signature for the PARylation reaction (Kleine, Poreba et al. 2008, Barkauskaite, Jankevicius et al. 2015). PARP-1, then, transfers the ADPr (①) to the side chain of lysine, aspartic acid, glutamic acid, serine and cysteine residues of the target proteins (Liu, Vyas et al. 2017) and also to terminal phosphate

residues at DNA breaks (Talhaoui, Lebedeva et al. 2016, Zarkovic, Belousova et al. 2018). Given the negative charge and the size of each PAR, PARylation may have effects on the amino acid acceptor, modifying its biological properties, chemical structure, function and localization (d'Amours, Desnoyers et al. 1999). In the elongation reaction, the ADPr units bound via a ribose-ribose glycosidic α(1”- 2’) bonds (②) (Kim, Zhang et al. 2005). The PAR chains comprise 200 to 400 units. In some cases, PAR branching takes place every 20-50 ADPr residues (③) (Kiehlbauch, Aboulela et al. 1993, Altmeyer, Messner et al. 2009, Liu, Vyas et al. 2017) (Figure 19).

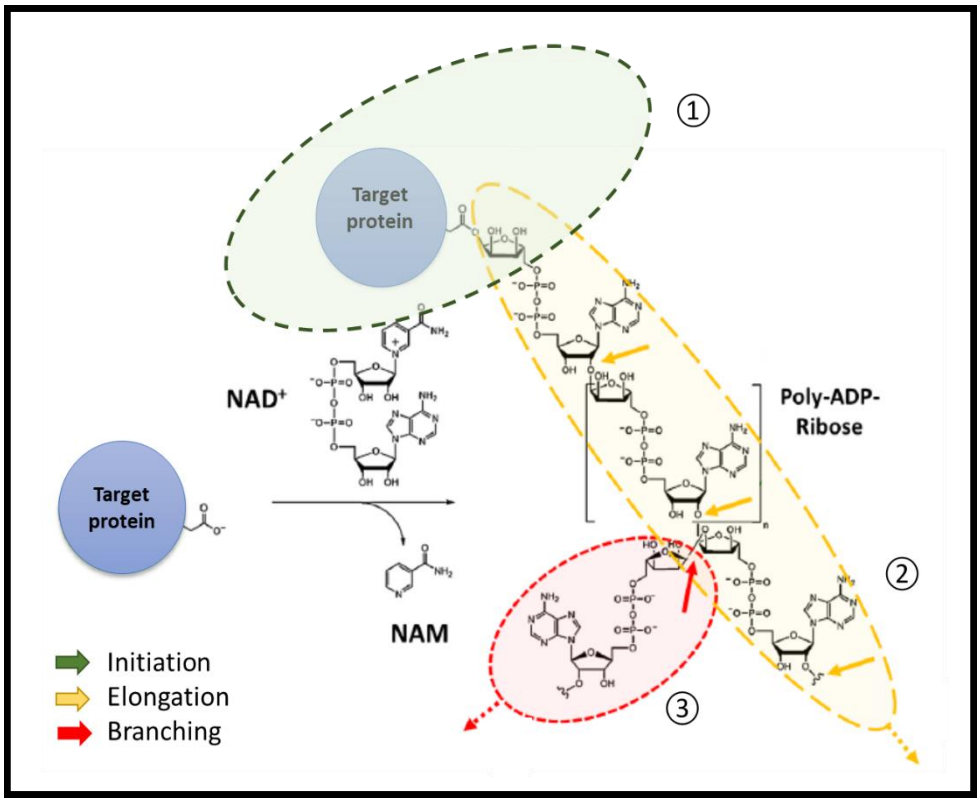


Figure 19. Poly (ADP-ribosyl)ation reaction. The PARP-1 activity starts by the hydrolysis of NAD^+ into a molecule of NAM and an ADPr unit. Then, ① PARP-1 transfers the ADPr unit into target proteins. ② ADPr units are bound via ribose-ribose glycosidic α (1”-2’) bond to form the PAR chain. ③ Some PARPs such PARP-1 and PARP-2 form branches of PAR on target protein.

Depending of the length of the branch, the polymers synthesis by PARPs can be mono-,-oligo- or poly (ADP-ribosyl)ation. The classification of PARP proteins in accord with their polymers synthesis:

- Mono (ADP-ribosyl)ation or MARylation: PARP-3, PARP-6 to PARP-16 in exception of PARP-9 and PARP-13 that lack catalytic activity.
- Poly (ADP-ribosyl)ation or PARylation: PARP-1, PARP-2, PARP-4 and Tankyrases
- Oligo (ADP-ribosyl)ation: Tankyrases

Finally, as already mentioned, PARP-1 and PARP-2 are responsible for 80-90% and 10-20%, respectively, of the total ADP-ribosylation (Amé, Rolli et al. 1999). Moreover, these proteins are the mainly enzymes that can catalyze the branching polymerization reaction (Vyas, Matic et al. 2014). At the cellular level, the majority of PARPs activity occurs in the nucleus, but PAR polymers can be localized in the cytoplasm (Bai 2015). In fact, some PARPs can be found in the cytoplasm such as TANK1, TANK2, PARP-7, PARP-10, PARP-12, PARP-13 and PARP-15, and PAR molecules and PARylated proteins can circulate between the nucleus onto the cytoplasm (Krietsch, Rouleau et al. 2013).

2.2. PAR degradation

PARylation reaction possesses a fast turnover, having the duration of minutes (Gagné, Hendzel et al. 2006). PAR is rapidly degraded into ADPr units. The catabolism of PAR is modulated by a serie of enzymes such as poly (ADP-ribose) glycohydrolase (PARG), ADP-ribosylhydrolase 3 (ARH3) and proteins with macro-domains (Kim, Zhang et al. 2005, Feijs, Verheugd et al. 2013, Mashimo, Kato et al. 2013). The rapid degradation of PAR allows an efficient temporal control of the recruitment and dissociation of proteins that are attached to PAR at specifics cellular localizations. Otherwise, extensive PAR synthesis can trap the DNA repair proteins and block the access to downstream repair factors. Therefore, an orderly and timely degradation of PAR by PARG is very important in the DDR (Liu, Vyas et al. 2017).

PARG is the most studied enzyme from PAR degradation reaction. In mammalian cells, PARG encodes three isoforms that result from alternative splicing: PARG99, PARG102 (isoforms of 99 and 102 kDa) that are located in the cytoplasm, and PARG110 (isoform of 110 kDa) that is

mainly located in the nucleus and is also the most active isoform (Kim, Zhang et al. 2005, Meyer-Ficca, Lonchar et al. 2013). The crystallography analysis showed that PARG contains a macrodomain in its catalytic domain, where the PAR binds. Some residues, including Phe 277 and Glu 115 that are present in this domain and are important for the interaction with the ribose group of PAR (Slade, Dunstan et al. 2011). This interaction allows glycohydrolysis of the glycosidic bond that links two consecutive ADPr units, thus, degrading PAR (Slade, Dunstan et al. 2011). PARG, as well as ARH3, mediates the glycohydrolysis poly(ADP)ribose polymers, but not the removal of MAR from proteins, but they are capable of removing MAR from DNA breaks (Munnur and Ahel 2017, Zarkovic, Belousova et al. 2018). ARH3 is also associated with the PAR degradation in mitochondria (Niere, Mashimo et al. 2012).

The enzymes that degrade mono-ADPr contain a macro-domain like MacroD1, MacroD2 and C6orf130 (TARG1): they catalyze the degradation of mono-ADPr by cleavage of the bond between the protein and the ribose sugar (Jankevicius, Hassler et al. 2013, Rosenthal, Feijs et al. 2013).

3. PARP-1 protein

I will now focus only on PARP-1 protein and its functions since my PhD project is to understand how CDA deficiency leads to weakened basal PARP-1 activity.

3.1. PARP-1 domains

PARP-1 is a highly conserved protein important for several physiological processes such as DNA damage response, inflammation and carcinogenesis. The PARP-1 structure comprises three functional regions: the N-terminal domain, which is responsible of the recognition of DNA structures, the central region necessary for the interaction of PARP-1 with other proteins and the C-terminal region, containing the catalytic domain (Figure 20).

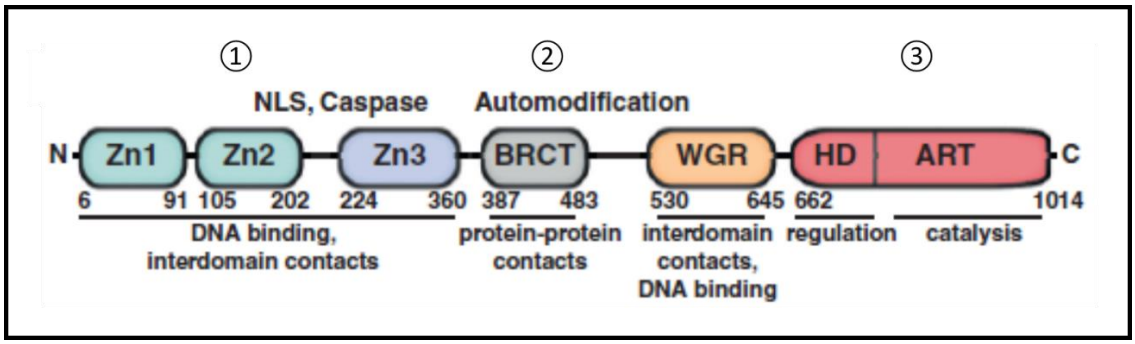


Figure 20. The modular domain architecturel of PARP-1. PARP-1 comprises three major regions: the N-terminal region that comprises three zinc-fingers domains, essential for PARP-1 binding to DNA (Zn1, Zn2) and for interdomain interaction (Zn3), a central region (BRCT domain) required for the interactions between PARP-1 and proteins, and the C-terminal region including the WGR domain, necessary for DNA binding and interdomain contacts and the CAT domain, required for PARP-1 catalytic activity (adapted from Langelier *et al*., 2013).

① N-terminal region consists in three zinc-binding domains Cys-Cys-His (Zn1, Zn2, Zn3) that allows the binding of PARP-1 to DNA. The first two domains, Zn1 and Zn2 allow PARP-1 to recognize DNA structures such blunt ends, 3'-single base overhangs, nick ends and single and double strand DNA (D'Silva, Pelletier et al. 1999, Pion, Bombarda et al. 2003, Langelier, Planck et al. 2011). The third domain, Zn3 (Langelier, Servent et al. 2008), as already mentioned, permits the interaction inter-domain between WGR and CAT necessary for PARP-1 catalytic activity (Tao, Gao et al. 2008, Langelier, Ruhl et al. 2010, Langelier, Planck et al. 2012). This domain also participates in the PARP-1-dependent activity of chromatin remodeling (Langelier, Ruhl et al. 2010). Finally, the N-terminal region contains a NLS sequence (Schreiber, Molinete et al. 1992).

② The central region corresponds to the automodification domain. This domain contains a BRCT motif that is responsible for the protein-protein interactions. It also contains several Glu, Aspartate (Asp) and Lysine (Lys) residues that are acceptors for PARP-1 auto-PARylation (Luo and Kraus 2012). Finally, this region also comprises a leucine zipper motif that favors homo- or hetero-dimerization of PARP-1, inducing the PARylation activity (Mendoza-Alvarez and Alvarez-Gonzalez 1993).

③ The C-terminal regions is the most conserved region of the PARP superfamily. The C-terminal region displays the WGR domain that is important to the binding of PARP-1 to DNA break by participating in interdomain interactions between Zn1, Zn3 and CAT (Langelier, Planck et al. 2012). In response to DNA damage, both Zn1 and Zn3 interact with one face of the WGR domain, which also binds to the DNA. Then, the other face of WGR domain interacts with the HD subdomain of the CAT domain, inducing its catalytic activity (Langelier, Planck et al. 2012, Steffen, Tholey et al. 2014). Finally, the C-terminal region also comprises the CAT domain, which is divided into two subdomains: the auto inhibitory helical (HD) and the ART subdomain, containing the highly conserved PARP signature (H-Y-E residues) which allows NAD^+ fixation and the initiation of the PARP-1 catalytic activity (Langelier, Planck et al. 2012).

3.2. PARP-1 functions in DNA Damage Response (DDR):

Several studies highlight the central role of PARP-1 and its PARylation activity in various DNA repair pathways and in the maintenance of genomic stability. PARP-1 is necessary for the repair of both SSBs and DSBs.

3.2.1. Single-strand breaks (SSB) repair

SSBs are disruptions in one of the strands of the DNA double helix and are the most frequent form of DNA damage. SSBs result from either oxidative damage and intermediates from other DNA repair pathways (Caldecott 2008).

When a SSB occurs, both Zn1 and Zn2 domains, from the N-terminal region of PARP-1, bind to the DNA break in a specific orientation, where Zn1 binds to the 5'-end and Zn2 binds to the 3'-end. The binding of both domains to the DNA, induces the interaction of the 3rd domain, Zn3, with Zn1 and the 5'-end, originating a binding site for the WGR domain, allowing then, a first interdomain contact between Zn1, Zn3 and WGR domains. This reduces the conformational space between all the different PARP-1 domains, decreasing the energy cost and allowing the second interdomain interaction between Zn3, WGR and CAT domains, which provides the energy necessary for destabilizing CAT domain. Finally, this leads to the unfolding and release of HD subdomain, exposing the NAD^+ binding site, initiating the catalytic activity of PARP-1 (Eustermann, Wu et al. 2015, Yang, Liu et al. 2018).

3.2.2. Double-stand breaks (DSB) repair

DSBs are formed after exposure to DNA-damaging agents, such as ionizing radiation, or due to endogenous cellular process such as replication fork collapse or during genome rearrangements, including class-switch recombination (CSR), variable diversity and joining recombinantion (V(D)J recombination) and meiosis. DSBs are the most deleterious DNA lesions and they must be quickly repaired. DSBs are commonly repaired through HR or NHEJ and the choice of what pathway is initiated is determined by the cell cycle phase and the chromatin context (Ray Chaudhuri and Nussenzweig 2017). PARP-1 can also recognize DSBs strands through the N-terminal DNA-binding domain (DBD) that consists of a tandem repeat of Zinc-finger (Zn) domains. In the case of DSBs, PARP-1 adopts a slightly different module of recognition in comparison to the SSB recognition. In the DSBs, the Zn2 motif is dispensable for the DNA breaks recognition. The recognition of DSBs by PARP-1 is performed as describe below: ❶A DSB generates two ends in the DNA-strand helix. The Zn1 motif is capable of recognize one of the ends. ❷Simultaneously, Zn3 associated with the WGR domain reside in the minor and major grooves of the DNA helix, respectively, by contacting each side with the backbone of the helix. This allows the stabilization of the interaction. Furthermore, the WGR domain stacks against the ribose at the 5'-terminal of the DNA strand. ❸ Then, the interaction of one end of the DSB with the N-terminal of PARP-1, promotes a conformational change of the catalytic domain. Indeed, the HD subdomain exerts an auto inhibitory effect by blocking the binding of NAD^+ binding. When PARP-1 is activated by DNA damage, HD undergoes a local unfolding, releasing the auto inhibitory effect and allowing the NAD^+ binding in the catalytic domain for PARylation activity (Dawicki-McKenna, Langelier et al. 2015). ❹Finally, the activation of PARP-1 by recognition of DNA breaks rapidly catalyzes PARylation at DNA damage sites. At the DNA damage sites can PARylate PARP-1 itself or others target proteins, in which mediates the early recruitment of several DNA damage repair proteins to repair DSBs (Yang, Liu et al. 2018) .

3.2.3. Homologous recombination

HR (already discussed in chapter 1) is a pathway that functions during the S and G2 phase of the cell cycle that allow the repair of DSBs or the restart of stalled replication forks.

Although PARP-1 seems not to be indispensable for the HR process, several studies support that PARP-1 can be important for the restart of stalled replication forks mediated by HR. Bryant *et al.* (2009) (Figure 21) showed that the stalling of replication forks resulting from HU treatment lead to activation of both PARP-1 and PARP-2 and binding of these proteins to the blocked replication fork (①). This promotes the recruitment of MRE11/RAD50/Nbs1 (MRN) complex, which is required for end processing (②). The resected DNA will be coated by RPA, which is then replaced by the recruitment and loading of RAD51 (③), to initiate HR and the restart of stalled replication fork. Inhibition or disruption of PARP-1 impairs the downstream steps of the HR pathway, indicating the essential role of PARP-1 in the HR process (Bryant, Petermann et al. 2009).

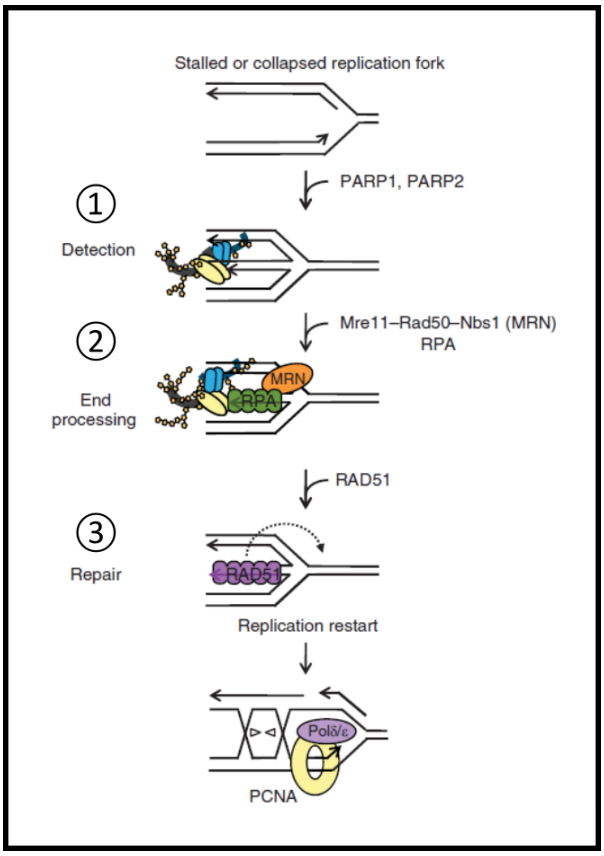


Figure 21. Role of PARP-1 and PARP-2 in the restart of stalled replication forks by HR. Blocked replication forks lead to the activation and binding of PARP-1 and PARP-2 to the fork. This promotes the recruitment of MRE11/RAD50/Nbs1 (MRN) complex, inducing DNA processing, which in turns leads to RAD51 recruitment and loading and initiation of the HR pathway (adapted from Bryant *et al.*, 2009).

PARP-1 also favors the HR instead of NHEJ (Sugimura, Takebayashi et al. 2008, Du, Amarachintha et al. 2016) by preventing the association of the Ku70/Ku80 and XRCC1/DNA ligase IV complex to the DNA breaks. This mechanism results from the PARylation of Ku70 that leads to its release from the DNA, promoting the DSBs repair by HR (Sugimura, Takebayashi et al. 2008). Conversely, in both basal and HU treatment conditions, it was observed an accumulation of RAD51 foci and increase in SCE frequency in PARP-1 deficient cells, indicating an elevated recombination activity, which suggests an anti-recombinogenic activity for PARP-1 (Yang, Cortes et al. 2004). Furthermore, Ying *et al.* (2012) proposed that in BRCA2-deficient cells, PARP-1 protects the replication fork from extensive resection due to Mre11 recruitment (Ying, Hamdy et al. 2012).

More recently, it was reported that Timeless, a protein that helps stabilizing replication forks during DNA replication, is recruited to DNA damage sites through its interaction with PARP-1, independently of its PARylation activity, suggesting that the interaction between Timeless and PARP-1 might facilitate HR repair (Xie, Mortusewicz et al. 2015).

PARP-1 inhibition presents a synthetic lethal interaction with HR (Fong, Boss et al. 2009). PARP inhibitors are currently used in clinical to selectively kill HR defective cancer, such as BRCA1 or BRCA2 mutated tumors, as a strategy named synthetic lethality. In this strategy deficiency of two or more genes, where in this case PARP-1 and BRCA1/BRCA2 leads to cell death. Actually, in HR-deficient tumors, treatment with PARP inhibitors, (①) inhibits PARP-1 to repair SSBs after DNA damage, leading to the formation of DSBs (②). As BRCA genes are mutated, tumor cells present a defective HR repair leading to the accumulation of DSBs (③), resulting, thus, in cell death (④) (Figure 22) (Dziadkowiec, Gąsiorowska et al. 2016)

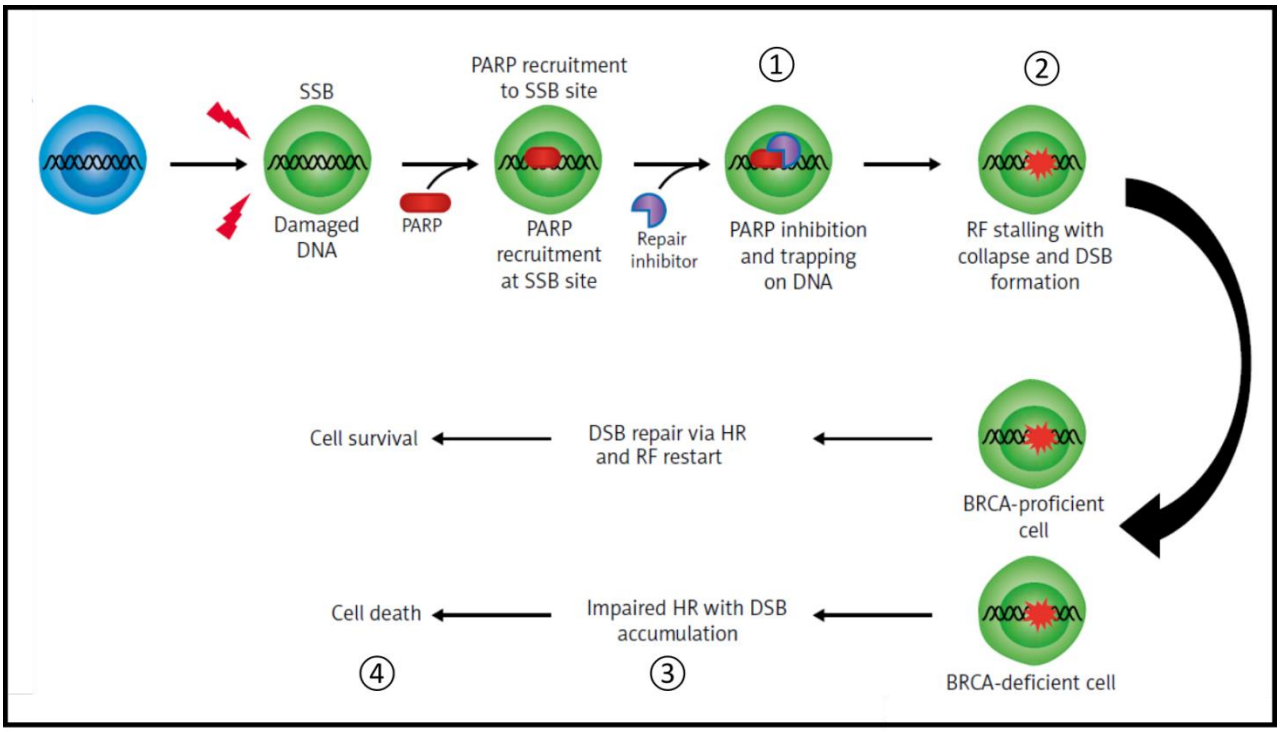


Figure 22. PARP-BRCA synthetic lethality strategy in cancer therapy. After DNA damage, PARP-1 is recruited to the damage site to repair SSB. Its activity is inhibited by PARP inhibitors, leading to the formation of DSBs during replication. As HR repair pathway is defective, DSBs accumulate in the cancer cells, resulting in cell death (adapted from Dziadkowiec *et al.*, 2016).

3.2.4. Non-homologous end-joining pathways

Non-homologous end-joining pathway (NHEJ) is another pathway that is activated in DSBs repair. The NHEJ comprises two sub pathways: the classical-NHEJ (C-NHEJ) and the alternative-NHEJ (Alt-NHEJ).

The role of PARP-1 in the C-NHEJ is not clear. Several studies report that PARP-1 is essential in this pathway to mediate the interaction between Ku70/Ku80 complex with DNA-PKcs. This interaction is crucial for the DSBs recognition and its stabilization during C-NHEJ (Couto, Wang et al. 2011, Paddock, Bauman et al. 2011, Patel, Sarkaria et al. 2011, Spagnolo, Barbeau et al. 2012). Moreover PARP-1 could have an important role in the choice between the C-NHEJ and the Alt-NHEJ pathways. PARP-1 can compete with the Ku70/Ku80 complex for binding to DNA breaks, and depending of which protein is recruited first, it favors one or the other pathway of the NHEJ. Ku presents a higher affinity for DNA ends and it is faster recruited to the DNA breaks than PARP-1, favorizing the C-NHEJ pathway (Wang, Wu et al. 2006). However, if the

C-NHEJ machinery is defective, PARP-1 is preferentially recruited and favors the activation of Alt-NHEJ (Wang, Wu et al. 2006, Mansour, Rhein et al. 2010). In the Alt-NHEJ, PARylation mediated by PARP-1 promotes the recruitment of MRE11 to the DNA damage site, initiating the short resections (Haince, McDonald et al. 2008). Also, the substrate of PARP-1, histone H1 can also stimulate DNA ligase III, an enzyme that is important in the restauration of the strand in the Alt-NHEJ (Rosidi, Wang et al. 2008).

Finally, PARP-1 can also facilitate Alt-NHEJ in CSR (class switch recombination), a process that occurs in lymphocytes B. CSR is a biological mechanism that allows proliferative lymphocytes B to rearrange the gene region encoding immunoglobulin heavy chains, allowing the B cells to change the immunoglobulins class for another (Stavnezer, Guikema et al. 2008). This mechanism starts by DNA breaks that consequently activates the NHEJ repair pathway. Briefly, the DNA breaks are induced by activation induced cytidine deaminase (AID), an enzyme that deaminates cytidine present in the DNA into uracil, which activates base excision repair (BER) or mismatch repair (MMR), leading to the formation of mutations or DSBs. This leads to the activation of DNA repair proteins, among PARP-1 that facilitates the repair of the DNA breaks by Alt-NHEJ (Robert, Dantzer et al. 2009), probably by competition with Ku (Wang, Wu et al. 2006).

3.2.5. Chromatin structure and remodeling

To an efficient DNA damage response, the re-organization of chromatin is required for facilitating the access of the DNA repair machinery to the DNA damage sites. The chromatin remodeling is regulated by several factors such as post-transcriptional modifications (PARylation, acetylation, phosphorylation and others) and by ATP-dependent chromatin remodelers (Tallis, Morra et al. 2014).

PARP-1 can regulate the condensation of DNA by different modes of action. One of the mechanisms consists in the modulation of the nucleosomes. PARP-1 can also regulate the interaction between histones and DNA. Actually, the attraction between the negatively charged phosphates of DNA and the alkaline residues of the histone is responsible for their association and regulates the chromatin condensation. Interestingly, the PARylation of the

histone H1 leads to the release of DNA and induces the relaxation of the chromatin, which allows the access of the DNA repair machinery to the damage sites. Other histones were reported as PARylation activity regulators such as H2A and H2B (d'Amours, Desnoyers et al. 1999). One example is the histone PARylation factor 1 (HPF1; C4orf27) that promotes PARP-1-dependent *trans* ADP-ribosylation of histones, which in turn, limits the hyper automodification of PARP-1 at DNA damage sites. Moreover, HPF1-deficient human cells are sensitive to DNA damaging agents and PARP inhibitors, suggesting a role of HPF1 in the genome maintenance and in the regulation of PARP inhibitors efficacy (Gibbs-Seymour, Fontana et al. 2016).

PARP-1 activity is also required for the recruitment of chromatin remodelers such as ALC1, a SNF2-like ATPase that contains a macrodomain (Pines, Vrouwe et al. 2012, Singh, Nardozza et al. 2017). The overexpression of ALC1 induces spontaneous tumors in mice (Chen, Huang et al. 2009). When PARylated, ALC1 is rapidly recruited to DNA breaks that leads to the formation of a stable nucleosome intermediate and, thus, promoting the chromatin relaxation at damage sites (Ahel, Hořejší et al. 2009, Gottschalk, Timinszky et al. 2009). PARP-1 is also capable of regulating SMARCA5/SNF2H, the catalytic subunit of chromatin remodeling complexes, ISWI. In fact, the recruitment of SMARCA5/SNF2H to the DNA damage sites its dependent of PARP-1, since in PARP-1-depleted cells, the accumulation of this catalytic subunit is significantly reduced in damage sites. Furthermore, PARP-1 is necessary for the interaction of SMARCA5/SNF2H with RNF168, an E3 ubiquitin ligase implicated in DSBs repair. Indeed, SMARCA5/SNF2H recognizes and binds PAR to RNF168, allowing their interaction with the chromatin (Smeenk, Wiegant et al. 2013). Remarkably, PARP-1 also regulates CHFR, another E3 ubiquitin ligase. CHFR contains a PBZ domain that interacts with PAR, modulating the early stages of DNA damage response by ubiquitination of PARP-1 and/or PARylated histones and, thus, promoting chromatin relaxation (Kashima, Idogawa et al. 2012, Liu, Wu et al. 2013, Brodie, Li et al. 2015). Regarding all these facts, it is suggested a physical and functional association between PARylation, chromatin remodeling and the signaling cascade that is initiated by ubiquitin ligases (Tallis, Morra et al. 2014).

PARP-1 also regulates:

❶ Chromatin condensation by recruiting histone chaperons, such APLF. PAR synthesis mediated by PARP-1 can interact with APLF via its PBZ domain, allowing its recruitment to DNA damage sites. This permits the removal of histones and the recruitment of histones variants such as Macro H2A1, promoting the DNA repair (Mehrotra, Ahel et al. 2011).

❷ Chromatin modifying proteins, such as CHD4, a member of the SNF2/RAD54 helicase family, to facilitate DNA repair. CDH4 is recruited to DNA damage sites in a PAR-dependent mode, establishing a transcriptionally repressed state and thereby facilitates the DNA repair (Polo, Kaidi et al. 2010).

Concerning these results, a model for chromatin remodeling by PARP-1 is proposed (Figure 23): PARP-1 activation after binding to nicked DNA (①), PARylation mediated-recruitment of chromatin-modifying proteins (②) that contributes to chromatin relaxation. In parallel, due to extensive PARylation, PARP-1 itself and other histones are release from DNA (③). This mechanism results in changes in the chromatin structure (chromatin relaxation) that facilitates the access and, thus, the recruitment of DNA repair machinery at DNA damage sites.

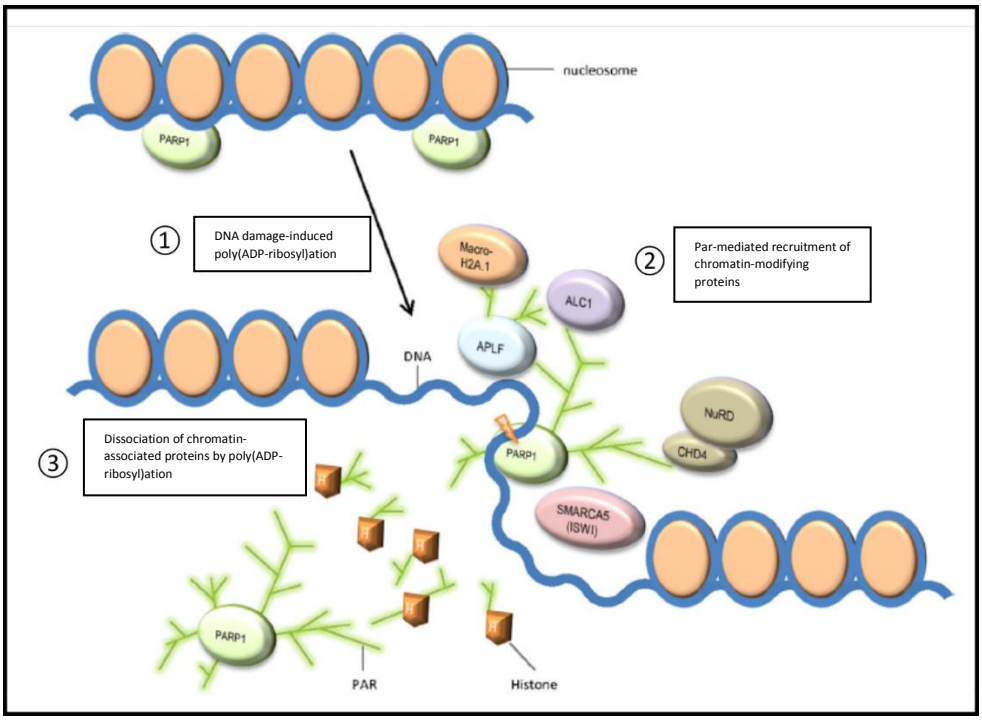


Figure 23. Role of PARP-1 in chromatin remodeling in response to DNA damage. ① PARP-1 is activated after binding to DNA damage sites, and ② promotes PARylation mediated-recruitment of chromatin-modifying proteins. In parallel, ③ due to extensive PARylation, PARP-1 itself and other histones are release from DNA (from Tallis *et al.*, 2014).

3.3. The physiological role of PARP-1:

In this part I will briefly present the role of PARP-1 in some physiological processes that are deregulated in cancer cells or involved in carcinogenesis.

PARP-1 activity is involved in several important cellular processes, such as cell cycle, DNA repair and cell proliferation. Dysfunctional regulation of these processes has serious outcomes as metabolic disorders, inflammatory diseases and cancer (Bai 2015).

3.3.1. Inflammation

Several studies reported that both inflammatory response and PARP-1 are activated in some inflammatory disease such as chronic obstructive pulmonary disease (COPD), acute lung injury (ALI) (Sethi, Dharwal et al. 2017), rheumatoid arthritis (García and Conde 2015). PARP-1 influences the expression of pro-inflammatory cytokines, such ILβ-1, TNF-1α in such central

nervous system (CNS) inflammation, by PARylating nucleosomal histones. This facilitates the access to nucleosomal DNA and the recruitment of NF-κB to the promoters of ILβ-1 and TNF-1α , inducing their transcription (Martínez-Zamudio and Ha 2014). PARP-1 also regulates the expression of inflammatory cytokines through PARylation of adenylateuridylate-rich element-binding protein embryonic lethal abnormal vision-like 1 (Elavl1)/human antigen R (HuR), leading to the increase expression of cytokines mRNA (Ke, Han et al. 2017).

PARP-1 also regulates the activation of the NF-κB pathway (Ba and Garg 2011, Weaver and Yang 2013), which is constitutively activated in several cancers. The activation of NF-κB pathway regulated by PARP-1 is performed by two mechanisms (Figure 24). Firstly, (①) in response to an inflammatory stimulus, PARP-1 interacts with the histone acetyl-transferase p300 and CREB-binding protein (CBP), leading to the acetylation of a specific lysine residue of PARP-1, mediating the interaction of PARP-1 with p50. This enhance the p300-p50 interaction, leading to the activation of NF-κB-mediated gene transcription (Hassa, Buerki et al. 2003, Hassa, Haenni et al. 2005), and secondly, (②) after DNA damage, PARP-1 autoPARylation promotes the formation of a “signalosome” that contains IKKγ (NEMO), the regulatory subunit of NF-κB inhibitory complex, PIASγ and ATM. Then, PAR from autoPARylated PARP-1 leads to the activation of PIASγ via its the PAR binding motif, that induces IKKγ SUMOylation and, consequently activates the NF-κB pathway (Stilmann, Hinz et al. 2009). Moreover, the interaction of PARP-1 with NF-κB was reported where increase PAR production leads to a conformational change and retaining of NF-κB subunit p65 in the nucleus, promoting the expression of NF-κB target genes such as intercellular adhesion molecule 1 (ICAM-1) and nitric oxide synthetase (NOS) (Zerfaoui, Errami et al. 2010, Hunter, Willmore et al. 2012).

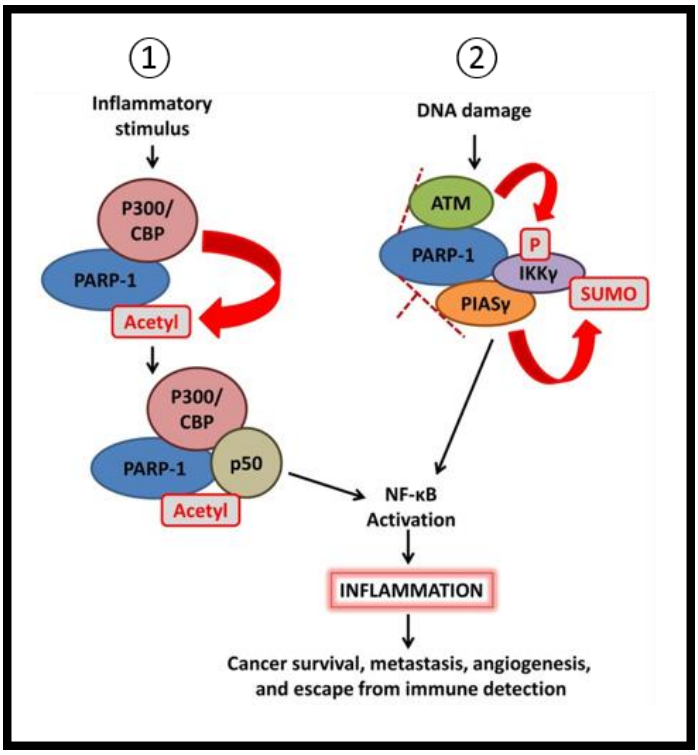


Figure 24. Mechanisms of PARP-1-mediated activation of NF-κB signaling pathway. ① Acetylation of PARP-1 by the p300/CBP mediates the p50-PARP-1 interaction and enhances p50-p300 interaction, leading to the activation of NF-κB signaling pathway. ② Formation of a signalosome complex (PARP-1, ATM, PIASγ and IKKγ interaction) where PAR formed during PARP-1 autoPARylation activates PIASγ via its PAR binding motif, inducing the SUMOylation of IKKγ and, consequently the activation of NF-κB pathway (adapted from Weaver and Yang, 2013).

Finally, PARP inhibition was reported to prevent the inflammation-associated side effects of chemotherapy treatment, suggesting that it might be helpful to use PARP-1 inhibitors in multidrug therapy (Weaver and Yang 2013).

3.3.2. Cell cycle

Cell cycle checkpoints are important for the maintenance of cell proliferation. Multiple checkpoints are deregulated in cancer, but the mitotic checkpoint gathers a great importance for both carcinogenesis and anticancer therapy. Cells with an impaired mitotic checkpoint can survive but they present defective chromosome segregation, resulting in genomic instability (Weaver and Yang 2013).

PARP-1 controls the cell cycle progression by regulating the expression of target genes, such as Sp1 transcription factor (Yang, Huang et al. 2013). Firstly, PARylation performed by PARP-1 is necessary for the assembly or function of the bipolar spindle. PARP-1 localized in the centromeres, where it PARylates proteins present at centromeres and centrosomes during mitosis such as Cenp-A, Cenp-B, and Cenp-C, and the spindle checkpoint protein, Bub3 (Saxena, Saffery et al. 2002, Kanai, Tong et al. 2003). PARP-1 is also responsible for the PARylation of p53, which regulates centrosome duplication and chromosome stability (Kanai, Tong et al. 2003). Furthermore, defective PARP-1 activity is associated with reduced Bub3 recruitment to centromeric domains, leading to defective mitotic checkpoint and failure of metaphase arrest and genomic instability (Kanai, Tong et al. 2003, Yang, Baumann et al. 2009, Weaver and Yang 2013).

In addition, PARP-1 can also interact with the E3 ubiquitin ligase, checkpoint with forkhead-associated and RING finger domains (CHFR), which is a tumor suppressor that participates in the early mitotic checkpoint (Yu, Minter-Dykhouse et al. 2005). The binding of these proteins leads to PARP-1 degradation and cell cycle arrest (Kashima, Idogawa et al. 2012, Weaver and Yang 2013).

Finally, PARP-1 can regulate the S-phase checkpoint through Chk1 activation. Chk1 and ataxia telangiectasia and Rad3-related (ATR), its kinase, are necessary for the regulation of the intra-S, S/M and G2/M checkpoint. In response to genotoxic stresses, Chk1 is phosphorylated by ATR inhibiting its auto-inhibition state and, allowing the activation of the downstream checkpoints (Aziz Sancar, Laura A. Lindsey-Boltz et al. 2004, Smith, Mun Tho et al. 2010, Smits and Gillespie 2015). In response to a stalled replication fork, PARP-1 is recruited to the fork where it PARylates itself and/or other target proteins such ATM and MRE11 (Haince, McDonald et al. 2008). Poly(ADP-ribose) polymers, that are formed due to PARP-1 activation (①), interact with Chk1 via the PAR-binding regulatory (PBR) motif. Although Chk1 is already bound to the stalled replication fork, its interaction with PAR leads to an efficient stabilization of Chk1 on the replication fork, facilitating its phosphorylation by ATR and consequently activating its kinase activity (②). Then, Chk1 is release from the replication fork to employ its checkpoint function (③) (Min, Bruhn et al. 2013) (Figure 25).

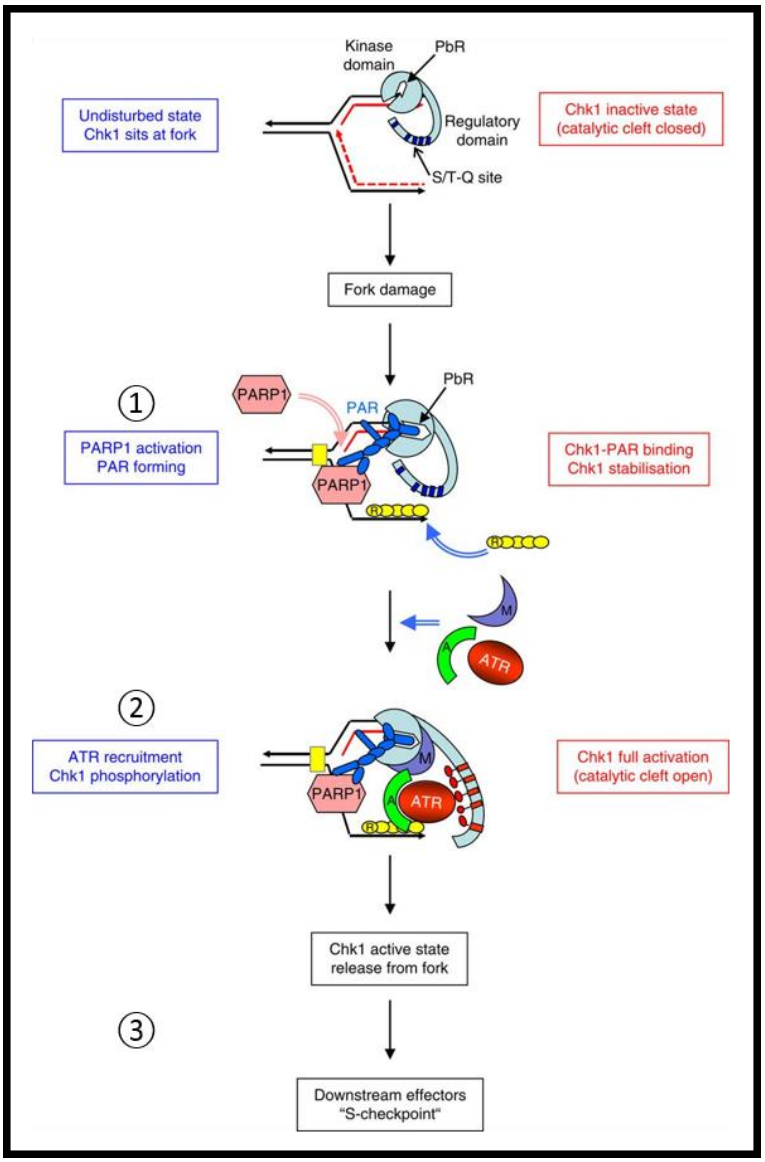


Figure 25. PARP-1 and PAR-mediated activation of Chk1 at blocked replication forks. PARP-1 is activated and binds to stalled replication forks. The PAR (dark blue dots) formed by PARP-1 activity interact with Chk1 (in light blue) via its PAR-binding motif (PbR) facilitating the stabilization of Chk1 in the replication fork. This leads to the recruitment of ATR that phosphorylates Chk1, that in turn activates Chk1 and allow its release from the fork to activate its downstream checkpoint (adapted from Min *et al.*, 2013).

3.3.3. Cellular energy and cell death

Impaired cell death signaling pathways, excessive proliferation and deregulated metabolism are the major characteristic of tumor cells. These phenotypes result in part from altered mitochondrial activity and inactivation of apoptotic signaling pathways by reduced expression of the pro-apoptotic factors p53. Both cell energetics and death are strongly regulated by

PARP-1, where PARylation activity may swift between cell fates by affecting both tumor progression and therapeutic response (Weaver and Yang 2013).

In response to DNA damage, the activated PARP-1 plays a role in the early steps of apoptosis by stabilizing p53 and facilitating its function as pro-apoptotic protein (Wesierska-Gadek J. 2005). PARP-1 binds to both central and carboxyl-terminal domains of p53 forming a tightly complex, where the latter one possesses a nuclear export signal (NES), responsible for the export of p53 from the nucleus to cytoplasm and allowing its degradation (Józefa, Jacek et al. 2003). Furthermore, the phosphorylation of Ser392 in the carboxyl-terminal region is responsible for the binding of PARP-1 to p53, covering the NES, allowing the retention p53 in the nucleus and preventing the export and consequent degradation of p53 (Józefa, Jacek et al. 2003). In the presence of extended DNA damage, PARP-1 is capable of inducing cell death by different mechanism instead of attempting repair (Figure 26):

① If the DNA damage is too extensive, PARP-1 will synthesize high levels of PAR, leading to depletion of NAD^+. This consequently leads to the depletion of ATP, resulting in energy deprivation that ultimately activates necrosis (Weaver and Yang 2013).

② Hyperactivation of PARP-1 and subsequent accumulation of PAR in the nucleus, cause the translocation of PAR into the cytoplasm, where it interacts with the apoptosis-induced factor (AIF), present in the outer mitochondrial surface. This interaction induces the translocation of AIF into the nucleus, potentially resulting in DNA fragmentation. This new type of PARP-1 - dependent cell death is known as parthanatos (Wang, Dawson et al. 2009).

③ PARP-1 PARylates the Asp216,374 and 384 residues from caspase-8, inhibiting pro-caspase-8 cleavage into the p18/p10 active form that is responsible for the initiation of apoptosis signaling, thus, inhibiting apoptotic signaling pathway (Yuan, Sun et al. 2013).

④ ATP depletion due to PARP-1 hyperactivation leads to the activation of AMP-activated protein kinase (AMPK), which inhibits mTOR and promotes autophagy (Virág, Robaszkiewicz et al. 2013).

⑤ Finally, to prevent the parthanatos or autophagy and the inhibition of caspase 8-mediated apoptosis, activated caspases -3 and -7 can cleave PARP-1 *in vivo* into two different fragments: an 89 kDa fragment with low PARylation levels and a second fragment of 24 kDa that can inhibit the activity of uncleaved PARP-1. This consequently leads to the conservation of NAD^+ and, thus, ATP levels, promoting cells to undergo the programmed cell death, known as apoptosis (Simbulan-Rosenthal, Rosenthal et al. 1998, Soldani, Lazzè et al. 2001, Soldani and Scovassi 2002, Ivana Scovassi and Diederich 2004, Chaitanya, Alexander et al. 2010).

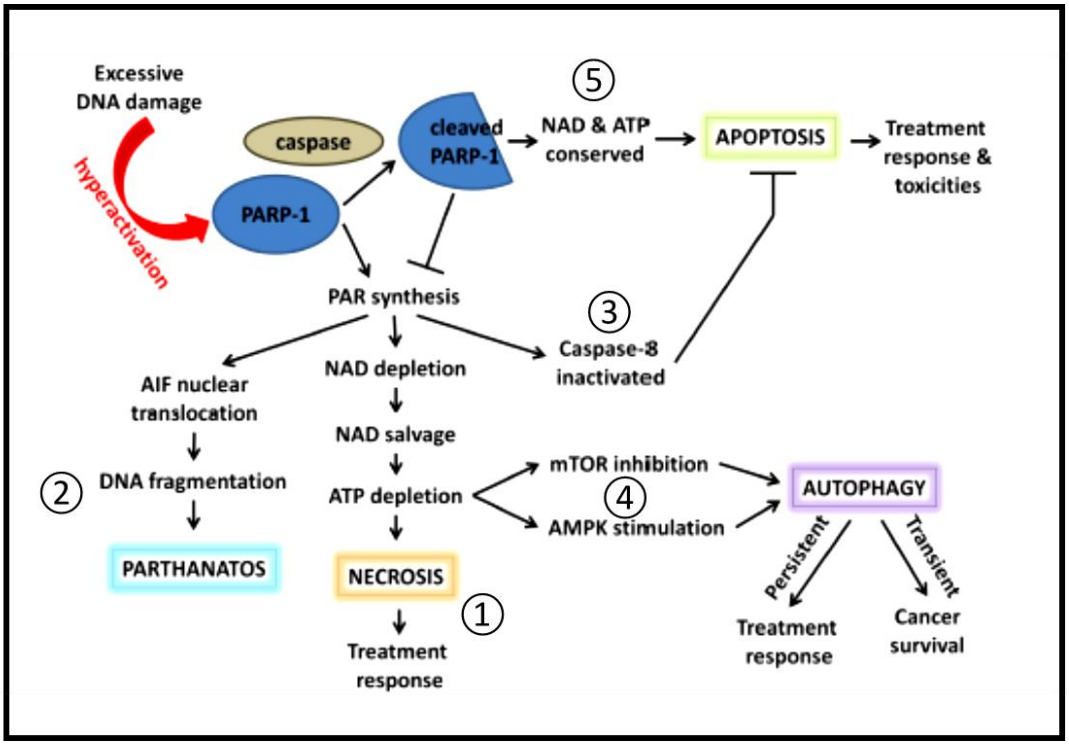


Figure 26. PARP-1 mediated cell death mechanisms. ① Hyperactivation of PARP-1 and PAR accumulation leads to NAD^+ and, thus, ATP depletion resulting in cellular energy failure and induction of necrosis. ② The accumulated PAR is translocated to the cytoplasm and interacts with AIF, leading to its translocation into the nucleus and parthanatos induction. ③ PARylaton can also inhibit caspase-8, leading to the inactivation of apoptosis signaling pathway. ④ ATP depletion as consequence of PARP-1 over-activation leads to the activation of AMPK that, in turn, inhibits mTOR, inducing autophagy. 5) Caspase -3 and -7 are capable of cleaving PARP-1 *in vivo*, leading to the conservation of NAD^+ and ATP levels, resulting in the induction of apoptosis. (from Weaver and Yang, 2013).

PARP-1 also creates a feed-forward regulatory cycle with the MAP kinase, c-jun N-terminal kinase (JNK), a protein present in the classical necroptotic pathway (Zhang, Lin et al. 2007). PARP-1 decreases the MAPK phosphatase (MPK-1) expression via PARylation of activating transcription factor 4 (ATF4), a c-AMP response element binding protein (CREB) transcription factor known to regulate MPK-1 expression (Hocsak, Szabo et al. 2017). This leads the inhibition of the AKT kinase, where both processes activate JNK proteins (Szanto, Hellebrand et al. 2009, Racz, Hanto et al. 2010). Conversely, JNK1 phosphorylates PARP-1, leading to its activation, creating a regulatory cycle between both proteins (Zhang, Lin et al. 2007). JNK proteins are activated in several cancers and might act as a driver for tumor development or treatment response (Bubici and Papa 2014), indicating that PARP inhibitors could be potential therapeutic treatment in JNK high activity tumors.

The mechanism underlying the choice of PARP-1 mediated cell death in response to genotoxic stress is not yet elucidated but may be influenced by the type and duration of the stress as well as cell type (Weaver and Yang 2013). One thing is sure, PARP-1 not only acts as DNA damage sensor, facilitating the DNA repair process but also acts a mediator of life-or death decisions of the cell.

3.3.4. Aging

Regarding PARP-1 and its function in the aging process, several studies showed a positive correlation between PARP-1 activity and mammalian longevity (Veith and Mangerich 2015). A study performed in human lymphocytes demonstrated that lymphocytes of centenarian (mean of 101 years old) presented higher PARylation activity compared to controls (age range from 20-70 years old) (Muiras, Müller et al. 1998). Additionally, a study performed in transgenic mice with an extra-copy of human PARP-1 (hPARP-1) confirmed the results observed in the human lymphocytes. Indeed, the overexpressed of hPARP-1 in these mice, prolonged disease-free life of male mice and decreased the tumor weight and even reduced the incidence of certain tumor types. Actually, it was suggested that enhanced PARP-1 activity leads to a more intensive and effective DNA repair machinery and, thus, preventing the accumulation of DNA damage (Mangerich, Herbach et al. 2010).

PARP-1 also positively regulates the NF-κB signaling pathway, which is an important key mediator in the aging process (Salminen and Kaarniranta 2009, Tilstra, Clauson et al. 2011). Moreover, PARP-1 hyperactivation leads to a rapid depletion of NAD^+ concentration in cells, leading to mitochondrial dysfunction, that is a common feature of aging, or to induction of cell death. Furthermore, the hPARP-1 mice used in the study mentioned earlier, and, where PARP-1 was overactivated, were overweight and presented glucose intolerance, suggesting that although mice live longer, they are susceptible to aging-associated metabolic disorders (Mangerich, Herbach et al. 2010). These results highlight the role of PARP-1 as an aging-promoting factor.

Noteworthy, PARP-1 is a double-edge sword: its hyperactivation is correlated with a longer life span due to an effective DNA repair system but inversely can lead to the development of metabolic disorders.

3.3.5. Carcinogenesis: PARP-1 as therapeutic target

Targeting DNA damage response mechanism seems to be an interesting strategy for cancer therapy. PARP-1 is notably vital for DNA damage repair process, so PARP-1 inhibitors are in the past few years the focus of several clinical trials (Ray Chaudhuri and Nussenzweig 2017).

PARP inhibitors are widely used in tumors that present reduction in the DNA repair capacity. PARP inhibitors showed to be very effective in killing cancer cells that present defective DNA repair machinery such as BRCA1 or BRCA2 mutations that are commonly present in familial breast cancer (Ray Chaudhuri and Nussenzweig 2017). As presented earlier, PARP inhibition in these types of tumors overcast two synergistic DNA repair pathways, the SSB pathway repair by PARP-1, and the HR by BRCA1 or BCRA2, that induce synthetic lethality (Veith and Mangerich 2015). The proposed mechanism is that in deficient DNA repair tumors, SSBs accumulate upon PARP inhibitors treatment and are converted into DSBs during DNA replication. As these tumors present ineffective homologous recombination pathway, cancers cells are sensitive to DSB leading to cell death (Schreiber, Dantzer et al. 2006). Therefore, PARP-1 inhibitors are being exploited for DNA repair-deficient ovarian, breast and prostate cancers (Bai 2015). One example is the PARP-1 inhibitor, Olaparib (Lynparza, AstraZeneca), that was approved by FDA in 2014 as a chemotherapeutic in germline BRCA-mutated ovarian

cancer (Kim, Ison et al. 2015) and in 2018 became the first PARP inhibitor approved, again, by FDA in germline BRCA-mutated metastatic breast cancer.
Moreover, PARP-inhibitors are being used in clinical as a mono-therapy or in combination with chemo- or radiotherapy. However, it was observed that in the same cancers that present defective DNA repair machinery, chemoresistance to PARP inhibitors can be developed. The most common mechanism for PARP inhibitors resistance is the reversion of BRCA1/2 mutation, which restores HR pathway (Edwards, Brough et al. 2008, Wang and Figg 2008).

Noteworthy, there are no PARP-1 specific inhibitor. As these inhibitors are designed to compete with the NAD^+, the substrate for PARP-1 activity, at its catalytic site, they are also capable of inhibiting other enzymes that depend of NAD^+, such as the other proteins from PARP family, mono-ADP-ribosyl-transferases and sirtuins (Rouleau, Patel et al. 2010).

Additionally, extensive cellular stress leads to uncontrolled PARP-1 hyperactivation and consequently activate inflammatory response, cell death and tissue damage. PARP inhibitors might also be used in other stress-related diseases such as cardiovascular diseases, metabolic disorders, diabetes, autoimmune diseases but these inhibitors are mainly used in cancer therapeutics (Hottiger 2015, Mukhopadhyay, Horváth et al. 2017, Ordway, Szebeni et al. 2017).

Understanding the roles of PARPs in maintaining genome integrity is not only important for the design of novel therapeutic agents but also crucial for gaining insights into the mechanism of chemoresistance in cancer cells (Ray Chaudhuri and Nussenzweig 2017).

4. PARP-1 in Bloom syndrome

As presented in chapter 2, Amor-Guéret's team discovered recently that the nucleotide pool disequilibrium resulting from CDA deficiency reduces the basal PARP-1 activity, leading to genetic instability (Gemble, Ahuja et al. 2015). However, it was unknown how the pyrimidine pool imbalance and subsequent reduced PARP-1 activity leads to the accumulation of unreplicated DNA sequences during mitosis. One potential explanation was through downstream targets of PARP-1, such Chk1, because in response to DNA damage, PARP-1

PARylates Chk1, allowing its stabilization and facilitating its phosphorylation and, subsequently the activation of the Chk1-dependent checkpoints, preventing cells from entering mitosis prematurely (Min, Bruhn et al. 2013). More recently, Gemble *et al.* (2016) demonstrated that Chk1-ATR pathway was compromised when CDA was deficient. The team showed that reduced PARP-1 activity due to CDA deficiency, impairs Chk1 and the S and G2/M downstream checkpoint activation, that consequently leads to the accumulation of unreplicated DNA sequences in mitosis, resulting in supernumerary UFB formation. Furthermore, culturing cells with dC, that results in intracellular accumulation of dC and dCTP, mimicking CDA deficiency, was sufficient to reproduce the reduction of basal PARP-1 activity and, thus, the poor activation of Chk1 in response to genotoxic stress. Treating CDA-deficient cells with low doses of camptothecin (CTP), leading to the activation of PARP-1, did not prevent the formation of supernumerary UFBs if either Chk1 or ATR were inhibited, indicating that the defective activation of Chk1 and of the downstream checkpoints was responsible of the excess UFB formation (Gemble, Buhagiar-Labarchède et al. 2016).

On the basis of these results, the team proposed the following model (Figure 27): (①) in CDA-proficient cells, PARP-1 activity ensures the optimal activation of Chk1, promoting checkpoint efficiency and preventing the accumulation of unreplicated DNA sequences and, consequently excess UFB formation during mitosis. However, (②) if CDA is deficient, reduced PARP-1 activity, impairs Chk1 activation, decreasing checkpoint efficiency, allowing the entry of cells containing unreplicated DNA into mitosis that consequently leads to supernumerary UFB formation (Gemble, Buhagiar-Labarchède et al. 2016).

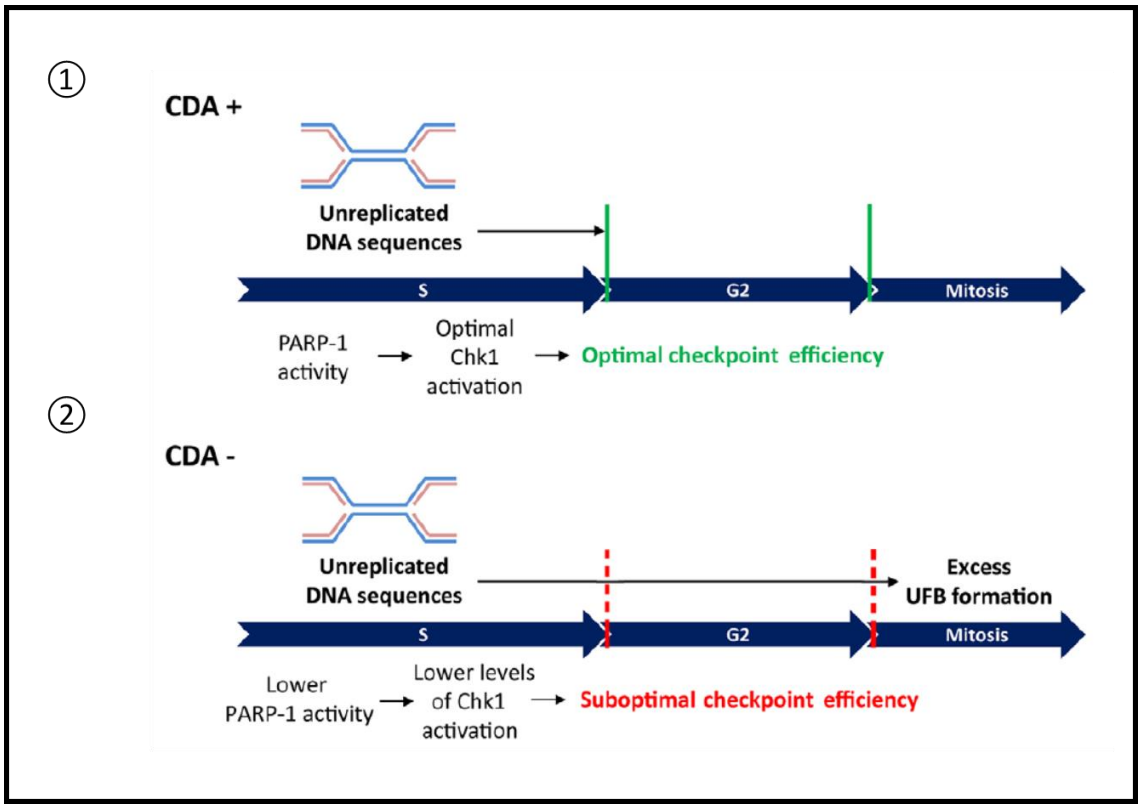


Figure 27. Balanced pyrimidine pool ensures optimal activation of chk1, preventing the formation of supernumerary UFBs. ① In CDA-proficient cells, basal PARP-1 activity ensures optimal activation of Chk1, resulting in checkpoint efficiency that prevents UFB formation. ② In cells lacking CDA, low PARP-1 activity, leads to a suboptimal activation of Chk1, leading to reduced checkpoint efficiency, resulting in the accumulation of unreplicated DNA sequences and excess UFB formation (from Gemble *et al.*, 2016).

The team revealed a new role of Chk1 in preventing chromosome segregation defects. Also, these results indicate ATR-Chk1 acts downstream from PARP-1, preventing the accumulation of unreplicated DNA in mitosis, and, thus, UFB formation.

These results indicate that the decrease in PARP-1 activity contribute to several cellular features associated with BLM deficiency.

Since NAD^+ is essential for PARP-1 activation and function, the next chapter is dedicated to the NAD metabolism.

The NAD metabolism

1. NAD^+ biosynthesis

Nicotinamide adenine dinucleotide (NAD) is a co-enzyme that plays an important role in redox reactions. It was discovered and characterized as a heat-stable factor during alcohol fermentation in yeast by Harden and Young in 1906. Then, it was chemically described as a dinucleotide by Hans von Euler-Chelpin in 1930 and Otto Warburg (1936) showing that NAD was involved in redox reactions essential for cellular metabolism (Berger, Ramírez-Hernández et al. 2004, Magali R. VanLinden 2015). NAD is required for several biological processes, from redox reactions as oxidative phosphorylation to non-redox reactions as gene transcription, cell differentiation and survival, apoptosis, chromatin stability, DNA repair and cellular metabolism (Chiarugi, Dölle et al. 2012, Di Stefano, Loreto et al. 2017). Moreover, NAD plays a fundamental role in cellular homeostasis: by being implicated in both bioenergetic and signaling pathways, NAD molecule is a key regulator of metabolism and cellular events (Nikiforov, Kulikova et al. 2015).

When NAD is used as a co-enzyme for the redox reactions (its oxidation leads to the formation of NAD^+ and its reduction to NADPH), its levels remain constant. However, if NAD is used for non-redox reactions, then NAD is cleaved and consequently its levels decrease. Hence, to prevent the complete depletion of the NAD levels, continuous biosynthesis of this molecule is necessary (Di Stefano, Loreto et al. 2017). The NAD biosynthesis is mainly performed by two major routes: the *de novo* pathway, that converts amino acid tryptophan into NAD^+ (Mattevi 2006, Nikiforov, Kulikova et al. 2015), and the salvage pathway, where three different building blocks ((nicotinic acid (NA), nicotinamide (NAM) and nicotinamide ribose (NR)) are used for NAD synthesis (Mattevi 2006).

1.1. The de novo pathway

The *de novo* pathway comprises a total of nine reactions steps that ultimately leads to the synthesis of NAD^+ (Figure 28). Tryptophan (Trp) which is supplied from the diet, is used as a substrate in this pathway that, after five reactions steps, leads to the formation of an unstable intermediate product, the α-amino-β-carboxymuconate-ε-semialdehyde (ACMS) (①). The formation of this metabolites is a branching point in the *de novo* pathway (Bender 1983). In

this point, ACMS can be further converted into intermediate substrates that are oxidized into water and carbon dioxide (CO_2) via the glutarate pathway and tricarboxylic acid (TCA) cycle (②) (Houtkooper, Cantó et al. 2010, Cantó, Menzies et al. 2015). Alternatively, if the enzymatic capacity of ACMS decarboxylase (ACMSD) to convert ACMS into its intermediate is surpassed (Ikeda, Tsuji et al. 1965), ACMS can be subjected to a spontaneous cyclization, leading to the formation of quinolinic acid (QA) (③). The degradation of tryptophan into QA is called the kynurenine pathway. Then, QA is converted into NA mononucleotide (NAMN) by QA phosphoribosyltransferase (QAPRT) (④) that is rapidly transformed into NA adenine dinucleotide (NAAD) by the ATP-dependent nicotinamide mononucleotide adenyltransferase (NMNAT) enzymes (⑤) (Bender 1983, Okuno and Schwarcz 1985, Houtkooper, Cantó et al. 2010, Cantó, Menzies et al. 2015). This step is common for both the *de novo* and NA salvage pathway. Finally, the intermediate product NAAD leads to the biosynthesis of NAD^+ by NAD^+ synthetases (NADS) (⑥) (Cantó, Menzies et al. 2015, Di Stefano, Loreto et al. 2017). The NAD^+ *de novo* pathway using L-tryptophan is performed in mammalian cells, fungi and bacteria. In some bacteria and plants, the *de novo* pathway uses L-aspartate as substrate to initiate this pathway, that after two reaction steps leads to the formation of QA that is converted at the end into NAD^+ (Mattevi 2006, Lin, Kwan et al. 2010) as previously described.

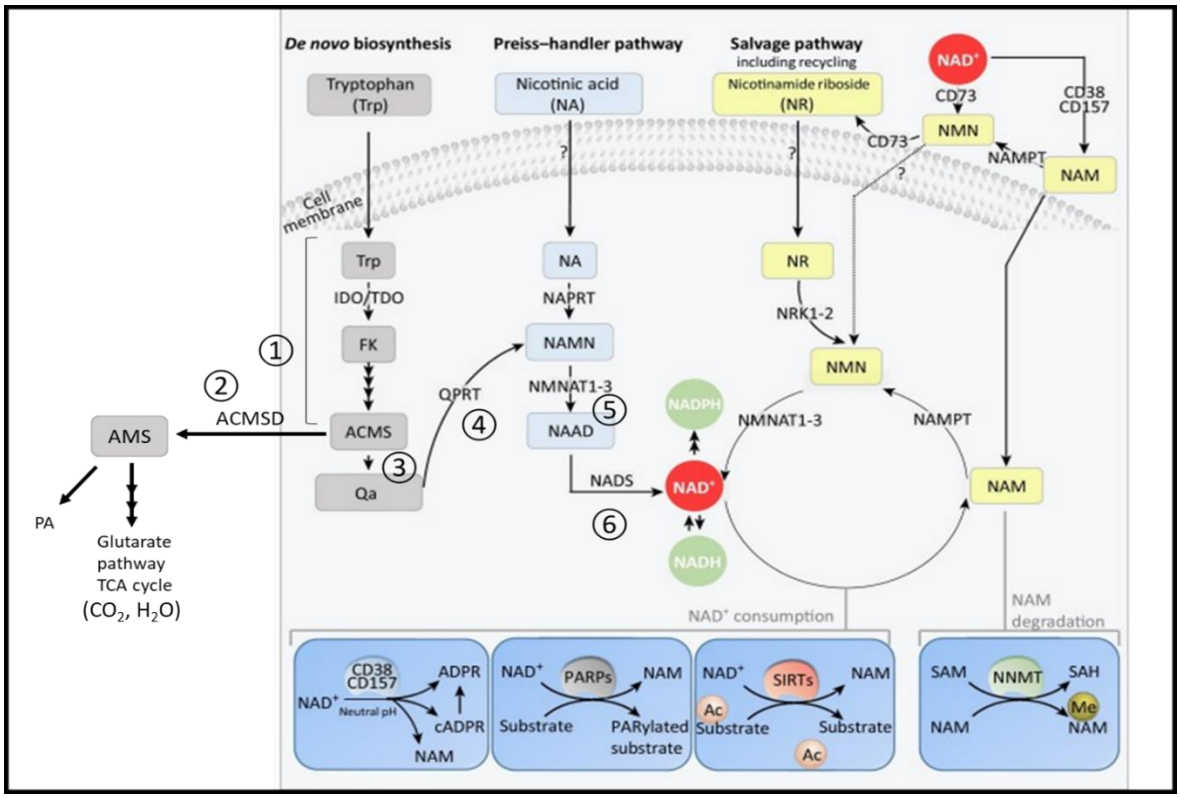


Figure 28. The NAD⁺ de novo and salvage biosynthesis pathways. The *de novo* pathway uses Trp as a substrate, that after 9 reactions steps leads to the formation of NAD^+. The salvage pathway uses NA (via NA salvage pathway), NAM (via NAMPT salvage pathway) and NR (via NR salvage pathway) to produce NAD^+ (adapted from Fang *et al.*, 2017).

As already mentioned, NMNATs are involved in both *de novo* and salvage pathways. They are responsible for the conversion of NAMN into NAAD in the *de novo* pathway and NA salvage pathway and for the conversion of nicotinamide mononucleotide (NMN) into NAD^+ in the other two salvage pathways (Figure 29) (Sorci, Cimadamore et al. 2007). Three mammalian isoforms were described and characterized, NMNAT1, NMNAT2 and NMNAT3 (Raffaelli, Sorci et al. 2002, Zhang, Kurnasov et al. 2003). These enzymes differ from their oligomeric state, subcellular localization and catalytic properties (Berger, Lau et al. 2005, Lau, Niere et al. 2009) and play an important role in neuroprotection (Brazill, Li et al. 2017).

❶ NMNAT1 is a nuclear enzyme that is ubiquitously expressed in all tissues, with highest levels in skeletal muscle, heart, kidney, liver and pancreas whereas it is almost undetectable in the brain (Emanuelli, Carnevali et al. 2001, Fernando, Conforti et al. 2002, Cantó, Menzies et al. 2015). However, NMNAT1 enzymatic activity is important for neurodegenerative

diseases. It was reported that overexpression of NMNAT1 plays a role in neuroprotection in human disorders such polyQ toxicity and hypoxic ischemia (Verghese, Sasaki et al. 2011, Ocampo, Liu et al. 2013) whereas its loss of function induces neurodegeneration (Rossi, Geiszler et al. 2018). In fact, mutations of NMNAT1 is responsible for the Leber congenital amaurosis disorder, an inherited retinal degradation characterized by vison loss that affects mainly children (Falk, Zhang et al. 2012). Finally, it was recently demonstrated a role of NMNAT1 as a neuroprotector in tauopathies such as Alzheimer's disease (Rossi, Geiszler et al. 2018).

❷ NMNAT2 is mostly located in the cytoplasm and Golgi apparatus (Raffaelli, Sorci et al. 2002, Yalowitz, Xiao et al. 2004, Berger, Lau et al. 2005) and is highly express in the brain (Raffaelli, Sorci et al. 2002). This enzyme is also involved in neuroprotection (Magali R. VanLinden 2015). NMNAT2 can be involved in the initiating event of Wallerian degeneration (Di Stefano, Loreto et al. 2017) and when associated with heat-shock protein 90 (HSP90), a protein chaperone, leads to the relieving of the toxic phosphorylation of Tau protein effects in both frontotemporal dementia and parkinsonism 17 (FTDP-17) (Ali, Allen et al. 2016).

❸ NMNAT3 is mainly localized in the mitochondria but can be also found in the cytosol. NMNAT3 is expressed in lung, spleen, red blood cells and in the tissues where the other two isoforms are also expressed (Zhang, Kurnasov et al. 2003, Nikiforov, Dölle et al. 2011, Felici, Lapucci et al. 2013, Hikosaka, Ikutani et al. 2014). Exactly as the other NMNATs, NMNAT3 has also an important role in neuroprotection. The overexpression of this protein was found to be essential in axonal protection against optic nerve degeneration due to TNF-1α induction and intraocular pressure (IOP) elevation (Kitaoka, Munemasa et al. 2013). NMNAT3 is also an important neuroprotective enzyme in the neonatal hypoxia ischemia (HI) by inhibiting the apoptotic and necrotic neurodegeneration and it plays a role in maintaining the survival of immature neurons (Rafael, Marianne et al. 2017).

Despite the *de novo* pathway being considered as the classical pathway for NAD^+ synthesis, this is not sufficient to maintain the physiological NAD^+ concentration in the cell. Thus, the salvage pathways are preferentially used to obtain the NAD^+ levels required for the cellular processes (Henderson 1997, Nikiforov, Dölle et al. 2011).

1.2. The Salvage pathways

NAD^+ can also be produced from metabolite recycling such NA, NAM and NR or by diet uptake (Houtkooper, Cantó et al. 2010). NA and NAM are also known as vitamin B3 and are commonly used as food supplement (Nikiforov, Kulikova et al. 2015). The salvage pathways are faster and less-energetic biosynthetic pathways. Moreover, these pathways are important because it recycles the products of NAD breakdown to synthetize, back, NAD^+ (Mattevi 2006). The salvage pathways comprise three substrate specificity pathways, the ① NA salvage pathway, the ② NAM salvage pathway and the ③ NR salvage pathway (Figure 29).

① The NA salvage pathway starts by the conversion of NA by the NA phosphoribosyltransferase (NAPRT) into NAMN. Then, this pathway shares the same reaction as in the Trp-derivated pathway, where the latter product is converted into NAAD by the NMNAT (Cantó, Menzies et al. 2015, Nikiforov, Kulikova et al. 2015). Finally, NAAD suffers an amidation reaction to form NAD^+ that is performed by the NADS (Nikiforov, Kulikova et al. 2015). This pathway is also called Preiss-Handler pathway in honor of its discoverers (Preiss and Handler 1958, Preiss and Handler 1958). Interestingly, this pathway is predominantly performed in lower organisms such as bacteria and yeast (Houtkooper, Cantó et al. 2010, Chiarugi, Dölle et al. 2012). In these organisms, NAM is converted into NA by nicotinamidase, where it is rapidly consumed by NAPRT (Michel, Emmanuel et al. 2002, Anthony, Fabienne et al. 2003, Sauve 2008). In mammals, this pathway acts primarily in liver and kidney, where NAPRT activity is higher (Hara, Yamada et al. 2007, Cantó, Menzies et al. 2015). Finally, mammalian cells lack nicotinamidase activity (Shibata, Hayakawa et al. 1986, Carson, Seto et al. 1987, Sauve 2008), indicating that in humans and other mammals, the NAD^+ biosynthesis is mainly performed through the recycling of NAM (Cantó, Menzies et al. 2015).

② In the NAM salvage pathway, NAM and phosphoribosylpyrophosphate (PRPP) serves as substrates for nicotinamide phosphoribosyltransferase ($NAMPT^4$) enzyme, to form NMN (Garten, Petzold et al. 2009, Yang and Sauve 2016). Then, the latter is converted into NAD^+ by NMNAT enzyme (Houtkooper, Cantó et al. 2010). This pathway is the most direct and economical route (Chiarugi, Dölle et al. 2012). Moreover, NAM salvage pathway is the principal pathway to the NAD^+ biosynthesis in humans (Cantó, Menzies et al. 2015, Di Stefano,

Loreto et al. 2017). This is supported by several observations where, first, NAM is the end-product of the NAD^+ catalyzation by NAD^+-dependent enzymes, such as PARPs and Sirtuins (SIRT). In fact, these proteins cleave the NAD^+, which is accompanied by the liberation of a NAM moiety (Nikiforov, Kulikova et al. 2015). Secondly, NAM is the most abundant NAD^+ precursor present in the bloodstream (Gross and Henderson 1983), third, NAMPT is expressed in all mammalian tissues (Shibata, Hayakawa et al. 1986) and NAMPT knockout is embryonic lethal (Revollo, Körner et al. 2007, Zhang, Van Haandel et al. 2017). NAMPT is the rate-limiting enzyme in this pathway (Revollo, Grimm et al. 2004) and it can be found as an extracellular form (eNAMPT) and intracellular form (iNAMPT) (Houtkooper, Cantó et al. 2010). The iNAMPT is located in the nucleus, cytosol and mitochondria (Kitani, SachikoOkuno et al. 2003, Yang, Lavu et al. 2006, Yang, Yang et al. 2007, Nakagawa, Lomb et al. 2009), although its location in the mitochondria is still largely debated (Pittelli, Formentini et al. 2010, Stefano and Conforti 2013).

[4] The NAMPT will be briefly presented in this chapter. NAMPT characterization, functions and its importance in physiological processes will be further discussed in the chapter dedicated to this protein. During my PhD project, several interesting results linked to NAMPT were found.

③ Finally, if cells are unable to metabolize both NA and NAM, the NAD^+ synthesis can be assured by the NR metabolite (Nikiforov, Dölle et al. 2011, Grozio, Sociali et al. 2013, Nikiforov, Kulikova et al. 2015). The NR salvage pathway, as the name said, converts NR into NMN by the nicotinamide riboside kinases (NKR). Then, as in the other pathways, NMN is adenylated into NAD^+ by NMNAT. In human, two NKR isoforms, NKR1 and NKR2, were identified (Bieganowski and Brenner 2004). Both enzymes are able to metabolize NR and nicotinic acid riboside (NAR), suggesting that these substrates are also NAD^+ precursors vitamins as NA and NAM (Tempel, Rabeh et al. 2007). NKR1 is ubiquitously expressed in all tissues, while NKR2 is expressed in the heart, skeletal muscle and liver (Cantó, Houtkooper et al. 2012, Stefano and Conforti 2013). Finally, NR and NAR are also produced in mammalian cells and then excreted to be transported to other cells (Kulikova, Shabalin et al. 2015). The uptake of theses substrates is through specific transporters such ENTs (Nikiforov, Dölle et al. 2011, Nikiforov, Kulikova et al. 2015).

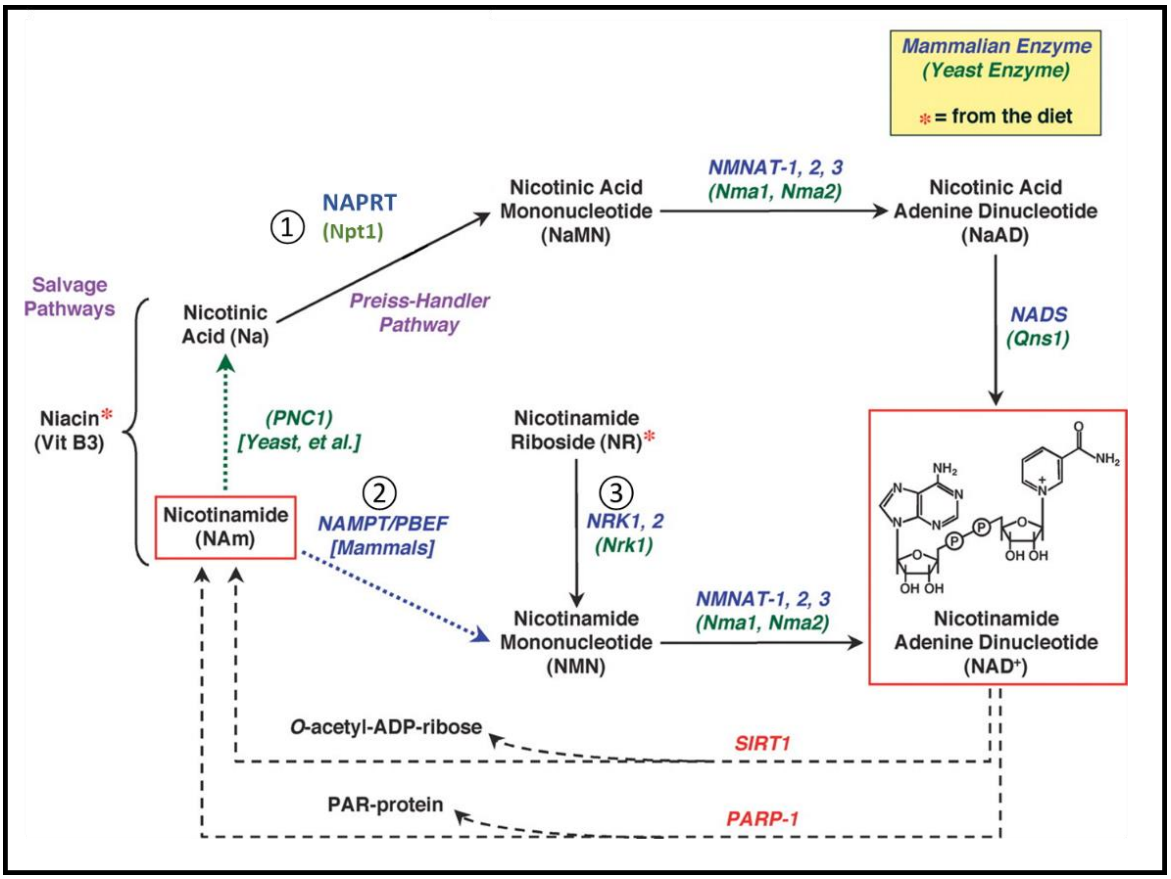


Figure 29. NAD⁺ salvage biosynthetic pathways. NAD⁺ salvage biosynthesis can be performed by three different pathways depending of the substrate. The ① NA salvage pathway that converts Na into NAMN by NAPRT, the ② NAM salvage pathway, where NAM is used as a substrate for NAMPT to produce NMN and ③ NR salvage pathway where NR is converted into NMN by NKR. Then, the intermediates products are adenylated into NAD⁺ by NMNAT, except NAMN that first is converted into NAAD by NMNAT and then into NAD⁺ by NADS. The enzymes represented in blue are the mammalian enzymes whereas the enzymes indicated in green and present in yeasts. The * indicates the substrates that are acquired from the diet. The dashed arrows represent reactions performed by the NAD⁺-dependent proteins such PARP-1 and SIRT-1, produces NAM that at the end of the reaction which is automatically recycled by NAMPT into NMN (adapted from Kim *et al.*, 2005).

The NAD⁺ pools are consumed and resynthesize several times in a day. Under normal conditions, NAD⁺ biosynthesis is affected by the availability of the precursors, which can alter the rate of NAD⁺ production.

1.3. NAD^+ cell distribution

The intracellular levels of NAD^+ are generally between 0.2 and 0.5 mM, depending on the cell type or tissue, but these levels can be increased up to ~2 fold in response to physiological stimuli (Cantó, Menzies et al. 2015). NAD^+ concentration may mirror the specific requirements for NAD of different cell types and tissues (Stefano and Conforti 2013). In particular, the liver seems to be the major core of the NAD metabolism since all the NAD^+-synthesis enzymes are expressed in the liver. In this organ, all the NAD^+ precursors can be converted to produce NAD^+. The liver might be a reservoir of NAD^+ precursors by extracting them from dietary sources and converting them into other NAD precursors that are, then, excreted into the bloodstream (Chiarugi, Dölle et al. 2012). The NAD^+ plasma concentration is between 10 and 50 nM (O'Reilly and Niven 2003, Stefano and Conforti 2013).

In mammalian cells, the NAD pools are compartmentalized, and the compartments are determined by the subcellular localization of the NMNATs isoforms (Stefano and Conforti 2013). Three major pools of NAD are found in the nucleus (where NMNAT1 is expressed), in the cytosol (where NMNAT2 is expressed) and in the mitochondria (where NMNAT3 is expressed). NAD pools compartments might also be determined by specific metabolic needs (Cantó, Menzies et al. 2015). Actually, the mitochondria are the largest NAD pool compartments (Nikiforov, Dölle et al. 2011, Stefano and Conforti 2013), where NAD^+ content is $\geq$ 250 µM (Yang, Yang et al. 2007, Nakagawa, Lomb et al. 2009, Cantó, Menzies et al. 2015). Numerous biological processes, where NAD plays an important role, such as oxidative phosphorylation and ATP biosynthesis are performed in the mitochondria, explaining why mitochondria is the largest NAD pool in cells (Yang, Yang et al. 2007). The NAD pool in the nucleus is around 70 µM, and most of the NAD^+-dependent reactions occurs in this compartment. NAD^+ biosynthesis performed in the nucleus directly supplies the NAD^+ levels necessary for NAD^+-dependent reactions. Interestingly, a relationship between PARP-1 and NMNAT 1 was highlighted: during DNA damage response, PARP-1 is activated, leading to the PARylation of NMNAT1 that is recruited to the damage site. There, NMNAT1 bind to PARP-1 and directly provides the NAD^+ necessary for the PARP-1 functions without completely depleting the NAD^+ level (Berger, Lau et al. 2007, Zhang, Berrocal et al. 2012).

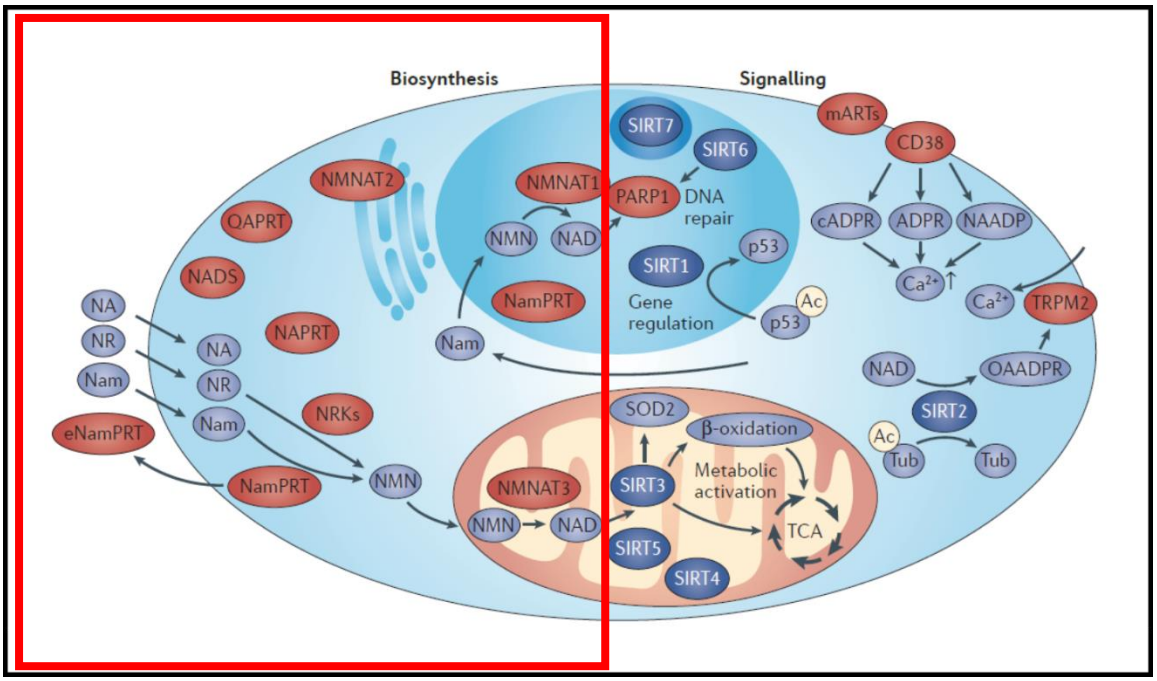


Figure 30. Subcellular compartmentation of NAD synthesis. The subcellular distribution of NAD pools and the NAD-synthesis enzymes in the mammalian cells are shown in the left side (red square). The major NAD pool are in the mitochondria, nucleus and cytosol. In these same compartments are present the NAD^+-synthesis enzymes such NAMPT and NMNAT, that are determining the subcellular NAD pool distribution (adapted from Chiarugi *et al.*, 2012).

It seems that the different compartments (cytosol, nucleus and mitochondria) expressed both NAMPT (although the presence in NAMPT in the mitochondrial still controversial) and NMNATs, thus, each compartment have autonomous NAD^+ biosynthesis mainly through the salvaging of NAM, the end product of NAD^+-dependent enzymes (Houtkooper, Cantó et al. 2010).

2. NAD^+-dependent enzymes

2.1. Sirtuins

Sirtuin proteins, are NAD^+-dependent enzymes that are homologous to the yeast silent information regulator 2 (Sir2). This factor was identified as regulator gene silencing that promotes lifespan in yeasts (North and Verdin 2004, Guarente 2007, Haigis and Sinclair 2010). In mammals, seven proteins (SIRT1-SIRT7) belonging to the Sirtuin family were identified based on the presence of a highly conserved catalytic domain. The N- and C-terminal domains

vary among the different members of this family accounting for their subcellular localization, enzymatic activity and binding targets (Table 2). SIRT1, SIRT6 and SIRT 7 are located in the nucleus, whereas SIRT7 was also found in the nucleolus (Shashi, Nirupama et al. 2013). SIRT2 is a cytoplasmic protein, but can be located in the nucleus in G2/M phase transition of the cell cycle (Vaquero, Scher et al. 2006), and SIRT3-SIRT5 are present in the mitochondria (Frye 2000, Michishita, Park et al. 2005, Verdin, Hirschey et al. 2010). However, these proteins can be translocated to other compartments under specific conditions (Vaquero, Scher et al. 2006, Iwahara, Bonasio et al. 2012). The main function of this family is the NAD^+-dependent deacetylation of histones and target proteins, which leading to the liberation of NAM and O-acetyl-ADP-ribose. Some sirtuins can present MARylation (SIRT4), lipoamidase (SIRT4, SIRT6), demalonylase and desuccinylase (SIRT5) activity (Liszt, Ford et al. 2005, Haigis, Mostoslavsky et al. 2006, Michishita, McCord et al. 2008, Du, Zhou et al. 2011, Mathias, Greco et al. 2014). Due to its functions, sirtuins are involved in several biological processes, such as DNA repair, post-transcriptional modifications regulation, lifespan regulation, energy metabolism and carcinogenesis (Guarente 2007, Imai and Guarente 2010, Houtkooper, Pirinen et al. 2012, Mei, Zhang et al. 2016).

Sirtuin	Class	Localization	Activity	Targets
SIRT1	I	Nucleus, cytosol	Deacetylation	PGC1α, FOXO1, FOXO3, p53, Notch, NF-κB, HIF1α, LXR, FXR, SREBP1c and more
SIRT2	I	Cytosol	Deacetylation	Tubulin, PEPCK, FOXO1, PAR3
SIRT3	I	Mitochondria	Deacetylation	LCAD, HMGCS2, GDH, OXPHOS complexes, SOD2, IDH2 and more
SIRT4	II	Mitochondria	ADP-ribosylation	GDH
SIRT5	III	Mitochondria	Deacetylation, demalonylation, desuccinylation	CPS1
SIRT6	IV	Nucleus	Deacetylation, ADP-ribosylation	H3K9, H3K56
SIRT7	IV	Nucleolus	Unknown	Unknown

Table 2. Localization and target of the different Sirtuins (adapted from Houtkooper et al., 2012).

❶ SIRT1, the most studied enzyme from this family, was demonstrated to regulate several physiological processes, such metabolism, DNA repair, aging, telomere length and carcinogenesis (Oberdoerffer, Michan et al. 2008, Palacios, Herranz et al. 2010, Zhang, Chen et al. 2014, Davinelli, Maes et al. 2016). SIRT1 can also regulate both mitochondrial function and biogenesis by deacetylating peroxisome proliferator-activated receptor-γ, co-activator 1α (PGC-1α), an important protein in the expression of mitochondrial genes, leading to its activation (Chalkiadaki and Guarente 2012). Overactivation of SIRT1 offsets the development of diabetes, obesity and other metabolic syndromes by mitochondrial activation or mitochondrial biogenesis through the activation of PGC-1α (Picard, Kurtev et al. 2004, Lagouge, Argmann et al. 2006). Regarding the DNA repair, SIRT1 deacetylates histones, such as H1 and H4, which contributes to the recruitment of DNA damage repair factors (Imai, Armstrong et al. 2000, Vaquero, Scher et al. 2004). SIRT1 promotes HR by deacetylating WRN, a RECQ helicase (Li, Casta et al. 2008, Uhl, Csernok et al. 2010). NHEJ can also be promoted by deacetylation of KU70, which enhances the Ku70-dependent DNA repair (Jeong, Juhn et al. 2007). Finally, SIRT1 can be recruited to the DSBs in an ATM kinase dependent manner (Dobbin, Madabhushi et al. 2013). In the carcinogenesis process, SIRT1 has a double role. SIRT1 can act as both a tumorigenic factor by regulating the transcription factors P53 and FOXO, leading to the reduction of their pro-apoptotic effects (Vaziri, Dessain et al. 2001, Brunet, Sweeney et al. 2004, Solomon, Pasupuleti et al. 2006, Hori, Kuno et al. 2013). Also, SIRT1 expression is increased in several human cancers, such hepatocarcinoma (Hao, Zhu et al. 2014), ovarian carcinoma (Mvunta, Miyamoto et al. 2017), breast cancer (Kuo 2013), colorectal carcinoma (Chen, Sun et al. 2014) and prostate cancer (Huffman, Grizzle et al. 2007). In breast cancer, SIRT1 also regulates the oncogenic signaling mediated by the estrogen receptor α (ERα) (Santolla, Avino et al. 2015).

❷ SIRT2 is a cell cycle regulator (Inoue, Hiratsuka et al. 2007, Nie, Li et al. 2014), since it is implicated in the mitotic progression and genome integrity (Dryden, Nahhas et al. 2003, Kim, Vassilopoulos et al. 2011). During mitosis, SIRT2 is responsible for the deacetylation of co-activators of anaphase, which promotes the APC complex (Kim, Vassilopoulos et al. 2011). SIRT2 is also essential for the ATR kinase pathway, an important pathway for genome stability, by playing a role in replication stress through deacetylating both CDK9 and ATR-interacting protein (ATRIP) (Zhang, Park et al. 2013, Zhang, Head et al. 2016, Head, Zhang et al. 2017).

SIRT2 expression is decreased in glioma, liver cancer and gastric adenocarcinoma and increased in non-small cell lung cancer (NSCLC) (Grbesa, Pajares et al. 2015), acute myeloid leukemia (AML) (Deng, Ning et al. 2016) and prostate cancer (Hou, Chen et al. 2012). SIRT2 might be linked to cancer metabolism and promotes tumor growth by regulating the activities of HIF-α (Lim, Lee et al. 2010), phosphoglycerate mutase (PGAM) and glucose-6-phosphate dehydrogenase (G6PD), where the latter enzymes being important for the glycolysis and biomass production (Wang, Zhou et al. 2014, Xu, Li et al. 2014, Xu, Wang et al. 2016).

❸ SIRT3 is implicated in the regulation of several aspects of the mitochondrial metabolism. It is involved in the regulation of ROS production by either activating manganese SOD (MnSOD) (SOD2) (Tao, Coleman et al. 2010) or by deacetylating factors present in the electron transport chain, which enhances antioxidants defenses (Sundaresan, Gupta et al. 2009) or represses ROS production (Bell, Emerling et al. 2011, Finley, Carracedo et al. 2011) . SIRT3 also deacetylates Ku70, indicating that this enzyme could be implicated in Ku70-dependent DNA repair (Sundaresan, Samant et al. 2008). In cancer, SIRT3 might function as a tumor suppressor by its ability to regulate ROS production. In fact, in oral squamous cell carcinoma (OSCC), overactivation of SIRT3 inhibits cell growth and decreases the ROS basal levels (Chen, Chiang et al. 2013).

❹ SIRT4 mRNA was found to be reduced small cell lung carcinoma, gastric cancer and breast cancer (Huang, Cui et al. 2015, Fu, Dong et al. 2016, Shi, Liu et al. 2016). Also, this lower expression of SIRT4 is associated with shorter survival in patients with lung cancer (Fu, Dong et al. 2016). A new role of SIRT4 as a stress resistant factor in cancer was demonstrated, where disruption of SIRT4 sensitizes tumor cells to DNA damage and ER stress (Jeong, Hwang et al. 2016).

❺ SIRT5 suppresses cellular respiration by inhibiting pyruvate dehydrogenase complex (PDC) and succinate dehydrogenase activities, probably facilitating cancer cells to performed aerobic glycolysis (Park, Chen et al. 2013). This repression also facilitates cell growth and drug resistance in NSCLC (Lu, Zuo et al. 2014). SIRT5 also plays a role as a tumor suppressor specially in oral squamous cell carcinoma (OSCC) and endometrial carcinoma (Lai, Lin et al. 2013, Bartosch, Monteiro-Reis et al. 2016).

❻ Interestingly, all SIRT6 functions were revealed from a SIRT6 knock-out mice. It was demonstrated that SIRT6 deficiency leads to genomic instability and hyper sensibility to certain genotoxic stress, indicating a role of SIRT6 in DDR (Mao, Hine et al. 2011). This is also supported by the fact that SIRT6 is implicated in the recruitment and stabilization of DNA-PKcs in DSBs, promoting the C-NHEJ repair pathway (McCord, Michishita et al. 2009). Moreover, SIRT6-mediated MARylation of PARP-1 on K521 stimulates PARP-1 activity, promoting either HR and/or NHEJ pathways (Mao, Hine et al. 2011). In carcinogenesis, SIRT6 is known as a tumor suppressor due to its role in cancer metabolism. SIRT6 represses aerobic glycolysis (Kim, Xiao et al. 2010, Wu, Seto et al. 2015) and absence of SIRT6 expression, leads to tumor formation (Sebastián, Zwaans et al. 2012). SIRT6 expression is decrease in pancreatic, liver, colon and small cell lung cancer (Lai, Lin et al. 2013, U., Kerstin et al. 2013, Zhang 2013, Kugel, Sebastián et al. 2016, Zhu, Yan et al. 2018), but not in prostate and breast cancers, where its expression is increased (Khongkow, Olmos et al. 2013, Liu, Xie et al. 2013).

❼ SIRT 7 plays a role in DNA repair by deacetylating the histone H3 at the DNA damage sites, which promotes the recruitment of 53BP1, a NHEJ repair factor (Paredes and Chua 2016, Vazquez, Thackray et al. 2016). SIRT7 is overexpressed in hepatocellular carcinoma, gastric and colorectal cancers (Kyu, Heon et al. 2013, Yu, Ye et al. 2014, Zhang, Chen et al. 2015, Lee, Jung et al. 2016, Tang, Lu et al. 2017, Deng, Wang et al. 2018). Notably, the overexpression of SIRT7 protects cancer cells from genotoxic stress and enables cell survival probably by enhancing genome integrity in cancer cells (Kiran, Oddi et al. 2015).

2.2. PARPs

The PARP superfamily was already presented in the previously chapter (chapter 3). So, I will briefly describe this family.

The PARP superfamily comprises 17 members in human and 16 members in mice (Hottiger, Hassa et al. 2010). PARPs are implicated in several physiological processes, such DNA repair, cell cycle regulation, gene transcription, inflammation, cell death and carcinogenesis (Bai 2015). PARP-1, is the most studied protein from this family, is essential for DNA repair and others processes and its inhibitor is widely used in cancer therapy (Mullard 2014).

The PARP proteins and their main functions are summarized in the Figure 31.

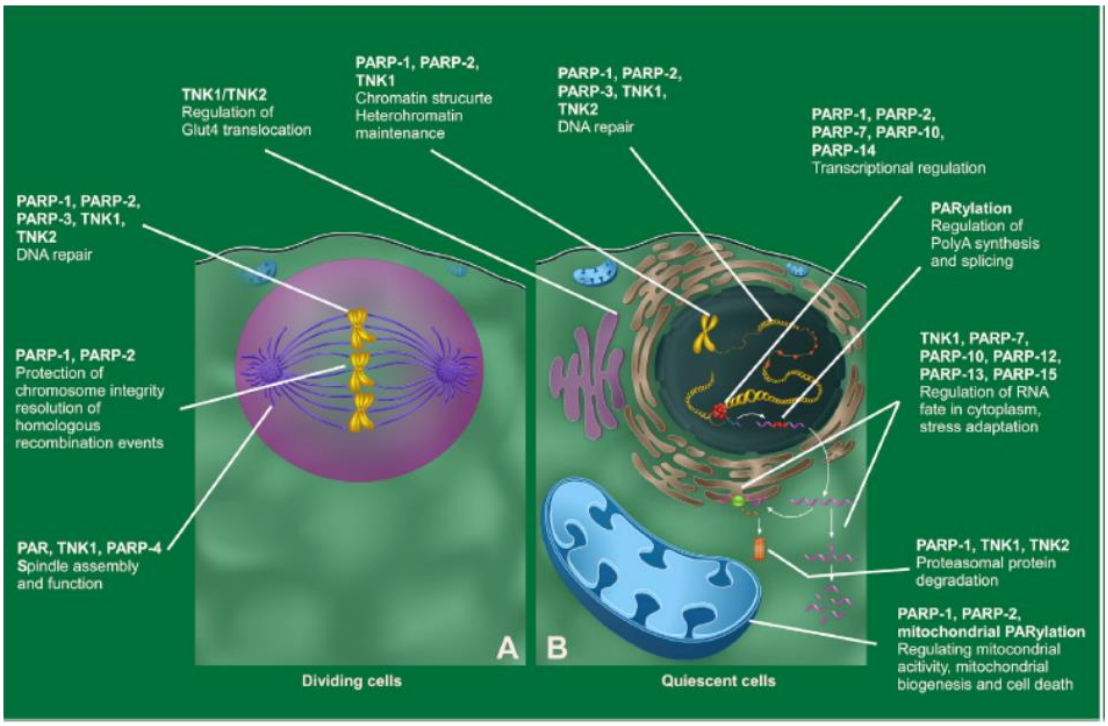


Figure 31. The different PARP proteins and their main functions. **A)** The PARP functions in dividing cells and **B)** the functions of PARP proteins in quiescent cells (from Bai *et al*., 2015).

2.3. Cyclic ADP-ribose synthetases

Cyclic ADP-ribose synthetases (cADPR synthetases), also known as NAD glycohydrolases, are another family of NAD^+-dependent enzymes that produce Ca^{2+}-mobilizing metabolites (cyclic adenosine diphosphate-ribose (cADPR), ADPR and NAAD phosphate (NAADP)) from both NAD^+ and NADP (Han, Lee et al. 1996). Only two enzymes were identified in this family, CD38 and its homolog CD157, that were first described as plasma membrane antigens in T lymphocytes and thymocytes (Valeria, Gianluca et al. 2013). These enzymes were also found in non-lymphoid tissues such as muscle, liver and brain (Aksoy, White et al. 2006) and have both intra- and extracellular enzymatic activity (Czura and Czura 2006, Lee 2012, Zhao, Lam et al. 2012). CD38 knockout mice present an increase in NAD^+ levels in several tissues (Young, Choleris et al. 2006), whereas overexpression of CD38 leads to the reduction of NAD^+ content (from 10-30-fold decrease). The reduction of CD38 expression is implicated in energy metabolism and antioxidant defenses (Hu, Wang et al. 2014), suggesting that this enzyme

mediates continuous NAD^+ consuming and might contribute to NAD homeostasis (Chiarugi, Dölle et al. 2012). CD38 overexpression has also be reported as a negative prognostic marker in chronic lymphocytic leukemia (CLL) (Dürig, Naschar et al. 2002, Pittner, Shanafelt et al. 2005, Malavasi, Deaglio et al. 2011). Recently, it has been shown that CD38 overexpression leads to tumor growth in lung cancer (Bu, Kato et al. 2018) and enhances cell proliferation and apoptosis inhibition in cervical cancer (Shan, Songshu et al. 2017). Although no specific CD38 inhibitors were found, CD38-specific antibodies were found to induce cell death in neoplastic B cell lines and, thereby, were used for clinical trials (van de Donk, Janmaat et al. 2016) where one antibody, daratumumab (DARZALEX®, Janssen Biotech, Inc.) was already approved by FDA for the treatment of multiple myeloma (Bhatnagar, Gormley et al. 2017, Frerichs, Nagy et al. 2018).

3. NAD^+ metabolism as a therapeutic target

NAD^+ is a cofactor for numerous enzymes that are implicated in cellular metabolism, cell bioenergetics and oxidative stress. NAD^+ deficiency was demonstrated to be implicated in several pathophysiological processes, such aging, diabetes, neurodegeneration, obesity and cancer (Figure 32).

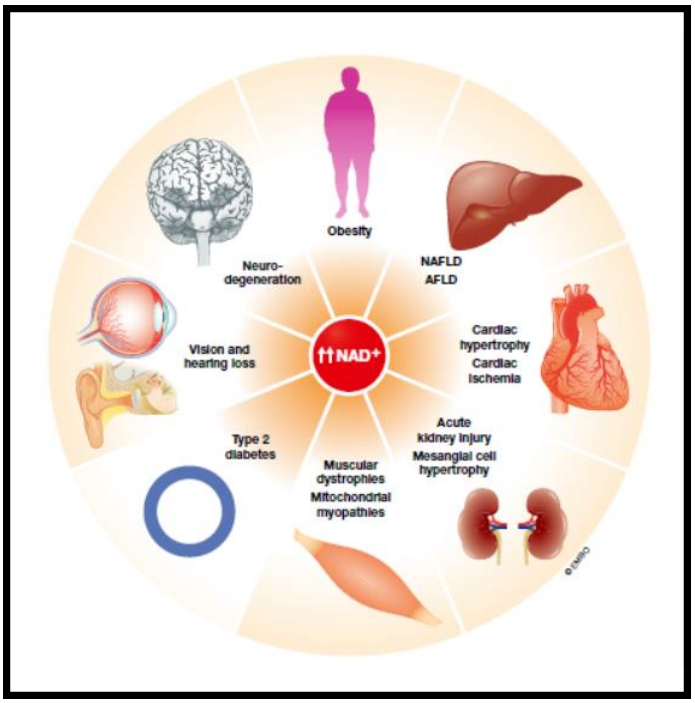


Figure 32. Human diseases in which NAD^+ deficiency is implicated, and its boosting used as therapeutic treatment (Katsyuba and Auwerx, 2017)***.***

3.1. NAD^+ in nutrition: supplementation of NAD precursors

The first evidence of a dietary deficiency of NAD^+ or NAD precursors was the development of Pellagra, a disease characterize by dermatitis, diarrhea and dementia that was thought to be an infectious disease. This disease was very common in rural poor regions in the United States (Bogan and Brenner 2008). In 1914, the epidemiologist Joseph Goldberger confirmed that Pellagra was a consequence of NAD precursors deficiency by substituting a corn-based alimentation by an animal-based nutrition, such eggs, milk and meat (these aliments contain higher amounts of NAM and NA) (Goldberger 2006). Several years later, the biochemist Conrad Elvehjem (1937) identified NAM and NA, as having antipellagragenic activity (Elvehjem, Madden et al. 1937). As observed in the case of pellagra, NAD precursors or NAD^+ itself can be implicated in other metabolic disorders. *Thus, I will briefly present the benefits and drawbacks of NAD precursors supplementation.*

Niacin or Vitamin B3 are the collective names of NAM and NA. NA has been widely used for the treatment of hyperlipidemia (Altschul, Hoffer et al. 1955, R. Crouse 1996, Digby, Ruparelia et al. 2012). Although dietary niacin is not associated with side effects, pharmacological NA induces negative secondary effects (Elhassan, Philp et al. 2017). Indeed, NA is a ligand for the G-protein-coupled receptor GPR109A. This protein is expressed on Langerhans cells, and mediates the production of prostaglandin, a hormone-like that functions as a vasodilator and inhibits the aggregation of blood platelets (Benyó, Gille et al. 2006). NA binds to GPR109A, leading to vasodilatory effects, such as hypotension, and headaches (Benyó, Gille et al. 2005, Bays and Rader 2009, Kamanna, Ganji et al. 2009, Rezq and Abdel-Rahman 2016). A NA analog, acipimox was discovered, but it is also a ligand for GPR109A, and, thus, presents the same side effects than NA treatment (Pike 2005).

NAM is the preferential NAD precursor for NAD^+ synthesis in mammalian cells. This metabolite has been used for the treatment of several metabolic disorders such diabetes mellitus with minimal side effects (Knip, Douek et al. 2000, Faris 2009). NAM does not binds to GPR109A protein (Pike 2005) but high doses of NAM can be toxic, especially for the liver (Knip, Douek et al. 2000). Moreover, long-term use of NAM inhibits the activity of sirtuins proteins (Bitterman, Anderson et al. 2002, Avalos, Bever et al. 2005).

NR is now recognized as NAD precursor vitamins. NR administration leads to the increase in NAD^+ levels, in a dose-dependent manner and no side effects have been reported to date. Several studies of NR supplementation were performed in mice. It was observed that NR treatment is beneficial in high-fat-diet (HFD)-fed mice. Actually, NR protects mice from weight gain, leads to insulin sensitivity and increases mitochondrial content in skeletal muscle and brown adipose tissue. Moreover, this metabolite increases the endurance and tolerance to cold in these mice (Cantó, Houtkooper et al. 2012). NR has been also associated to therapeutic effects in muscle disorders and in mitochondrial myopathies (Cerutti, Pirinen et al. 2014, Khan, Auranen et al. 2014). Finally, NR can play a beneficial role in neurogenerative diseases, since the treatment with this metabolite in an Alzheimer's diseases mice model (Tg2576) demonstrated an improvement in the progression of this disease (Gong, Pan et al. 2013).

Finally, the NMN, an intermediate product of the NAD^+ salvage pathway, was shown to increase the NAD^+ levels both *in vivo* and *in vitro*. NMN administration also has a therapeutic effect in several disorders. NMN treatment enhances insulin secretion and action by restoring NAD^+ biosynthesis, which leads to SIRT1 activation (Caton, Kieswich et al. 2011, Yoshino, Mills et al. 2011). NMN treatment also inhibits age-associated disorders, such as inflammation (Mills, Yoshida et al. 2016). This metabolite also improves several neuronal functions (Long, Owens et al. 2015, Wang, Hu et al. 2016, Wei, Kong et al. 2017, Yao, Yang et al. 2017). However, *in vitro* experiments that NMN local accumulation due to supplementation of high doses of NMN leads to local accumulation of NMN, in the brain and could, thus, promote the development of Wallerian degeneration (Di Stefano, Nascimento-Ferreira et al. 2014).

3.2. NAD^+ and Metabolic diseases

3.2.1. NAD^+ and obesity

Obesity is a common Western countries disease, characterized by the pathological expansion of adipose tissue and its dysfunction. Obesity leads to the deregulation of the production adipose tissue - secreted proteins, insulin-sensitizing adipokines such adiponectin, lipids, pro-inflammatory cytokines, free fatty acids (FFA) and other metabolites. This disease is also

associated with the development of metabolic disorders, such as multi-organ insulin resistance, in which the latter is implicated in the development of type 2 diabetes, atherogenic dyslipidemia, non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases. Adipose tissue can maintain the functional integrity by modulating the production of the proteins and metabolites mentioned above. However, disruption of the adipose tissue is involved in metabolic disorders not only in this tissue but in other organs such liver and skeletal muscle (Yamaguchi and Yoshino 2017).

Recently it was reported that NAD^+ metabolism regulate the adipose tissue function and the insulin sensitivity. In fact, in HFD-fed mice, a decrease in NAMPT-mediated NAD^+ biosynthesis leads to an increase in phosphorylation of Ser273 of peroxisome proliferator-activated receptor gamma (PPARγ) (Stromsdorfer, Yamaguchi et al. 2016) (Figure 33), an important regulator of the adipocyte metabolism (Schug and Li 2011). The phosphorylation of this serine impairs the PPARγ activity leading to the disruption of genes where its expression is altered in obesity, including adiponectin, that is associated to the insulin resistance in obesity (Yadav, Kataria et al. 2013). This study also showed that NAMPT-mediated NAD^+ synthesis regulates the phosphorylation of CDK5, a regulator of phosphorylation of PPARγ at ser273 (Choi, Banks et al. 2010). However, the regulation mechanism is unclear, probably through modulation of SIRT1 activity, since it was found that in SIRT1 -/- mice an increase in the acetylation of lys293 leads to the phosphorylation of ser273, impairing PPARγ (Qiang, Wang et al. 2012, Mayoral, Osborn et al. 2015). Nevertheless, administration of NMN replenish the effects of the decrease of NAD^+ concentration in the adipose tissues. This study was important because it highlighted a new pathophysiological role of NAD^+ as a regulator of PPARγ in adipocytes (Stromsdorfer, Yamaguchi et al. 2016).

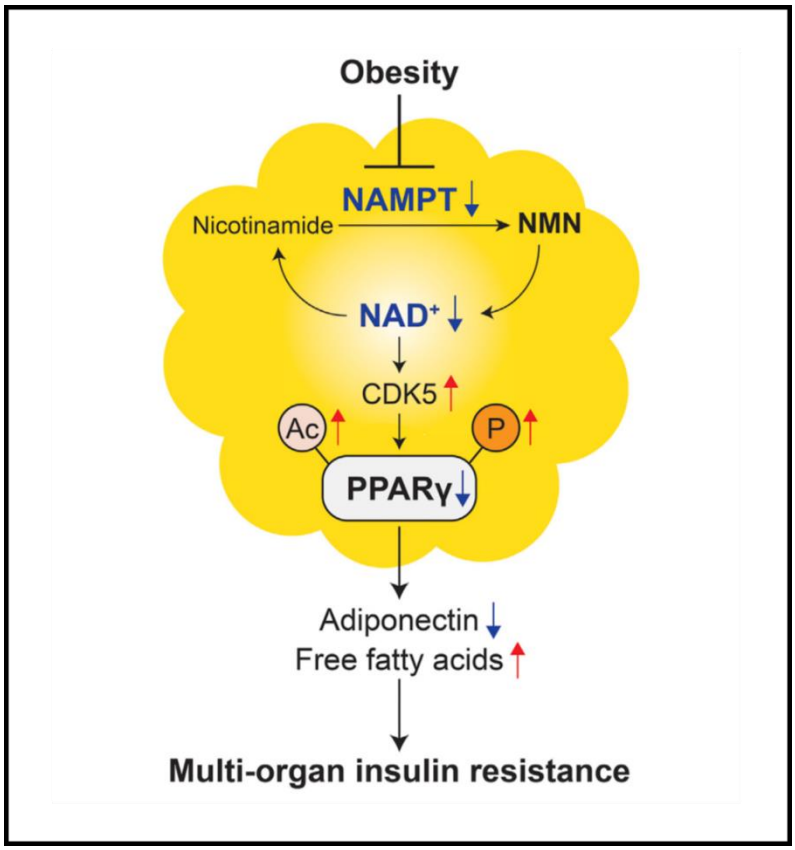


Figure 33. Mechanism of adipose tissue dysfunction and subsequent insulin resistance. Decrease in NAMPT activity leads to reduced levels of NAD^+ and, thus, to the activation of CDK5 and phosphorylation of PPARγ which, in turn, leads to a reduced production of adiponectin, that is essential for insulin sensitivity, and to an increase in the production of free fatty acids that leads to insulin resistance in tissues (adapted from Stromsdorfer *et al.*,2016).

NAD^+ deficiency was also found in other models of obesity, which might result from reduced activity of NAMPT due to a high fat alimentation, and increased activity of PARP-1. In fact, a study in human patients, showed that the expression of NAMPT, SIRT1, SIRT3 and SIRT7 were decreased whereas the activity of PARP-1 was increased in these obese human patients compared to controls (Zhang and Ying 2018).

All these studies highlighted the pathophysiological consequences of NAD^+ deficiency in obesity. Thus, increasing the NAD^+ availability by either NAD precursors supplementation or overexpression of enzymes that participates in the NAD^+ biosynthesis is an excellent therapeutic approach to prevent obesity progression.

3.2.2. NAD^+ and diabetes

Type 2 diabete mellitus (T2DM) is one of the most common diseases in the XXI century around the world. The development of this disease can lead to important health problems such as heart disease, kidney disease, neuropathy, blindness, amputations (most common limb amputations) and stroke (Chawla, Chawla et al. 2016). T2DM is known to be a consequence of both obesity and sedentary life style but is primary cause is unknown (Lowell and Shulman 2005). However, it is established that insulin resistance plays a role in the early set of this disorder whereas progression of TD2M leads to defects in insulin secretion from pancreatic β cells and development of hyperglycemia (Lowell and Shulman 2005). Moreover, the insulin resistance, which is mentioned as state of tissues presenting a weakened response to the normal insulin levels (Saltiel 2000), is due to defective functions and structure of mitochondria due to intracellular accumulation of fatty acid metabolites and ROS (Houstis, Rosen et al. 2006, Holland, Brozinick et al. 2007, Koves, Ussher et al. 2008, Toledo 2014). These alterations are common in skeletal muscle and liver. Skeletal muscle is a tissue that presents a high concentration of mitochondria and is dependent on oxidative phosphorylation for energy production and, together with the liver, is responsible for the glucose homeostasis. The transition from most insulin-responsive to insulin-resistance organs accounts for the major alterations seen in T2DM (Kelley, He et al. 2002, Lowell and Shulman 2005).

The mechanism for insulin-resistance in these organs are slightly different (Figure 34). In muscle (Figure 34A), reduced mitochondrial fatty acid oxidation due to mitochondrial defect or to reduced mitochondrial content (①) is responsible for the intracellular accumulation of long chain coenzyme A (LCCoA) and diacylglycerol (DAG) (②). These metabolites activate PKCs (③) that consequently induces a serine/threonine kinase activity cascade (④). The induction of this cascade leads to the increase in phosphorylation of the Ser residues (Ser 302 and 307) of insulin receptor substrate-1 (IRS-1) (Werner, Lee et al. 2004), that inhibits the phosphorylation of IRS-1 Thr residues (Tyr 1158, 1162, and 1163) by the insulin receptor (Draznin 2006). This, ultimately, leads to inhibition of phosphatidyl inositol 3-kinase (PI 3-kinase) (⑤) which, in turn, suppresses the insulin-stimulated glucose transport (⑥) (Okada, Kawano et al. 1994), and reduced glycogen synthesis (⑦) (Shulman 2000, Yu, Chen et al. 2002, Morino, Petersen et al. 2006, Savage, Petersen et al. 2007). In the liver (Figure 34B),

accumulation of DAG due to increased lipogenesis and/or reduced fatty acid mitochondrial oxidation (❶) activates the PKC-ε (❷) which, in turn, binds to insulin receptor and inhibits the Thr phosphorylation of both IRS-1 and IRS-2 (❸). This is leads to the weakened activation of the PI 3-kinase and AKT2 (❹). The defective activation of AKT2 (that also occurs in the muscle), results in a reduced phosphorylation of GSK3 and FOXO (❺) leading to a reduced insulin-stimulated glycogen synthesis (❻) and a lower inhibition of the hepatic gluconeogenesis (❼) (Samuel, Liu et al. 2004, Morino, Petersen et al. 2006, Savage, Petersen et al. 2007).

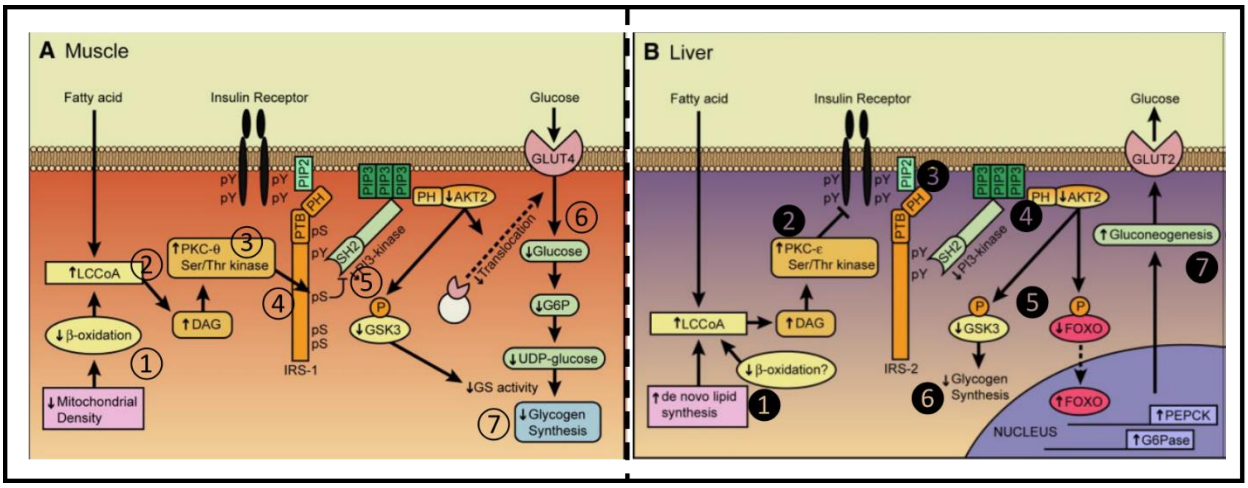


Figure 34. Mechanism of insulin-resistance in skeletal muscle (A) and liver (B). **A)** Reduced β-oxidation due to mitochondrial dysfunction leads to the accumulation of LCCoA and DAG, resulting in the activation of PKC that inhibits the phosphorylation of Thr residues (Tyr 1158, 1162, and 1163) of IRS-1 by the insulin receptor. This leads to the lower activation of PI 3-kinase and AKT2. Weakened activation of AKT2 is responsible for lower phosphorylation of GSK3 that results in reduced insulin-stimulated glucose transport and glycogen synthesis. **B)** Increased lipogenesis activity and reduced β-oxidation leads to the accumulation of LCCoA and DAG that in turn activates PKC. This lead to the inhibition of the same Thr residues phosphorylation of IRS-1 and IRS-2 by the insulin receptor and, consequently, to the lower activation of PI 3-kinase and AKT2. The reduced activation of AKT2 leads to the decrease phosphorylation of GSK3 resulting in the reduced glycogen synthesis and weakened gluconeogenesis inhibition (from Savage *et al.*, 2007).

The insulin resistance can also result from impaired oxidative phosphorylation (OXPHOS). A studied performed in mice demonstrated that a defect in the activation of CRIF, a protein essential for the production of mtDNA that encodes OXPHOS proteins, leads to the reduced activity of OXPHOS, causing inflammation and insulin resistance (Kim, Kwon et al. 2012, Ryu, Kim et al. 2013)

In addition, the development of hyperglycemia as a consequence of a defect in insulin secretion from pancreatic β cells is due to the weakened adaptation of pancreatic β cells to meet the body increase in insulin demand. In obese individuals, the pancreatic β cells can both adapt to the increased demand of insulin by expansion of the β cells mass and by maintaining the glucose response of these cells, preventing the development of T2DM. However, inadequate β cells expansion to compensate the increase in insulin demands or decreased response to glucose, leads to the weakened insulin secretion by pancreatic β cells, resulting in hyperglycemia (Rhodes 2005).

The role of NAD^+ metabolism in the T2DM development is mainly due to impaired NAMPT-mediated NAD^+ biosynthesis which, in turn, leads to a reduced activity of SIRT1 (S. and J. 2013). The decrease in SIRT1 activity leads to 1) insulin resistance due to reduced deacetylation of PGC-1α, which is responsible for mitochondrial biogenesis and activation of genes implicated in fatty acid oxidation process (Gerhart-Hines, Rodgers et al. 2007, Xu, Bai et al. 2013), 2) to overproduction of glucose levels by impaired mTorc2/Akt signaling, that is implicated in the regulation of gluconeogenesis (Wang, Kim et al. 2011), 3) defective hepatic glucose uptake (HGU) as consequence of a decrease deacetylation of glucokinase regulatory protein (GKRP), an enzyme required for glucose uptake (Watanabe, Inaba et al. 2018) and 4) weakened insulin secretion in pancreatic β cells (Revollo, Körner et al. 2007, do Amaral, Ueno et al. 2011, Luu, Dai et al. 2013).

Thus, increasing NAD^+ biosynthesis seems as an attractive approach for T2DM treatment. NMN supplementation in HFD-fed mice leads to an amelioration of glucose intolerance in pancreatic β cells, thus to an increase in insulin secretion, a decrease in lipogenesis and in an enhancement of hepatic insulin sensitivity (Yoshino, Mills et al. 2011, Stromsdorfer, Yamaguchi et al. 2016). In addition, treatment with acipimox, a SIRT1 activator, was shown to improve the tissue insulin sensitivity (Worm, Henriksen et al. 1994, Santomauro, Boden et al.

1999, Daniele, Eldor et al. 2014) and pancreatic β cells function when combined with dapagliflozin, a drug that is used for diabetes treatment (Merovci, Abdul-Ghani et al. 2016). Resveratrol treatment also leads to decrease glucose and insulin levels in patients that present TD2M (Brasnyó, Molnár et al. 2011, Crandall, Oram et al. 2012).

3.3. NAD^+ in Aging

It is well established that NAD^+ levels decrease during the aging process. Several studies performed in mice, showed that mice present age-related NAD^+ levels decrease in numerous organs, such as brain, liver, muscle, pancreas, adipose tissue and skin (Yoshino, Mills et al. 2011, Zhu, Lu et al. 2015, Can-Can, Xi et al. 2016, Mills, Yoshida et al. 2016, Zhang, Ryu et al. 2016). The NAD^+ deficiency during the aging process results from several mechanism that includes excessive consumption of NAD^+ by NAD^+-dependent proteins or by defects in the NAD^+ biosynthesis pathways (Zhang and Ying 2018).

❶ One of the mechanisms of age-related decrease in NAD^+ levels is due to accumulation of DNA damage. It is already established that ROS production and oxidative stress increase with age (Gil del Valle 2011). ROS lead to DNA damage that consequently activates DNA repair proteins such as PARP-1. Overactivation of PARP-1 results in depletion of NAD^+ (Braidy, Guillemin et al. 2011, Mouchiroud, Houtkooper et al. 2013), that leads to defective SIRT1 activity, resulting in a decreased deacetylation of FOXO-3A and DAF-16, leading to a reduced antioxidant production and, thus, to an increased in oxidative stress (Kobayashi 2005, Wook Oh, Mukhopadhyay et al. 2005, Sun, Chen et al. 2017). Treatment with PARP inhibitors has been reported to be sufficient to restore NAD^+ levels in aged organisms (Gomes, Price et al. 2013).

❷ Another mechanism resulting in age-related decrease in NAD^+ levels is a defect in NAD^+ biosynthesis pathway. Indeed, age-related decrease in NAMPT levels, results in a defective regeneration of NAD^+ concentration in cells (Yoshino, Mills et al. 2011). Overexpression of NAMPT or administration of NMN, the product of NAMPT, in aged mice are sufficient to increase the NAD^+ at the levels similar to that found in young mice (Yoshino, Mills et al. 2011). Furthermore, NAMPT is regulated by the circadian transcriptional activators BMAL-1 and

CLOCK that are the master regulators of the circadian clock machinery (Borut , Ramsey, Yoshino et al. 2009). Disruption of the circadian rhythm leads to defective NAMPT activity and, thus, reduced NAD^+ production (Brown, Schmitt et al. 2011, Mattis and Sehgal 2016). Oxidative stress and inflammatory cytokines such TNF-α reduces NAMPT and NAD+ levels in hepatocytes (Imai and Yoshino 2013). TNF-α can suppresses the BMLA-1/CLOCK complex in the liver (Cavadini, Petrzilka et al. 2007). Since both oxidative stress and TNF-α leads to the development of chronic inflammation during aging, they might be responsible for the decrease in NAMPT activity and the subsequent NAD^+ production and for the defective circadian clock machinery during aging (Imai and Guarente 2014). Finally, the activity of NMNAT3 and nicotinamide nucleotide transhydrogenase (NNT), enzymes that control the NAD^+ homeostasis in the mitochondria, has been reported to decrease during aging, leading to a decrease in mitochondrial NAD^+ levels, impairing SIRT3 activity (Jin, Youjeong et al. 2016).

❸ Defects of mitochondrial functions are the hallmark of the aging process. Reduced NAD^+ levels during aging, affect the activation of NAD^+ consuming proteins such as SIRT1. Weakened of SIRT1 activity leads to a reduced mitochondrial biogenesis through the decrease in deacetylation of PGC -1α, a SIRT1 substrate, resulting in the damage of the complex I of the electron transport chain (ETC), and in the decline of mitochondrial activity (Imai and Guarente 2014). In addition, reduced activity of SIRT1 is responsible of the reduction of mitochondrial gene expression in mice (Wang, Zhao et al. 2014). Moreover, weakened SIRT1 activity leads to the overexpression of HIF-1α acetylation. The pseudohypoxic state due to high levels of HIF-1α leads to the recruitment of c-myc that inactivates the promoter of the mitochondrial transcription factor TFAM, showing how the defective nuclear NAD^+ pools impaired the mitochondrial functions (Lim, Lee et al. 2010, Zwaans and Lombard 2014).

❹ Finally, complex I of the ETC is the major acceptor of the reduced form of NAD^+, NADH. Inactivation of the complex I by inhibition of one of its subunit, such as ndufs4, leads to increase in NADH levels and decrease in NAD^+ levels (Karamanlidis, Lee et al. 2013). Consequently, this leads to reduced SIRT3 activity, that is associated to the induction of a pseudohypoxic state that impairs mitochondrial function (Gomes, Price et al. 2013).

3.4. NAD^+ in neurodegeneration

Neurodegeneration is known as a progressive deterioration and loss function of neurons that ultimately leads to neurons death. The most common neurogenerative diseases are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), frontotemporal dementia and spinocerebellar ataxias. Some of these diseases lead to memory loss and cognitive defects that when aggravated results in loss of people's ability to do simple things like speaking, walking and even breathing (Bertram and Tanzi 2005). Axonal degeneration is considered as the early mechanism for neurodegenerative diseases. Axon degeneration is a controlled process that leads to axon self-destruction. It is already established that NAD^+ and enzymes that are implicated in its synthesis play a role in neuroprotection. This is well illustrated in the Wallerian degeneration, studied to elucidate the process of traumatic nerve injury and axon degeneration in both central and peripheral nervous system (CNS and PNS) (Rotshenker 2011). A mouse model called "Wallerian degeneration slow mice (WLD^S)" that contains a dominant mutation of the slow Wallerian degeneration (*WLDS*) gene (Lunn, Perry et al. 1989, Mack, Reiner et al. 2001) was found over 20 years ago. In these mice, it was observed that Wallerian degeneration was delayed. This phenotype was due to a overexpression of the WLDS protein, a chimeric protein, containing a short fragment of the ubiquitin factor, ube4b, and the full-length of NMNAT1 (Conforti, Tarlton et al. 2000, Fernando, Conforti et al. 2002), that involved in NAD^+ production in both *de novo* and salvage pathways. They also reported that the activity of NMAT1 leads to the NAD^+-mediated activation of SIRT1, that plays a role in neuroprotection (Rossi, Geiszler et al. 2018). NMNAT1 also prevent axon degeneration by inhibiting the sterile alpha and Toll/interleukin receptor motif-containing protein (SARM1) (Conforti, Gilley et al. 2014). Finally, since NAD^+ levels are decreased during axon degeneration, supplementation with NAD^+ precursors or overexpression of the NAD^+-synthesis enzymes are showed to delay axon degeneration (Araki, Sasaki et al. 2004, Wang, Zhai et al. 2005, Sasaki, Araki et al. 2006).

Another evidence to support the role of NAD^+ metabolism in the neuroprotection was the discovery of a small molecule through an *in vivo* screen to identify molecules that enhance hippocampal neurogenesis in adult mice (Pieper, Xie et al. 2010). This compound is called PC73 and was shown to prevent the death of newborn neurons and to present neuroprotective

activity in neurodegenerative diseases such PD and ALS (De Jesús-Cortés, Xu et al. 2012, Tesla, Wolf et al. 2012, Yin, Britt et al. 2014). PC73 was found to be an activator of NAMPT, resulting in an increased production of NAD^+, delaying neurodegeneration (Wang, Han et al. 2014).

NAMPT activity is also important for cerebral ischemia. Overexpression of NAMPT results in neuroprotection in models of cerebral ischemia, highlighting the role of NAD^+ metabolism in the ischemic brain injury (Zhang, Xie et al. 2010).

NAD^+ deficiency might be a major pathological factor in AD. NMN administration in AD rat models increases the cognitive functions. Restoration of NAD^+ and ATP levels resulting from NMN administration led to a decrease in neurotoxicity and cell death induced by amyloid-β oligomeric (Wang, Hu et al. 2016).
Neurodegenerative diseases are also associated with mitochondrial dysfunction that leads to generation of ROS. SIRT1 activation mediated by increase NAD^+ levels improves the outcome of these pathologies, due to the increase in PGC-1α mediated by SIRT-1 activity (Lin and Beal 2006, Johri and Beal 2012, Min, Sohn et al. 2013).

Finally, the depletion of NAD^+ in neurodegenerative diseases is also associated with the activation of PARP enzymes. Some DNA repair disorders, including AT, xeroderma pigmentosum group A (XPA) and cockayne syndrome (CS) are associated with neurodegeneration. These disorders display mitochondrial deregulation as a consequence of SIRT1 inhibition, which reduce mitophagy, an autophagic process (Vander Heiden, Cantley et al. 2009, Fang, Scheibye-Knudsen et al. 2014). However, increase in NAD^+ levels enhances the lifespan and healthspan in different worm (*Caenorhabditis elegans*) and mice AT models and attenuates AT neuropathologies, by normalizing muscular function and delaying memory loss (Fang, Kassahun et al. 2016).

3.5. NAD^+ in cancer

As already mentioned in this chapter, NAD^+ is essential for PARPs and SIRTs activity, which are NAD^+- consuming enzymes implicated in DNA damage response and carcinogenesis (Bai 2015, Mei, Zhang et al. 2016). Also, tumor cells required high amounts of NAD^+ to overcome the increase activity of these same enzymes. Thus, impairing NAD^+ availability can play a role in both DNA repair and tumor progression. As already showed, cancer cells are associated with PARP-1 overactivation. So, reducing NAD^+ levels in tumor cells impaired the activity of PARP-1 to repair DNA lesions, ultimately leading to cell death (Bajrami, Kigozi et al. 2012). One strategy to decrease the NAD^+ levels by inhibiting the NAD^+-synthetic enzymes. Several tumors present overexpression of NAD-synthesis enzymes such NMNAT and NAMPT. Therefore, treating cancer cells with NAD^+-generating enzymes inhibitors seems to be a good therapeutic target.

Regarding NMNAT, few inhibitors were described. The most common inhibitor of NMNAT is gallotanin, that can inhibit the three NMNAT isoforms (Berger, Lau et al. 2005). NMNAT is also important for the activation of antineoplastic prodrugs, like tiazofurin. These drugs are then converted into their respective dinucleotide by the NRK and NMNAT through NR salvage pathway. In consequence, these nucleotides interfere with both NAD metabolism and purine nucleotide synthesis due to inhibition of IMP dehydrogenase (Jayaram 1985, Jayaram, Kusumanchi et al. 2011). However, tiazofurin is highly toxic and is limited in cancer treatment (Guido, N. et al. 1990, L. Grem, Rubinstein et al. 1990, Vranić, Savovski et al. 2000).

NAMPT, is a very interesting target because increase levels of this protein were found in several tumors, such prostate cancer (Wang, Hasan et al. 2010) and its implicated in physiological roles such as vasculogenesis (Kim, Bae et al. 2007) and inflammation (Aller, de Luis et al. 2009). Also, NAMPT is the only NAD^+ biosynthesis enzyme for which highly specific inhibitors are available. The most common is FK866 (also known as APO866) and GMX1777. FK866 is a specific inhibitor that binds to NAMPT active site leading to its inhibition (Hasmann and Schemainda 2003), whereas GMX1777 is converted into its active form, GMX1778, which, in turn, is phosphorylated by NAMPT. The resulting compound leads to the inhibition of

NAMPT, but it cannot leave the cells, which results in permanent inhibition of NAD^+ synthesis, leading to cell death within 24h in mice (Hasmann and Schemainda 2003).

As already discussed, NAD^+ levels are compartmentalized in cells and NAD^+ concentration is not the same between all the compartments. Moreover, it is difficult to known if the NAD^+-generating enzymes inhibitors are capable of entering in these compartments, which if not, could lead to inefficient cancer treatment. Therefore, taking account the difference in the NAD metabolism might be helpful to improve cancer treatment through NAD targeting.

NAMPT is the rate-limiting enzyme for the NAD^+ synthesis that is overexpressed in several cancer. Furthermore, this enzyme can regulate PARP-1 activity. Taking into account these aspects and knowing that results of my PhD project revealed new link between NAMPT and PARP-1 in the context of CDA deficiency, the final chapter of this introduction section is dedicated to NAMPT.

Nicotinamide phosphoribosyltransferase (NAMPT)

1. Nicotinamide phosphoribosyltransferase

Nicotinamide phosphoribosyltransferase (NAMPT) is an enzyme that participates in the NAD^+ biosynthesis. This protein has been extensively studied due to its function as NAD^+-generating enzyme as well as its functions in several physiological process. The NAMPT enzymatic activity was reported in 1957 by Preiss and Handler, the same researcher that reported the salvage pathway by NAPRT and was nominated NMN pyrophosphorylase (Preiss and Handler 1957). After being isolated from activated peripheral blood lymphocytes (PBLs), NAMPT was identified as pre-B-cell colony-enhancing factor (PBEF), acting as a cytokine that promotes the pre-B-cell colony formation in the presence of interleukins (Samal, Sun et al. 1994). In 2001, Martin *et al.* found that the gene *nadV*, a NAD phosphoribosyltransferase that when expressed, allows the grow of *Haemophilus ducreyi* in NAD-free media, was presenting homology with PBEF (Martin, Shea et al. 2001). Later, the murine NAMPT was cloned in *Actinobacillus pleuropneumoniae*, a bacterium that lacks *nadV*, allowing the growth of this bacteria (Anthony, J. et al. 2002). In 2005, NAMPT was identified as an adipokine functioning as an insulin mimic, called Visfatin. However, due to problems to reproduce the results, the scientific article was retracted.

Finally, NAMPT was approved as the official name of this protein by HUGO gene nomenclature committee and by the mouse genomic nomenclature committee, although the other two names, PBEF and vistafin, can be found in the literature.

1.1. NAMPT structure

NAMPT is a homodimer protein that belongs to the type II phosphoribosyltransferase group (Khan, Tao et al. 2006, Kim, Lee et al. 2006, Wang, Zhang et al. 2006). NAMPT has two active sites, one in each monomer. When the monomers bind together, they form a long and narrow tunnel, called the “tunnel region” (Sampath, Zabka et al. 2015). The active sites lie in the end of the dimer interface (Figure 35). Autophosphorylation of the NAMPT residue His247, leads to at least 1100-fold increase in its enzymatic activity (Burgos and Schramm 2008), induces the stabilization of the NAMPT-PRPP complex, allowing an efficient capture of NAM (Burgos, Ho et al. 2009). This His247 residue is located in the conserved cluster in the active site and is

essential for NAMPT enzymatic activity. Indeed, several studies reported that mutations of this residue lead to a decrease or loss of its enzymatic activity. The H247E mutation causes a decrease in NAMPT enzymatic assay, whereas His247A mutation abolish completely its enzymatic activity (Wang, Zhang et al. 2006, Zhang, Heruth et al. 2011).

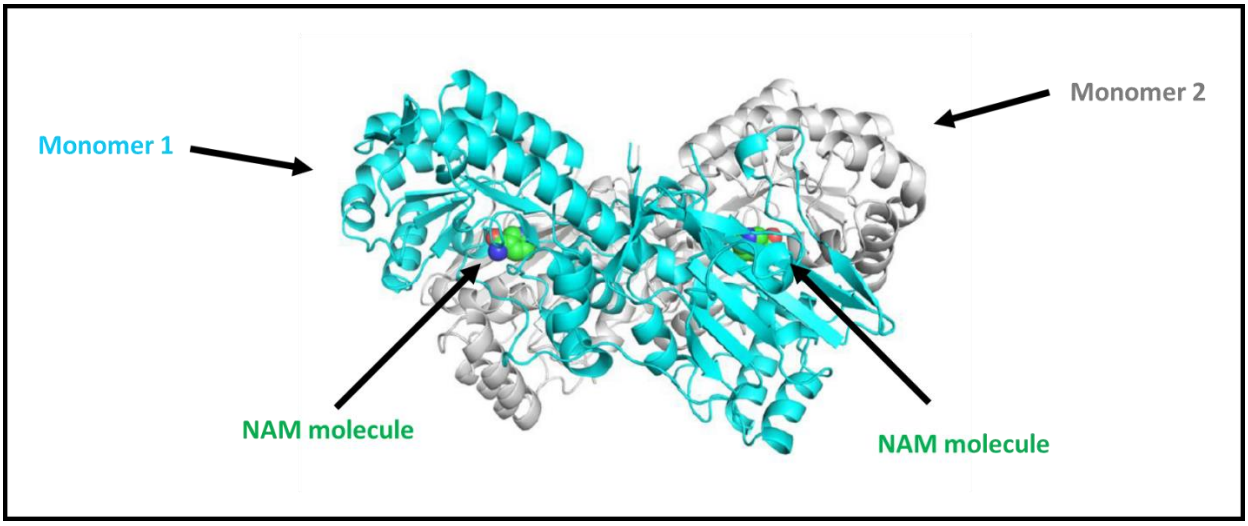


Figure 35. NAMPT crystallography structure with its substrate NAM. The two monomers are colored in blue and grey (from Sampath *et al*., 2015).

The NAMPT inhibitor, FK866, function as a substrate mimic that competes directly with NAM in the NAMPT active site.

1.2. NAMPT gene and protein

As already mentioned above, *NAMPT* gene was first isolated from a cDNA library of human PLBs (Samal, Sun et al. 1994). *NAMPT* gene is located in chromosome 7 within the 7q22.1 and the 7q31.33 (Jia, Li et al. 2004) and is composed of 11 exons and 10 introns (S Ognjanovic 2001). *NAMPT* gene is highly conserved and human *NAMPT* share homology across mammalian species such mice (95%) (Anthony, J. et al. 2002), canines (96%) (R McGlothlin, Gao et al. 2005) and with lower organisms like prokaryotes, including *H. ducreyi* (30%) (Martin, Shea et al. 2001), primitive metazoan like marine sponges (58%) (Muller, Perovic et al. 1999, Anthony, J. et al. 2002), insects (Tracy, Zeenat et al. 2008) and fish (86%) (Fujiki, Shin et al. 2000, Anthony, J. et al. 2002). NAMPT gene is ubiquitously expressed in all tissues, revealing a vital role of this protein in cellular processes. Homozygous NAMPT mice knockout is

embryonic lethal (Zhang, Heruth et al. 2011). NAMPT protein comprises 491 amino acids with a molecular weight of approximately 55 kDa. In mammals, NAMPT can be found in intracellular (iNAMPT) and extracellular (eNAMPT) forms. Both iNAMPT and eNAMPT suffers post-transcriptional modifications such as phosphorylation, ubiquitination and acetylation (Shackelford, Mayhall et al. 2013).

1.2.1. Intracellular NAMPT (iNAMPT)

1.2.1.1. iNAMPT in the salvage pathway of NAD^+ biosynthesis

The major function of iNAMPT is the synthesis of NAD^+ through the salvage pathway (Figure 36). The NAD^+ pathway depending from NAMPT activity is the more efficient and faster to produce NAD^+. NAMPT produces NMN by transferring the phosphoribosyl group of the substrate PRPP into the other substrate NAM (Anthony, J. et al. 2002). Then, NMN is converted into NAD^+ by the NMNAT enzymes (Houtkooper, Cantó et al. 2010) (Figure 36). In this pathway the catalytic activity of NAMPT is lower compared to NMNAT enzymatic activity, thus NAMPT is the rate-limiting enzyme in the NAD^+ salvage pathway. Small changes in NAMPT levels are sufficient to affect the NAD metabolism and the events that are dependent on this metabolite (Revollo, Grimm et al. 2004).

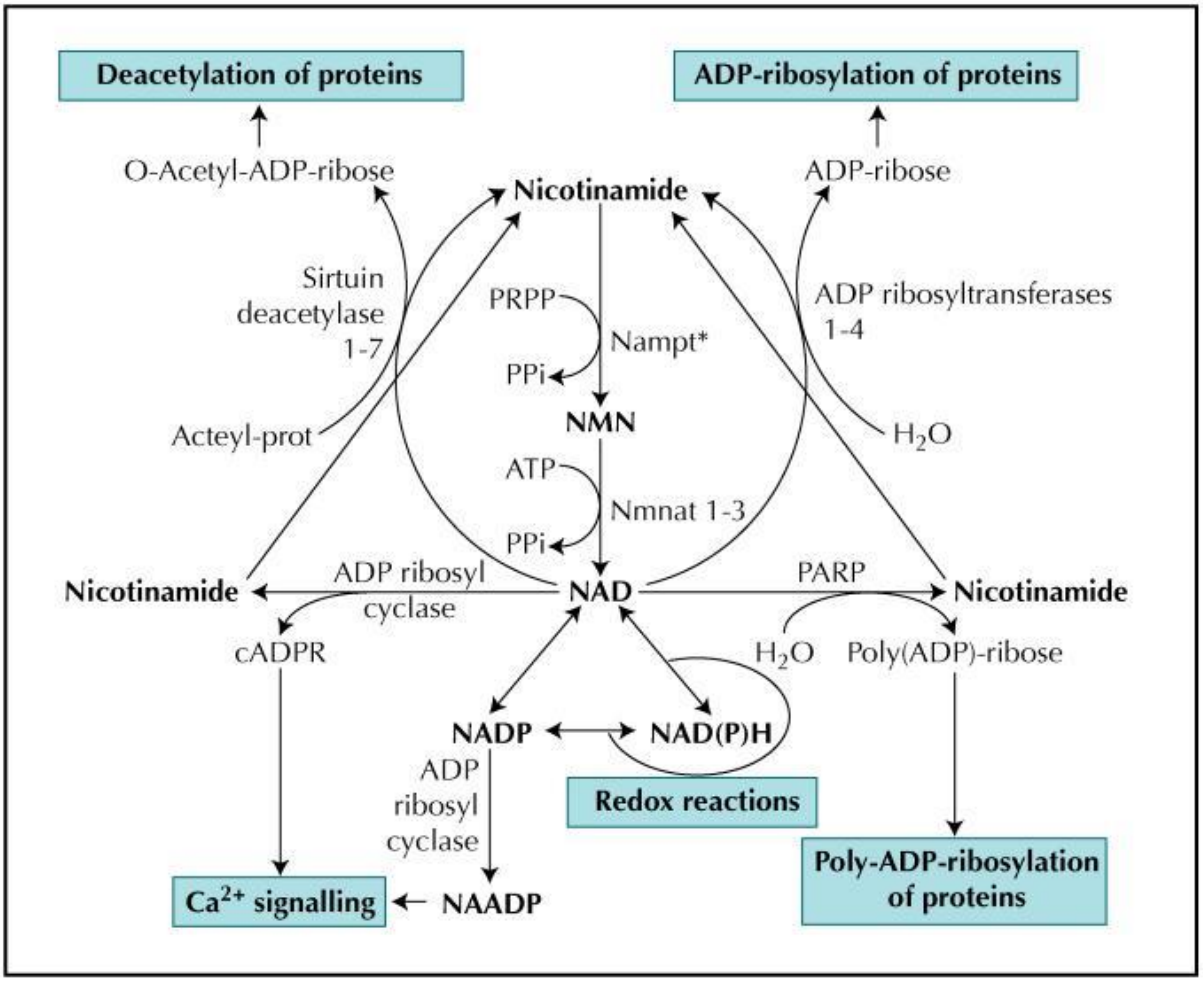


Figure 36. NAMPT salvage pathway. NAMPT converts the NAM recycled from NAD-dependent enzymes activity and PRPP into NMN. Then, NMNAT enzymes produce NAD^+ from NMN. As NAMPT is the rate-limiting enzyme in this pathway, this protein regulates both the NAD cellular pools and consequently the activities of Sirtuins and PARPS (from Sethi, 2007).

Due to its NAD-synthetic enzyme function, NAMPT regulates the intracellular NAD pools and consequently influences the activity of NAD-dependent enzymes such PARPs and Sirtuins (Figure 36) and ultimately plays an important role in cellular metabolism, mitochondrial functions oxidative stress and others.

1.2.1.2. iNAMPT cell distribution and expression

INAMPT is predominately located in the cytoplasm (❶), and in the nucleus (❷), and may be in the mitochondrial (❸) (Figure 37). The localization of NAMPT in the mitochondria is controversial. One study reported that cell survival after a genotoxic stress was dependent on

mitochondrial NAD^+ pool and associated with an increased expression of iNAMPT and mitochondrial SIRT3 and SIRT4, indicating that mitochondrial NAMPT-mediated NAD^+ synthesis plays a role in cell survival (Yang, Yang et al. 2007). However, another study did not detect iNAMPT expression in the mitochondria (Nikiforov, Dölle et al. 2011). To my knowledge, no study dedicated to cytoplasmic and nuclear NAMPT, separately, have been reported.

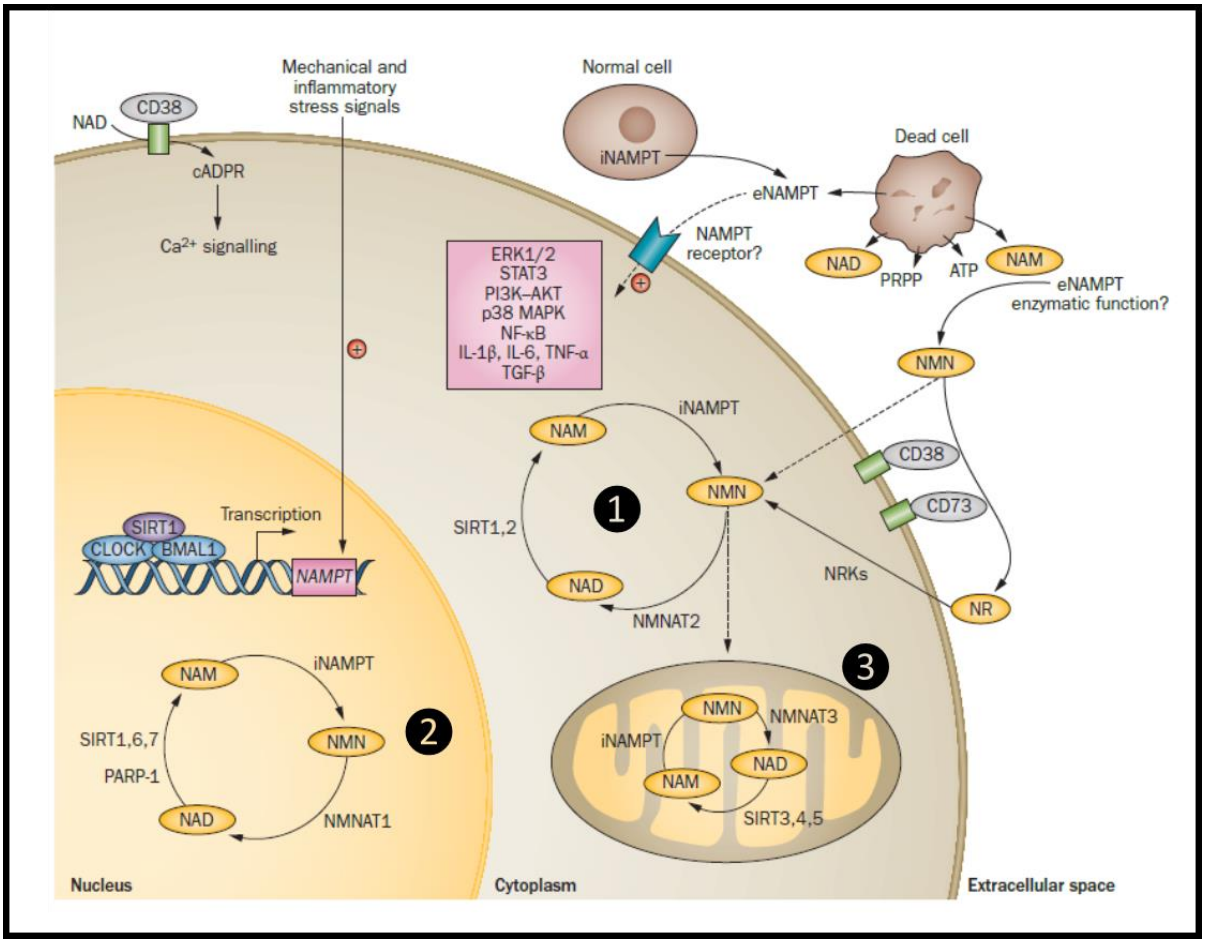


Figure 37. iNAMPT cell distribution. iNAMPT is mainly localized in the ❶ cytosol and and in the ❷ nucleus. NAMPT is also suspected to be located in the ❸ mitochondria but this localization is controversial (from Garten *et al.,* 2015).

iNAMPT is expressed in human heart, placenta, brain, lungs, liver, skeletal muscle, kidney and pancreas (Samal, Sun et al. 1994). Yet, in mice, iNAMPT is highly expressed in brown adipose tissues (BAT), liver and kidney, is moderately expressed in heart and weakly expressed in white adipose tissue (WAT), lung, spleen, testis and skeletal muscle. No iNAMPT was detected in mice pancreas and brain (Revollo, Körner et al. 2007). Cellular stress and nutrient restriction

leads to an increase iNAMPT levels (Yang, Yang et al. 2007). iNAMPT levels are also increase in several human tumors, which is considered as a negative prognostic.

1.2.1.3. iNAMPT physiological functions

The major function of iNAMPT is the NAD^+ biosynthesis from NAM. Thus, iNAMPT is capable of regulating the NAD^+ intracellular pools. By regulating the NAD^+ pools, iNAMPT also plays a role in the regulation of NAD^+ consuming enzymes, including PARPs, Sirtuins and cADPR synthetases. Therefore, iNAMPT is important for the physiological processes where NAD^+-consuming enzymes are involved:

① **iNAMPT and PARP-1 in carcinogenesis:** one physiological role of iNAMPT is to protect cells from NAD^+ depletion resulting from PARP-1 overactivity. Indeed, extensive DNA damage leads to PARP-1 activation that can consume until 80-90% of the intracellular NAD^+, leading to cell death (d'Amours, Desnoyers et al. 1999, Hassa, Haenni et al. 2006, Alano, Garnier et al. 2010). Cancer cells present an increased PARP-1 activity (Pignochino, Capozzi et al. 2017, Wang, Xiong et al. 2017). Thus, cancer cells need a mechanism to overcome the NAD^+ depletion and prevent cell death. This mechanism is mediated by the overexpression of iNAMPT (Venkateshaiah, Khan et al. 2013). The increase in PARP-1 activity is also due to SIRT1 deacetylation (Rajamohan, Pillai et al. 2009). Noteworthy, PARP-1 and iNAMPT overexpression are regularly observed in the same human tumors (Ossovskaya, Alvares et al. 2007, Shackelford, Bui et al. 2010, Wang, Hasan et al. 2010, Zhou, Bi et al. 2018). In myeloma cells, inhibition of iNAMPT leads to a reduced PARP-1 activity and cell viability. Furthermore, supplementation of myeloma cells with NAD^+ precursors in the culture medium abolish the effects of iNAMPT inhibition, indicating a role of iNAMPT in cancer cell survival (Venkateshaiah, Khan et al. 2013).

❷ **iNAMPT and CD38 in carcinogenesis:** CD38 belongs to the cADPR synthetases family. The functions of this protein are poorly understood but its overexpression is both a marker for advanced stage and for a negative prognostic for CLL and myeloma (Dürig, Naschar et al. 2002, Costa, Toscani et al. 2017). CD38 is an important NAD^+ consuming enzyme and CD38-expressing tumor cells are sensitive to iNAMPT activity (Nahimana, Attinger et al. 2009).

❸ **iNAMPT and SIRT1 in carcinogenesis:** overexpression of iNAMPT activates SIRT1 leading to oxidative stress resistance and cell survival in prostate cancer cells due to the deacetylation of SIRT1 downstream target FOXO3a, increasing its expression and consequently inducing antioxidant defenses. Both NAMPT knockdown or inhibition lead to a decrease in SIRT1 activity and consequently, to a reduction of antioxidant defenses, sensitizing prostate cancer cells to oxidative stress caused by H_2O_2 or chemotherapeutic treatment (Wang, Hasan et al. 2010). Thus, iNAMPT plays an important role in carcinogenesis by regulating SIRT1 and its dependent processes.

❹ **iNAMPT and SIRT1 in cell survival:** the conversion of vascular smooth muscle cells (SMCs) from a proliferative into a non-proliferative contractile state attributes to these cells the vasomotor function to develop and remodel blood vessels. This conversion is associated with an increase in iNAMPT activity, which in turn leads to an intracellular production of NAD^+ that activates SIRT1, promoting SMC maturation (van der Veer, Nong et al. 2005). In these cells SIRT1 activation leads to P53 deacetylation and inhibition of apoptosis (van der Veer, Ho et al. 2007). In cardiac myocytes, PARP-1 overactivation leads to cell death due to depletion of NAD^+: this cell death is inhibited by the restoration of NAD^+ levels through the overexpression of NAMPT, that subsequently activated SIRT1. Lastly, this study highlight the role of iNAMPT in the protection of cells from cell death (Pillai, Isbatan et al. 2005).

❺ **iNAMPT and SIRT1 in TD2M:** increase activity of SIRT1 in pancreatic β cells enhances glucose tolerance by promoting glucose-stimulated insulin secretion (GSIS) in β cell-specific Sirt1-overexpressing (BESTO) transgenic mice (Moynihan, Grimm et al. 2005). With the advance of age, a decrease in SIRT1 activity, leads to a reduced GSIS and insulin resistance, an important feature of T2DM development. NAD^+ levels also decrease in an age-dependent manner in BESTO transgenic mice. NMN administration was sufficient to restore both NAD^+ levels and SIRT1 activity, enhancing again the glucose tolerance in BESTO mice and preventing the development of diabetes. These results were also confirmed in NAMPT-heterozygous mice, where GSIS is also impaired (Moynihan, F. et al. 2008). These studies highlight an important role of iNAMPT in preventing TD2M development.

1.2.2. Extracellular NAMPT (eNAMPT)

Extracellular NAMPT (eNAMPT) was first described as a cytokine (PBEF) secreted from pre-B cells to promote pre-B cell colony formation in association with stem cell factor and interleukin Il-7 (Samal, Sun et al. 1994). Then, eNAMPT was reported as an adipocytokine (Vistafin) that functions as an insulin mimetic (Fukuhara, Matsuda et al. 2005). However, this study was later retracted due to problems of reproducibility. Although eNAMPT is produced by the same gene than iNAMPT, its molecular weight is slightly higher due to post transcriptional modifications (Revollo, Grimm et al. 2004). The physiological functions of eNAMPT are controversial, but, it is undiscussable that eNAMPT is detectable in the media of most cell types and can be secreted from most studied cancer cell lines, indicating that eNAMPT is likely necessary for some biological processes (Grolla, Travelli et al. 2016).

1.2.2.1. Extracellular NAMPT distribution

eNAMPT was found in human and mouse circulation (Körner, Garten et al. 2007, Revollo, Körner et al. 2007), in human cerebrospinal fluid (Hallschmid, Randeva et al. 2009), seminal plasma (Thomas, Kratzsch et al. 2013) and in the supernatant of differentiated adipocytes (Tanaka, Nozaki et al. 2007, Yoon, Yoshida et al. 2015), hepatocytes (Garten, Petzold et al. 2010, Schuster, Penke et al. 2014), leucocytes (Friebe, Neef et al. 2011), cardiomyocytes (Pillai, Sundaresan et al. 2013), neurons (Zhao, Zhang et al. 2013, Jing, Xing et al. 2014), pancreatic β cells (Kover, Tong et al. 2013), lipopolysaccharide-activated monocytes (Schilling and Hauschildt 2012, Van den Bergh, Morin et al. 2012), amniotic epithelial cells (Ognjanovic and Bryant-Greenwood 2002) and cancer cells (Grolla, Travelli et al. 2016). The eNAMPT secretion induces several intracellular signaling pathways that consequently triggers the release of other cytokines that are responsible for several physiological and pathological processes (Grolla, Travelli et al. 2016) as summarized in Figure 38.

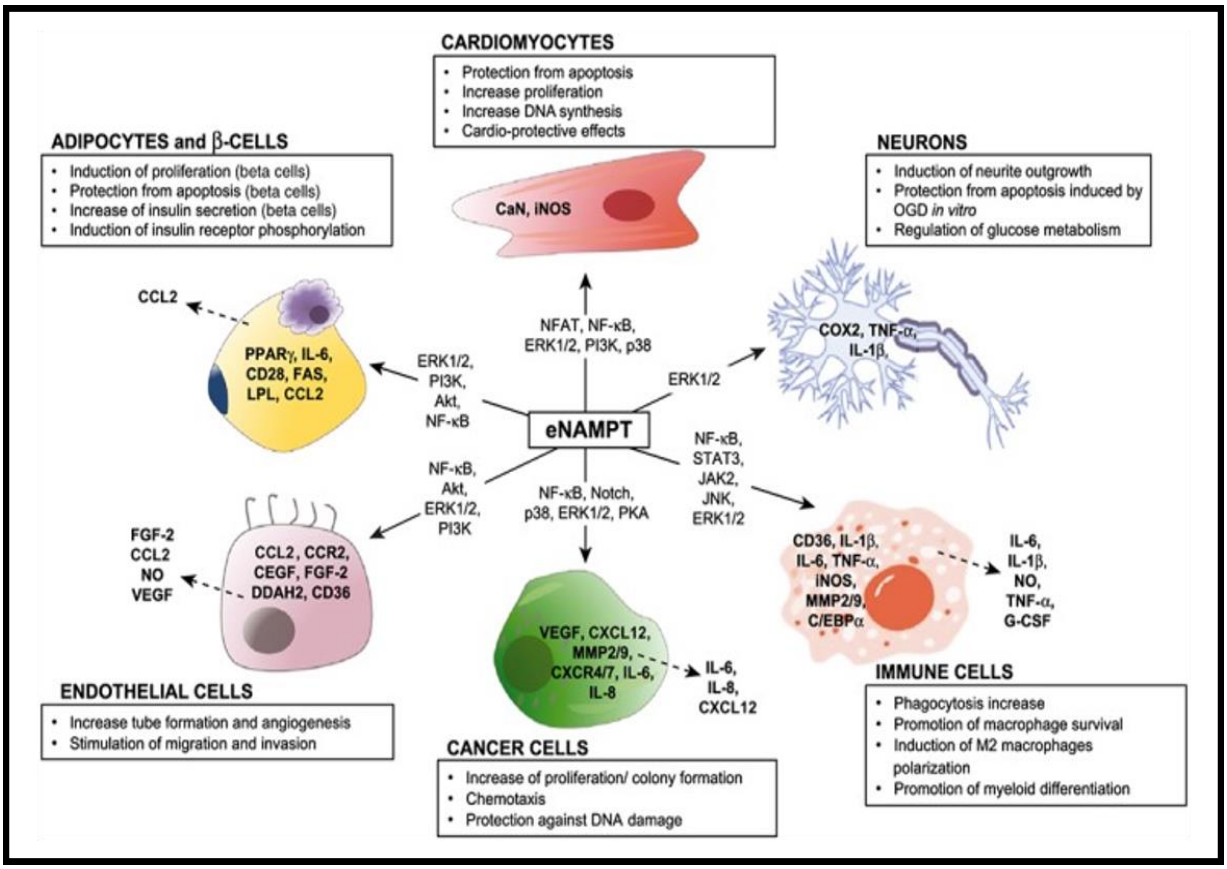


Figure 38. Example of cell types that secrete eNAMPT. *The secretion of eNAMPT leads to the induction of signaling pathways that triggers the production of cytokines that are involved in several physiological and pathological processes (from* Grolla *et al., 2016).*

I*n vivo* and *in vitro* experiments showed eNAMPT is secreted from cells after cellular, nutritional stress and inflammatory signals. Whether eNAMPT can be released under basal conditions remains unclear (Grolla, Travelli et al. 2016).

The mechanism of eNAMPT secretion was not yet elucidated but several studies provide the evidence that eNAMPT release in not a consequence of cell death or cell lysis but is positively regulated by secretory process in a cell-type-dependent manner (Wang, Xu et al. 2009, Fan, Meng et al. 2011). All the experiments to measure eNAMPT production were performed in serum-free media, which does not induce cell death but leads to nutritional and metabolic stress, that triggers eNAMPT release from cells (Garten, Petzold et al. 2010, Pillai, Sundaresan et al. 2013).

In addition, it was demonstrated that in human and mouse fully differentiated adipocytes, eNAMPT is released via a non-classical secretory pathway. The authors reported that eNAMPT

release was not inhibited when cells were treated with the classical ER-Golgi secretory pathway inhibitors such as brefeldin A and monensin (Tanaka, Nozaki et al. 2007). However, this mechanism was not observed in melanoma cells. Chloroquine treatment also increases eNAMPT secretion, indicating that it could be mediated by lysosomal trafficking (Tanaka, Nozaki et al. 2007, Grolla, Travelli et al. 2016).

Secretion of eNAMPT could also be the consequence of iNAMPT acetylation/deacetylation. Indeed, cardiomyocytes treatment with trichostatin A, a deacytelase inhibitor, or with NAM, an inhibitor of NAD^+-dependent enzymes such SIRT1, leads to a decrease in iNAMPT levels in cells and inhibits completely eNAMPT release (Pillai, Sundaresan et al. 2013). Thus, deacetylation of iNAMPT by SIRT1 influences NAMPT release, increasing both eNAMPT secretion and activity in adipocytes (Yoon, Yoshida et al. 2015).

1.2.2.2. eNAMPT physiological functions

At least three different functions were attributed to eNAMPT: a cytokine (PBEF), an insulin mimetic (Vistafin) and NAD biosynthesis enzyme (Stephens and Vidal-Puig 2006, Revollo, Grimm et al. 2007, Sethi 2007, Tracy, Zeenat et al. 2008). Regarding the possible function of eNAMPT as a NAD-synthetic enzyme, it still very controversial since Revollo *et al.* (2007) reported that eNAMPT was capable of NAD^+ synthesis (Revollo, Grimm et al. 2007), whereas Hara *et al.* (2011) demonstrated that C57/BL6 mice plasma presented low concentration of PRRP and ATP, indicating that eNAMPT is not enzymatically active the plasma (Hara, Yamada et al. 2011).

❶ **eNAMPT and obesity:** several studies reported that eNAMPT levels were increase in the plasma of obese and T2DM individuals (Berndt, Klöting et al. 2005, Haider, Holzer et al. 2006, Taşkesen, Kirel et al. 2012, Belo, Luizon et al. 2013). Lin *et al.* (2008), reported that eNAMPT allows macrophages survival, which plays a role in obesity-associated pathologies such inflammation, atherosclerosis and cancer. Moreover, inflammatory macrophages are increased in adipose tissue of obese individuals (Weisberg, McCann et al. 2003). eNAMPT protects macrophages from ER stress-induced apoptosis. During an ER stress-induced apoptosis, eNAMPT leads to the secretion of IL-6, which in turn activates the pro survival signal

transducer STAT3 in an autocrine/paracrine mode, promoting macrophage survival. Moreover, the activation of the IL-6 and downstream targets was independent of the NAMPT substrate, NAM, in the medium, and was not mimicked by the presence of NAMPT product NMN, and was not inhibited by FK886, a NAMPT inhibitor, indicating that the activation of this pathway is via a nonenzymatic mechanism. Thus, eNAMPT plays an important role in the balance of survival and death of macrophages and, thus, in obesity and its associated diseases (Li, Zhang et al. 2008).

❷ **eNAMPT and diabetes:** The role of eNAMPT in the T2DM is complex and complicated. Numerous studies have reported an increase in eNAMPT levels in both obese and T2DM individuals (Chen, Chung et al. 2006, Dogru, Sonmez et al. 2007, Ravi, Byung-Soo et al. 2008, Kieswich, Sayers et al. 2016). Other studies reported that eNAMPT was not associated to diabetes (Pagano, Pilon et al. 2006, Takebayashi, Suetsugu et al. 2007, Akturk, Altinova et al. 2008, Ravi, Byung-Soo et al. 2008). eNAMPT has also been reported to be an adipocytokine (Visfatin) that functions as an insulin mimetic that could bind to insulin receptor (IR) and promote insulin sensitivity (Fukuhara, Matsuda et al. 2005). Unfortunately, this article was retracted, as already mentioned. In contrast, *Xie et al.* (2008) demonstrated that eNAMPT could act as an insulin mimetic in osteoblasts but the binding to IR was not reported. They showed that eNAMPT, exactly like insulin, leads to the activation of IR, which in turn phosphorylates the tyrosine residues of IRS-1 and IRS-2, stimulating glucose uptake, a hallmark of insulin action. In addition, eNAMPT also induces osteoblast proliferation and type I collagen production, that is important for bone strength (Saito, Kida et al. 2014), and osteoblast matrix mineralization, favorably affecting bone metabolism. However, eNAMPT downregulates osteocalcin secretion, that was recently showed to promote insulin sensitivity (Kanazawa 2015). Xie *et al.* (2008) highlighted that eNAMPT acts as an insulin mimetic in osteoblasts, promoting insulin sensitivity by glucose transport as well as by enhancing osteoblast synthesis (Xie, Tang et al. 2007), preventing the osteoporosis development, which is associated with diabetes (Kanazawa and Sugimoto 2018). In contrast, glucose uptake enhanced by eNAMPT can have a negative effect in diabetic nephropathy (Song, Lee et al. 2008). Indeed, eNAMPT synthesis and secretion was increased in kidney mesangial cells (MCs) upon high glucose stimuli (Figure 39). eNAMPT secretion (①) activates IR that in turn leads to phosphorylation and activation of PI 3-kinase and its downstream target AKT (②),

favorizing glucose uptake (③). eNAMPT also leads to the increase in GLUT-1 protein expression and its translocation from cytoplasm to cellular membrane to facilitate the glucose transport (④). Finally, eNAMPT exogenous treatment also leads to the increased production of profibrotic molecules such TGF-β1, PAI-1 and type I collagen (⑤), accelerating diabetic nephropathy (⑥) (Song, Lee et al. 2008).

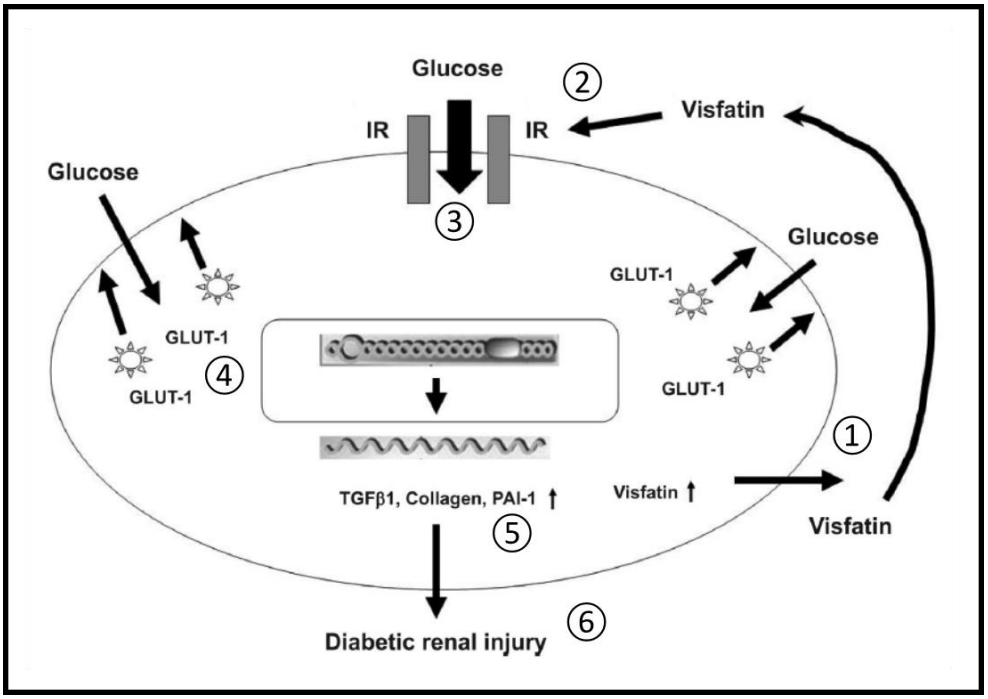


Figure 39. Kidney mesangial cells injury mediated by eNAMPT. eNAMPT production and secretion from kidney MCs leads to the activation of IR, stimulating glucose uptake. eNAMPT is responsible for the translocation of GLUT-1 to cellular membrane, favorizing glucose transport. Finally, eNAMPT endogenous treatment leads to the production of profibrotic molecules that favorizes MCs injury (from Song *et al.*, 2008).

❸ **eNAMPT and inflammation:** The expression of eNAMPT is increased upon numerous types of inflammation, including acute lung injury (Ye, Simon et al. 2005), inflammatory arthritis (Fabia, Olivier et al. 2007) and inflammatory kidney disorders (Yilmaz, Saglam et al. 2008). eNAMPT treatment (injections of murine eNAMPT recombinant protein) in mice leads to the up-regulation of inflammatory cytokines such as IL-1β, IL-1Ra, IL-6, Il-10 and TNF-α in human monocytes in a p38 and MEK-1 dependent manner. These cytokines are involved in several inflammatory diseases. eNAMPT is also a chemotactic factor that recruits both CD14+ monocytes and CD19+ B cells, and promotes T cells activation by inducing CD54 binding to

LFA-1, that is responsible for T cells activation (Moschen, Kaser et al. 2007). Another study reported that eNAMPT exhibits a proinflammatory effect in THF-1 derived macrophages by dose-dependent increase production of EMMPRIN and MMP-9 expression and MMP-9 activity through phosphorylation of ERK1/2 and p38 MAPK (Fan, Meng et al. 2011). eNAMPT pro-inflammatory role was dependent of iNAMPT enzymatic activity (Fan, Meng et al. 2011). Finally, eNAMPT contributes to vascular inflammation and endothelial dysfunction, inducing production of ROS in endothelial cells, which in turn induces the IκBα, leading to the translocation of the p65 subunit of NF-κB to the nucleus, activating the inflammatory pathway NF-κB. The activation of NF-κB by eNAMPT production is responsible for leukocyte adhesion to endothelial cells, that usually occurs in the early steps of vascular inflammation process, and to aortic endothelium through induction of cell adhesion CAMs molecules, ICAM-1 and VCAM-1. This study highlighted the role of eNAMPT as a vascular inflammatory molecule that increases the expression of CAMs molecules, leading to endothelial dysfunction (Kim, Bae et al. 2008). All these studies provide a new role for eNAMPT in vascular inflammation that is associated with obesity, diabetes and cancer (Dalamaga, Christodoulatos et al. 2018).

2. NAMPT and cancer

Both forms of NAMPT, iNAMPT and eNAMPT are expressed in numerous human cancers, where their higher expression are associated to malignant progression.

eNAMPT: plasmatic levels of eNAMPT are elevated in several human cancers, such as astrocytomas (Sreekanth Reddy, Srikantha et al. 2008), male oral squamous cell (Yu-Duan, Chao-Ping et al. 2013), gastric (Mohammadi, Zarghami et al. 2015), endometrial (Tian, Zhu et al. 2013), hepatocellular (Ninomiya, Shimizu et al. 2011), colorectal carcinomas (Fazeli, Dashti et al. 2013) and invasive breast cancer (Hung, Lo et al. 2016). eNAMPT levels are also associated with patient survival and metastasis formation (Grolla, Travelli et al. 2016). Indeed, high levels of eNAMPT correlates with both myometrial invasion and shorter survival in women with endometrial carcinoma (Tian, Zhu et al. 2013). In addition, in invasive breast cancer, high levels of eNAMPT also correlates with lymph node metastasis and the absence of both hormones estrogen and progesterone receptors (Dalamaga, Archondakis et al. 2012). Increased eNAMPT levels are also associated with the development of postmenopausal breast

cancer (Dalamaga, Archondakis et al. 2012). eNAMPT levels influences tumor progression, by leading to the transformation and polarization of macrophages into M2 phenotype that induces immunosuppression and tumor progression. Audrito *et al.* (2015) revealed that eNAMPT is secreted from CLL lymphocytes through Toll-like receptor, B-cell receptor and NF-κB signaling stimuli, resulting in the differentiation of macrophages into tumor M2-supporting macrophages. This process is also responsible for inducing an immunosuppressive and tumor-enhancing microenvironment in chronic lymphocytic leukaemia (Audrito, Serra et al. 2015).

iNAMPT: numerous studies demonstrated that iNAMPT is overexpressed in several tumors, including ovarian (Shackelford, Bui et al. 2010), breast (Zhou, Bi et al. 2018), colorectal (Lucena-Cacace, Otero-Albiol et al. 2018), prostate (Wang, Hasan et al. 2010), gastric cancers (Long, Che et al. 2012), endometrial carcinomas (Shackelford, Mayhall et al. 2013), myeloma (Venkateshaiah, Khan et al. 2013) and melanoma (Elena, Cristina et al. 2013). iNAMPT overexpression is also present in malignant lymphomas, such as diffuse B and follicular B-cell lymphoma, Hodgkin's lymphoma and peripheral T-cell lymphoma (HØGH, NINA et al. 2011). These high levels of iNAMPT expression are also correlated with increased tumor growth, cell differentiation, metastasis and a vertical growth phase in melanoma and with a negative prognostic in endometrial adenocarcinoma and astrocytoma (Shackelford, Mayhall et al. 2013). Up-regulation of iNAMPT also leads to resistance to chemotherapeutic agent such as fluorouracil, doxorubicin, paclitaxel and etoposide. Conversely, NAMPT knockdown or inhibition is associated with anti-neoplastic actions both *in vitro* and *in vivo* tumor models, such as colon, gastric, breast, pancreatic and others (Koike Folgueira, Carraro et al. 2005, Bi, Che et al. 2011, Espindola-Netto, Chini et al. 2017, Grohmann, Penke et al. 2018).

2.1. NAMPT as a new target for cancer treatment

As presented above NAMPT expression is increased in several human cancers, and NAMPT inhibition exerts anti-cancer effects, indicating that NAMPT is a potential therapeutic target for several human tumors (Wosikowski, Mattern et al. 2002, Nahimana, Attinger et al. 2009, Bi, Che et al. 2011, Bajrami, Kigozi et al. 2012, Okumura, Sasaki et al. 2012). Indeed, since NAMPT is important for NAD^+ and ATP synthesis, and these metabolites are increased in cancer cells, tumors cells are more sensitive to these inhibitors than normal cells (Bi and Che

2010). One specific NAMPT inhibitor was found. FK866 is highly specific inhibitor that leads to the reduction NAD^+ biosynthesis impairing PARP-1 and SIRT1 activity and ultimately leading to cancer cell death (Hasmann and Schemainda 2003). NAMPT inhibitors are also used in combination with other chemotherapeutic agents as synthetic lethality strategy. For example, in triple-negative breast cancer, FK866 was administered in combination with olaparib treatment, leading to a decrease in tumor growth to a greater extent than each treatment alone (Bajrami, Kigozi et al. 2012).

2.1.1. NAMPT inhibitors

The first generation of NAMPT inhibitors (NAMPTi), FK866 (APO866 and WK175), GMX1778 (CHS-828) and GMX1777 (EB1627), the soluble pro-drug GMX1778 (Binderup, Björkling et al. 2005), are the most characterized and well documented (Roulston and Shore 2016). New and recent NAMPTi, GNE-617, GNE-618, AU-771 and STF-118804 were also developed (Roulston and Shore 2016). These inhibitors have shown *in vivo* efficacy in several tumor xenografts models, including prostrate, ovarian, pancreatic, colorectal, small cell lung cancer, NSLCC, glioblastoma, fibrosarcoma and acute myeloid leukemia (Olesen, Christensen et al. 2008, Beauparlant, Bédard et al. 2009, Wang, Hasan et al. 2010, Matheny, Wei et al. 2013, O'Brien, Oeh et al. 2013, Tan, Young et al. 2013).

NAMPT inhibition leads to metabolic dysfunctions in tumor cells as a consequence of reduce NAD^+ biosynthesis, that ultimately results in cell death (Sampath, Zabka et al. 2015). *In vitro* experiments with NAMPTi lead to a rapid decrease in intracellular NAD^+ levels that consequently impairs glycolysis and nucleotide metabolism in the first 6 to 10h (Watson, Roulston et al. 2009, Xiao, Elkins et al. 2013). Then, a profound inhibition of glycolysis, reduced TCA cycle and subsequent ATP synthesis inhibition are observed. Inhibition of aspartate and alanine metabolism, nucleotide (purine and pyrimidine) metabolism and guanylate nucleotide synthesis occurs within 24h of treatment (Tan, Young et al. 2013, Tolstikov, Nikolayev et al. 2014). All these metabolic perturbations were also observed *in vivo* in xenografted tumors on mice treated with NAMPTi (Chan, Gravel et al. 2014) . When ATP depletion reach $>$ 90% of the total levels, cancer cell death is observed (Watson, Roulston et al. 2009, Tan, Young et al. 2013).

NAMPTi-induced cell death by either by apoptosis or autophagy mechanism. One study reported that cell death pathway induced by NAMPT inhibitors depend on the rate of ATP depletion. Indeed, when NAD^+ depletion is more than 95%, cells lose the ability to regenerate ATP. If a delay between the reduction of NAD^+ and ATP depletion is observed in cell treated with NAMPTi (t1/2 of 45h), then apoptosis and autophagy occur. If ATP depletion occurs more rapidly (about t1/2 of 32h) after NAD^+ biosynthesis inhibition then cell death occurs by oncosis, that is characterize by cellular swelling and formation of blisters in plasma membrane. Oncosis-mediated cell death is induced because after ATP depletion, cells quickly lose their ability to regulate the ion transport across the membrane, which leads to the loss of membrane potential in the cells (Del Nagro, Xiao et al. 2014).

Although NAMPTi can be used as a monotherapy, combination treatment of these inhibitors with other chemotherapeutic agents, as synthetic lethality strategy, enhancing NAMPTi effectiveness. The synergy mechanism between the two treatments results in the increase in consumption of NAD^+ by NAD^+-consuming enzyme and inhibition of NAD^+ production. Several studies demonstrated a synergistic toxicity in tumor cells when combination treatment with NAMPT inhibitors and DNA damage-inducing chemotherapies, including temozolomide (Goellner, Grimme et al. 2011), 5-fluorouracil (5-FU) (Bi, Che et al. 2011), fludarabine (Gehrke, Bouchard et al. 2014), pemetrexed (Chan, Gravel et al. 2014) and β-lapachone (Moore, Chakrabarti et al. 2015). Moreover, NAMPTi can also be combinated with DNA repair proteins inhibitors such PARP-1. It was already evidenced that tumor cells present overactivity of PARP-1. Combination of NAMPTi with PARP-1 inhibitors like olaparib results in enhanced inhibition of DNA repair proteins that lead to increase in DSBs and consequent results in cell death.

Unfortunately, drug resistance is a common feature of anti-cancer agents (Sampath, Zabka et al. 2015) and NAMPT inhibitors does not escape from this process. Numerous unique point mutations have been reported in cell lines treated with NAMPTi. These mutations occur within or near the substrate binding site (Wang, Elkins et al. 2014). In addition, these mutations are not found in normal and non-treated tumor tissues, indicating that the mutations appear after treatment (Duarte-Pereira, Silva et al. 2014).

Knowing that cancer cells have a great plasticity, it is expected that cancer cells can induces adaptative responses to the decrease in NAD^+ intracellular levels and enhances NAMPTi resistance (Roulston and Shore 2016).

2.1.1.1. NAMPT inhibitor clinical trials

FK866 and GMX1778 were the first NAMPT inhibitors to proceed into clinical trial. More than 100 individuals were treated with these two inhibitors, but they were discontinued due to dose-limiting toxicities or withdraw due to lack of financial support.

GMX1778 was administrated orally whereas GMX177 (the soluble form of GMX1778) and FK866 where administered intravenously during 24h or 96h, respectively. Dose-limiting toxicities for all inhibitors were observed including thrombocytopenia and gastrointestinal (GI) effects such as diarrhea, vomiting and esophagitis (Hovstadius, Larsson et al. 2002, Ravaud, Cerny et al. 2005, von Heideman, Berglund et al. 2010). Other symptoms were observed in patients treated with FK866 and GMX1777, such skin rash and lymphopenia (Holen, Saltz et al. 2008, Pishvaian, Marshall et al. 2009). Unfortunately, no objective responses, which means no objective tumor remission, were observed in advanced solid tumors and advance malignancies, but stable diseases were noted in patients treated with these NAMPT inhibitors (Figure 40).

Phase 1 clinical trial data for NAMPT inhibitors.

Drug[a]	n[b]	Dose/Admin/Frequency/Route	Efficacy[c]	DLTs[d]	Other G3+ toxicities[e]
GMX1778	16	6–26 mg QD for 5 days once every 4 weeks PO	Stable disease (n = 7)	Thrombosis Vomiting Diarrhea Esophagitis Thrombocytopenia	Nausea Fatigue Lymphocytopenia Anemia Leukopenia
GMX1778	37	50–500 mg once every 3 weeks PO	Stable disease (n = 11)	Mucositis Thrombocytopenia Leucopenia Diarrhea	Anemia Abdominal Pain Anorexia Asthenia Balanoposthitis Fever Gait Abnormal Haematuria Neutropenia Malaise Muscle Weakness NauseaParesthesiaPulmonary OedemaStomatitisVaginitisVomiting
GMX1778	7	20–80 mg once/week for 3 weeks, then 1 week off PO	None reported	Fatigue Hypokalemia Subileus Gastric Ulcer Hyperuricemia Anemia Dehydration	Diarrhea TIA[f] Vomiting
GMX1777	19	60–200 mg/m^2 24 hour IV infusion once every 3 weeks	Stable disease (n = 5)	None reported	GI hemorrhage Thrombocytopenia Rash
APO866	24	0.018–0.144 mg/m^2 96 hour IV infusion once every 4 weeks	Stable disease (n = 4)	Thrombocytopenia	Nausea Lymphocytopenia Fatigue Lymphopenea Hyperglycemia

QD, daily; PO, per os (oral); IV, intravenous.
[a] Test molecules GMX1778 is also referred to as CHS 828 and APO866 as FK866.
[b] Number of patients receiving at least one dose of indicated test molecule.
[c] Most favorable patient outcomes reported for given study.
[d] Dose-limiting toxicities.
[e] Other reported toxicities of grade 3 or higher.
[f] Transient ischaemic attack.

Figure 40. Clinical results from APO866, GMX1777 and GMX1778 (adapted from Sampath *et al.*, 2015).

To overcome the toxic effects of NAMPT inhibitors, it was proposed a co-administration of NA that allows higher NAMPTi to be tolerate. Indeed, depletion of NAD^+ levels and cytotoxicity from NAMPT inhibitors can be rescue by the NAPRT salvage pathway through NA supplementation. Moreover, almost all human tumors lack NAPRT enzyme, providing a strategy where patients treated with a combination of NAMPT inhibitors and NA, lead to an effective targeting of tumor cells (that are NAPRT negative) while protecting normal cells (that are NAPRT positive) from the toxic effects of NAMPT inhibitors (Watson, Roulston et al. 2009, Shames, Elkins et al. 2013).

Finally, a new phase I clinical trial for KPT-9274, a dual inhibitor for serine/threonine kinase 4 (PAK4), that participates in the actin and cytoskeleton reorganization and NAMPT is going to start in patients with advanced solid tumors and non-Hodgkin lymphoma.

The rest of this manuscript will be dedicated to the presentation of my PhD project, results, discussion and future perspectives.

Project presentation

When I arrived in Mounira Amor-Guéret's lab in November 2014, the team had just found that the excess UFB formation during mitosis in BLM-deficient cells was entirely due the pyrimidine pool imbalance resulting from CDA deficiency and, more specifically, to the decrease in basal PARP-1 activity induced by the excess of intracellular dC and dCTP (Gemble, Ahuja et al. 2015) (Figure 41).

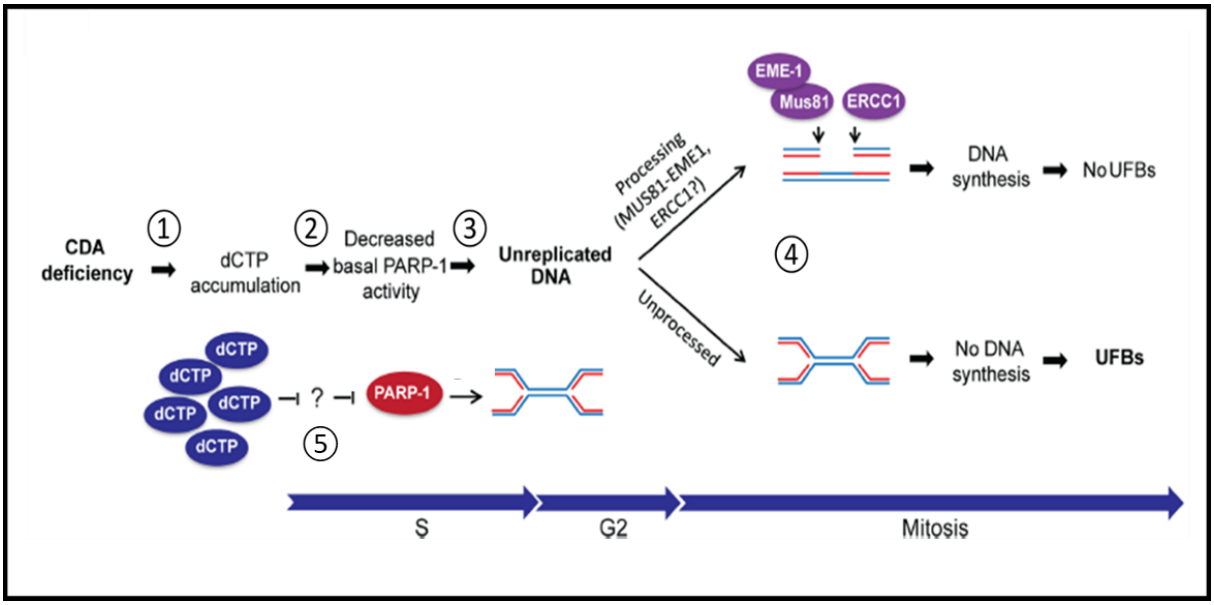


Figure 41. CDA deficiency leads to the decrease in PARP-1 activity and consequently, to excess UFB formation (from Gemble *et al.*, 2015).

At this time, the team had just demonstrated that the intracellular accumulation of dC/dCTP (①) resulting from CDA deficiency is responsible for a decrease in basal PARP-1 activity (②). This decrease in PARP-1 activity leads to the under-replication of some "difficult to replicate" loci in the genome, such as centromeres and common fragile sites (③). During late G2/early mitosis, some of these unreplicated DNA sequences are likely processed by MUS81-EME1 and ERCC1 nucleases (④) (Naim, Wilhelm et al. 2013, Ying, Minocherhomji et al. 2013). However, some of the excess unreplicated DNA sequences are not processed by nucleases, leading to excess UFB formation (④) (Gemble, Ahuja et al. 2015). The important question of how the intracellular accumulation in dC/dCTP leads to reduced PARP-1 activity (⑤) was not clarified. The team concludes that PARP-1 was inhibited by the intracellular accumulation of dC/dCTP, because increasing the intracellular dCTP pool *in vivo* by culturing CDA-proficient cells in the presence of dC was sufficient to impair PARP-1 in CDA-proficient cells (Figure 42A). Moreover,

they found that dCTP was the only dNTP inhibiting PARP-1 *in vitro*, although this inhibition was observed only at high dCTP concentrations, between 6.5 and 10 mM dCTP (Figure 42B). However, since only 100 µM NAD^+ was sufficient to fully reverse PARP-1 inhibition by 10 mM dCTP, it appears very unlikely that dCTP competes with NAD^+ *in vivo* to inhibit PARP-1 (Figure 42C). These results excluded an attractive hypothesis based on structural similarities between dC/dCTP and NAD, dC and dCTP presenting a domain very similar to the NAM domain present in NAD^+.

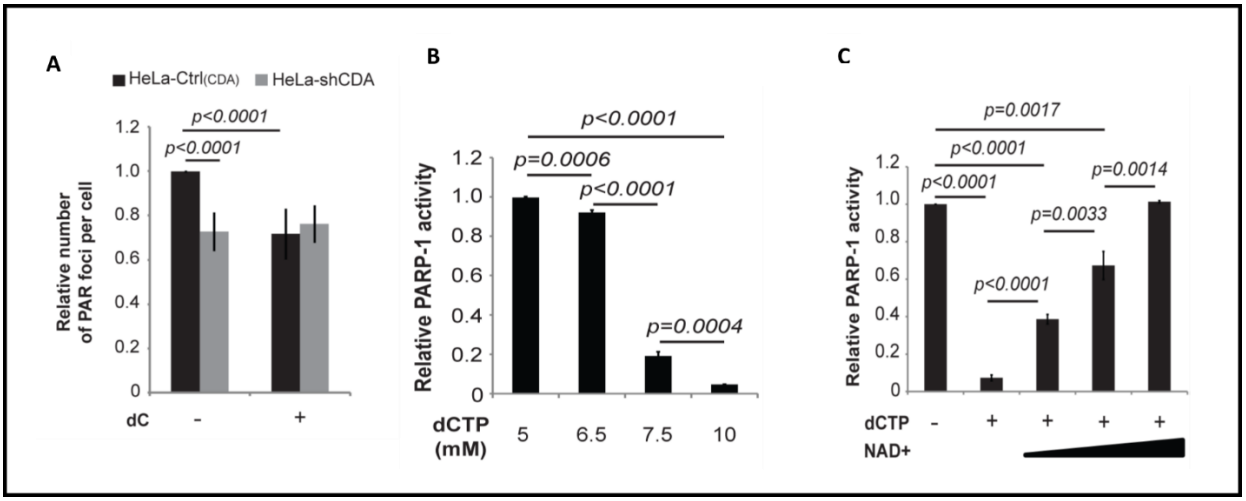


Figure 42. CDA deficiency leads to dC and dCTP intracellular accumulation, leading to both in vivo and in vitro PARP-1 inhibition (from Gemble *et al.,* 2015).

Thus, the team hypothesized that CDA deficiency and subsequent dC/dCTP accumulation inhibited PARP-1 indirectly. Therefore, the objective of my PhD project was to decipher the mechanism leading to the reduction of basal PARP-1 activity in the absence of CDA. This question is of major importance since the team demonstrated the loss of CDA expression in a large fraction of cancer cells and tumor tissues, mainly through an epigenetic mechanism (Mameri, Bièche et al. 2017). Moreover, as presented in the introduction section, PARP-1 inhibitors are now used for the treatment of several cancers, in particular those resulting from mutation in BRCA1 and BRCA2 (Dziadkowiec, Gąsiorowska et al. 2016). In this context, it is crucial to understand how the nucleotide pool disequilibrium resulting from CDA deficiency could impair PARP-1 activity, leading to a genetic instability.

Results

Pyrimidine pool disequilibrium reduces NAMPT activity, thereby lowering PARP1 activity

(Article in preparation)

Sandra Cunha Silveira[1,2,3,9,], Géraldine Buhagiar-Labarchède[1,2,3,9,], Rosine Onclercq-Delic[1,2,3,9,], Simon Gemble[1,2,3], Elias Bou Samra[1,2,3], Hamza Mameri[1,2,3], Patricia Duchambon[4,5], Christelle Machon[6,7], Jérôme Guitton[6,8], Mounira Amor-Guéret[1,2,3,10*].

The main objective of this work was to decipher how CDA deficiency leads to a decrease in basal PARP-1 activity.

Note: Some experiments need to be confirmed and others are ongoing.

The main results we obtained are presented below:

- **CDA deficiency leads to an increase in NAM levels in BS cells, and a decrease in NMN levels in both CDA-deficient BS and HeLa cells, suggesting that NAMPT activity, that catalyzes the conversion of NAM to NMN, is weak in CDA-deficient cells.**
- **The intracellular accumulation of dC/dCTP resulting from CDA deficiency decreases nuclear NAMPT activity.**
- **The inhibition or depletion of NAMPT in CDA-proficient cells decreases the basal activity of PARP-1 and increase the frequency of UFBs in these cells to levels similar to those observed in CDA-deficient cells, thereby mimicking CDA deficiency.**
- **The decrease in PARP-1 activity resulting from NAMPT inhibition is completely independent of cellular NAD^+ levels.**
- **The basal PARP-1 activity is fully restored in CDA-deficient cells by the overexpression of an exogenous wild-type NAMPT, whereas the inactive NAMPT mutant has no effect in these cells.**

These results demonstrate that the lower basal PARP-1 activity in CDA-deficient cells results from the lowers levels of NAMPT activity in these cells, regardless of total NAD^+ levels. This work reveals an unexpected link between CDA and NAMPT and thus, between pyrimidine pool and NAMPT-mediated NAD^+ salvage pathways.

Pyrimidine pool disequilibrium reduces NAMPT activity, thereby lowering PARP1 activity

Sandra Cunha Silveira[1,2,3,9,], Géraldine Buhagiar-Labarchède[1,2,3,9,], Rosine Onclercq-Delic[1,2,3,9,], Simon Gemble[1,2,3], Elias Bou Samra[1,2,3], Hamza Mameri[1,2,3], Patricia Duchambon[4,5], Christelle Machon[6,7], Jérôme Guitton[6,8], Mounira Amor-Guéret[1,2,3,10*].

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ABSTRACT

Cytidine deaminase deficiency causes pyrimidine pool disequilibrium. The excess cellular dC and dCTP jeopardizes genome stability decreasing basal poly (ADP-ribose) polymerase 1 (PARP-1) activity and increasing ultrafine anaphase bridge (UFB) formation. We investigated the mechanism of PARP-1 inhibition by CDA deficiency, in a large-scale metabolomics study. We found that CDA deficiency was associated with an increase in levels of nicotinamide (NAM), the substrate of nicotinamide phosphoribosyltransferase (NAMPT), and a decrease in levels of nicotinamide mononucleotide (NMN), the product of NAMPT. Excess dC/dCTP decreased NAMPT activity in CDA-proficient cells, whereas NAMPT inhibition or depletion decreased the basal PARP-1 activity and increased UFB frequency to levels similar to those in CDA-deficient cells. The overexpression of exogenous wild type NAMPT, but not of the catalytic mutant, abolished the decrease in basal PARP-1 activity and the increase in UFB frequency in CDA-deficient cells. These results reveal an unexpected link between the pyrimidine and NAD salvage pathways.

INTRODUCTION

The maintenance of genome stability is of major importance to prevent various aging-related diseases, including cancer (Veith and Mangerich 2015). We reported that cytidine deaminase (CDA) plays an essential role in maintaining genome integrity. CDA is an enzyme of the pyrimidine salvage pathway catalyzing the hydrolytic deamination of cytidine (C) and deoxycytidine (dC) to uridine (U) and deoxyuridine (dU), respectively (Nygaard 1986). The pyrimidine pool disequilibrium resulting from CDA deficiency contributes to the genetic instability associated with Bloom syndrome (BS) (Chabosseau et al. 2011), a human autosomal recessive disease displaying one of the strongest known correlations between chromosomal instability and a high risk of cancer, and resulting from mutations of both copies of the *BLM* gene encoding BLM, a 3'-5' RecQ DNA helicase (Cunniff, Bassetti et al. 2017). Moreover, CDA expression is downregulated in about 60% of cancer cells and tissues, mostly due to DNA methylation (Mameri et al., 2017); overexpression of microtubule-associated protein Tau (MAPT) in cancer cells and tissues with little or no CDA favors their survival since CDA and Tau deficiencies present a synthetic lethal interaction (Bou Samra et al., 2017).

We also reported that CDA deficiency results in significantly lower levels of PARP-1 (poly (ADP-ribose) polymerase 1) activity in basal conditions and in response to genotoxic stress, leading to the accumulation of unreplicated DNA during mitosis and, thus, to high levels of ultrafine anaphase bridge formation (UFB), regardless of BLM expression status (Gemble, Ahuja et al. 2015, Gemble, Buhagiar-Labarchède et al. 2016, Gemble, Buhagiar-Labarchède et al. 2017). PARP-1 is a multifunctional enzyme that mediates several aspects of the DNA-damage response through its poly(ADP-ribosyl)ation (PARylation) activity, involving the transfer of PAR units from nicotinamide-adenine-dinucleotide (NAD^+) to diverse acceptor proteins, including histones (Ray Chaudhuri and Nussenzweig 2017). PARP-1 thus plays a key role in preventing genetic instability, and its activity is dependent on intracellular NAD^+ concentration. Nicotinamide phosphoribosyltransferase (NAMPT) is a rate-limiting enzyme in the NAD biosynthesis via the salvage pathway. It catalyzes the transfer of a phosphoribosyl group from 5-phosphoribosyl-pyrophosphate (PRPP) to nicotinamide (NAM), generating the NAD^+ intermediate nicotinamide mononucleotide (NMN), which is converted to NAD by nicotinamide mononucleotide adenylyltransferase (NMNAT) (Sommer, Garten et al. 2008, Garten, Petzold et al. 2009). Through this function in NAD biosynthesis, NAMPT activity is crucial to regulate the activity of NAD-dependent enzymes such as Sirtuins and PARPs (Revollo, Grimm et al. 2004).

In this study, we aimed to determine the mechanism accounting for reduced PARP-1 activity in CDA-deficient cells. Using a large scale metabolomic analysis, we found that CDA deficiency was associated with an increase in levels of NAM, the substrate of NAMPT, and a decrease in levels of NMN, the product of the reaction catalyzed by NAMPT, suggesting that NAMPT activity is probably weak in CDA-deficient cells. We confirmed a slight but significant decrease in NAMPT activity in CDA-deficient cells. We showed that NAMPT inhibition led to a decrease in basal PARP1 activity, regardless of NAD^+ levels. Finally, we found that the expression of exogenous wild-type NAMPT, but not of a mutated NAMPT protein, fully restored basal PARP-1 activity, and prevented the increase in UFB frequency in CDA-deficient cells. These findings demonstrate that the decrease in basal PARP-1 activity in CDA-deficient cells results from a decrease in NAMPT activity, thus revealing the first link between cytidine deaminase deficiency and nicotinamide metabolism.

RESULTS

CDA deficiency alters NAMPT nuclear activity

We searched for metabolic pathways altered in CDA-deficient cells by conducting a large-scale metabolomic study comprising 531 compounds (Metabolon, Inc., USA). We analyzed the metabolome of two pairs of isogenic cellular models of CDA deficiency: the GM8505B-derived BS cell line, which has a mutated *BLM* gene and strongly downregulated CDA expression (BS-$Ctrl_{(BLM)}$; BLM^-/CDA^-), and its counterpart stably expressing an exogenous GFP-BLM construct restoring the expression of both BLM and CDA (BS-BLM; BLM^+/CDA^+) (Chabosseau, Buhagiar-Labarchède et al. 2011, Gemble, Ahuja et al. 2015); and a HeLa cell line stably expressing an adenoviral short hairpin RNA (shRNA) specific for CDA, which therefore displayed strong CDA downregulation (HeLa-shCDA: BLM^+/CDA^-), and its control counterpart expressing CDA (HeLa-$Ctrl_{(CDA)}$; BLM^+/CDA^+) (Gemble, Ahuja et al. 2015). Among the detectable metabolites differentiating between CDA-deficient and CDA-proficient cells and common to both cell models were the pyrimidine metabolites cytidine and 2'-deoxycytidine. These metabolites were undetectable or present in very small amounts in CDA-expressing cells and were detected in significantly larger amounts in CDA-deficient cells (6-fold increase and 34-fold increase in cytidine in BS and in CDA-depleted HeLa cells, respectively; 3-fold increase and 1.28-fold increase in 2'-deoxycytidine in BS in CDA-depleted HeLa cell, respectively) (Figure S1A). Uridine and 2'-deoxyuridine levels were significantly lower in CDA-depleted HeLa cells (3-fold decrease and almost fully decrease, respectively)

than in control cells, but were present in similar amounts (uridine) or were undetectable (2'-deoxyuridine) in BS and BS-BLM cells (Figure S1B and data not shown). Thus, CDA deficiency led to an increase in cytidine and deoxycytidine levels, as expected, validating our approach and confirming our previous results (Chabosseau, Buhagiar-Labarchède et al. 2011, Gemble, Ahuja et al. 2015).

In this study aiming to identify the mechanism jeopardizing basal PARP-1 activity, we carefully investigated the NAD metabolism pathways to determine whether CDA deficiency affect the use of NAD^+ by PARP-1. By examining metabolomic data, no significant difference in NAD^+ levels were observed between CDA-deficient cells and control cells, suggesting that the reduced PARP-1 activity in CDA-deficient cells was independent of NAD^+ levels (Figure 1A). However, a significant change in NAD-related metabolites in BS cells relative to control cells were a 20% increase in NAM levels and a decrease of 18% and 30% in NMN levels were observed in CDA-deficient BS and HeLa cells, respectively (Figures 1B and 1C). As NAM is the substrate of NAMPT and NMN is the product of the reaction catalyzed by NAMPT, these data suggest that NAMPT activity might be weak in CDA-deficient cells, leading to an accumulation of NAM and a decrease in NMN levels.

In mammalian cells, NAMPT is present in intracellular (iNAMPT) and extracellular (eNAMPT) forms (Shackelford, Mayhall et al. 2013). The major function of iNAMPT is the NAD^+ biosynthesis from NAM (Garten, Petzold et al. 2009). Intracellular NAMPT is present mostly in the cytoplasm and nucleus, and its possible presence in mitochondria remains a matter of debate (Yang, Yang et al. 2007, Nikiforov, Dölle et al. 2011). The functional differences between nuclear and cytoplasmic NAMPT are unclear. For validation of the above results, we used an assay that measures the conversion of ^{14}C-NAM to ^{14}C-NMN by NAMPT (Elliott, Ajioka et al. 1980). Since PARP-1 is a nuclear protein and it performs PARylation within the nucleus, the NAMPT activity was assessed within the protein nuclear fraction of HeLa-Ctrl$_{(CDA)}$ cells treated with FK866, a specific NAMPT inhibitor (Hasmann and Schemainda 2003), to validate the assay (Figure S1C), and of untreated HeLa-Ctrl$_{(CDA)}$ and HeLa-shCDA cells (Figure 1D). We found that NAMPT activity was inhibited by about 60% in nuclear extracts from FK866-treated cells, as expected (Figure S1D). Moreover, we found that nuclear NAMPT activity levels were decrease by 23% in HeLa-shCDA cells relative to HeLa-Ctrl$_{(CDA)}$ cells (Figure 1E and 1G). Collectively, these results provide further support for a reduced NAMPT activity in CDA-deficient cells.

We previously reported that the main consequence of CDA deficiency was a pyrimidine pool disequilibrium consisting of an excess of dC and dCTP, accounting for several aspects of the

phenotype associated with CDA deficiency (Chabosseau, Buhagiar-Labarchède et al. 2011, Gemble, Ahuja et al. 2015). To investigate the potential relationship between pyrimidine pool imbalance and NAMPT activity, we cultured HeLa-Ctrl$_{(CDA)}$ and HeLa-shCDA cells in the presence of dC, which is known to induce dCTP accumulation (Chabosseau, Buhagiar-Labarchède et al. 2011). We found that dC treatment decreased NAMPT activity by 26% and 33% in HeLa control cells and in CDA-depleted HeLa cells, respectively (Figure 1F and 1G), suggesting that excess of dC/dCTP resulting from CDA deficiency decreases NAMPT activity.

NAMPT inhibition decreases basal PARP-1 activity, mimicking CDA deficiency

We then investigated whether decreased NAMPT activity could mimic CDA deficiency by reducing basal PARP-1 activity, by treating the two pairs of isogenic cell models of CDA deficiency with the NAMPT inhibitor FK866. We ensured that FK866 treatment did neither changed PARP-1, NAMPT or CDA protein levels (Figure 2A and S2A), nor inhibit recombinant PARP-1 protein activity (Figure S2B). We then assessed basal levels of PARylation by measuring the relative number of PAR foci (Gemble, Ahuja et al. 2015) (Figure S2C). FK866 treatment led to a significant decrease in PAR focus frequency in both CDA-proficient BS-BLM (26% decrease) and HeLa cells (30% decrease), down to the levels observed in CDA-deficient cells (Figures 2B and S2D). FK866 treatment also slightly but significantly decreased the frequency of PAR foci in both cell lines lacking CDA (20% decrease in BS cells and 29% decrease in CDA-depleted HeLa cells) (Figures 2B and S2D). We previously reported that the decrease in basal PARP-1 activity resulting from CDA deficiency leads to an increase in the frequency of UFB formation (Gemble, Ahuja et al. 2015). We therefore analyzed the frequency of UFBs in these cells by staining them with antibodies specific for the helicase-like protein PICH (Plk1-interaction checkpoint “helicase”), the only way to detect the total UFB population (Baumann, Körner et al. 2007) (Figure S2E). We found that FK866 treatment increased UFB frequency by 38% and 30% in the CDA-expressing cells BS-BLM and HeLa control cells, respectively, to levels similar to those in CDA-deficient cells but did not increase UFB frequency in CDA-deficient cells (Figures 2C and S2F).

For confirmation of these results, we transiently depleted NAMPT from the two pairs of isogenic cell lines by transfecting them with a pool of four NAMPT-targeting siRNAs. After siRNA transfection, we assessed changes in NAMPT protein levels (Figures 2D and S2G) and the frequencies of PAR foci and UFBs (Figures 2E, 2F, S2H and S2I). NAMPT depletion had no impact on PAR foci or UFBs frequencies in CDA-deficient cells (Figures 2E, 2F, S2H and S2I). However, it decreased PAR focus frequency by 23% and 30% in the CDA-proficient cells

BS-BLM and HeLa control cells, respectively (Figure 2E and S2H) and increased the frequency of UFBs by 60% and 22%, respectively in these cells to levels similar to those observed in CDA-deficient cells (Figures 2F and S2I). These results demonstrate that inhibiting or depleting NAMPT in CDA-proficient cells decreases the basal activity of PARP-1 and increases the frequency of UFBs to levels similar to those in CDA-deficient cells, thereby mimicking CDA deficiency. Since no additive effect of CDA deficiency and NAMPT inhibition or depletion on UFB frequency was observed, these results indicate that CDA and NAMPT likely act in the same pathway to prevent UFB formation.

The decrease in basal PARP-1 activity resulting from NAMPT inhibition is independent of NAD^+ levels

As NAMPT converts NAM to NMN, which is in turn converted to NAD^+ by NMNAT, the decrease in NMN levels resulting from NAMPT inhibition or depletion was expected to result in a decrease in NAD^+ levels. We therefore checked whether the decreased basal PARP-1 activity in response to NAMPT inhibition could be due to a decreased NAD^+ biosynthesis. NAD^+ levels, PAR foci and UFB frequencies were thus evaluated in CDA-proficient cells and CDA-deficient cells after NMN and/or FK866 treatments (Figure 3A). NMN treatment tends to increase NAD^+ levels in the four cell lines, whereas FK866 treatment decreased NAD^+ levels (49% and 43% in BS-BLM and BS cells, respectively; 57% and 49% in HeLa control and CDA-depleted HeLa cells, respectively) in these cells, as expected (Figures 3B and 3C). Moreover, addition of FK866 to NMN-treated cells did not decrease NAD^+ levels in both BS-BLM and BS cell lines, compared to FK866-treated cells that were not treated with NMN, and slightly decreased NAD^+ levels in both HeLa control and CDA-depleted HeLa cells (Figures 3B and 3C). Importantly, although the presence of NMN allowed the maintenance of high levels of NAD^+ in the four cell lines treated with FK866, it did not prevent the decrease in PAR focus frequency resulting from NAMPT inhibition by FK866 in the same cells, and the subsequent increase in UFB frequency in FK866-treated CDA-proficient cells (Figures 3D, 3E, 3F and 3G). As expected, the decrease in PARP-1 activity resulting from FK866-induced NAMPT inhibition did not further increased UFB frequency in CDA-deficient cells (Figures 3D, 3E, 3F and 3G). These results demonstrate that the decrease in basal PARP-1 activity resulting from NAMPT inhibition is, thus, completely independent of overall cellular NAD^+ concentration.

The decrease in basal PARP-1 activity in CDA-deficient cells is rescued by the overexpression of an exogenous wild-type NAMPT, but not by a mutated NAMPT

We investigated whether the levels of PARP-1 activity in CDA-deficient cells could be restored by the overexpression of an exogenous NAMPT, using a construct expressing either a wild-type (WT) NAMPT or the NAMPT protein with a mutated catalytic site (H247A). In parallel, two recombinant proteins — WT and H247A-mutated NAMPT proteins — were produced and their activity was assessed with the assay measuring the conversion of ^{14}C-NAM to ^{14}C-NMN. The wild-type NAMPT was highly active, whereas the mutated protein had much lower levels of activity (93% decrease), as expected (Figure S3A). The number of cells transfected with the wild-type or mutated NAMPT construct was similar for CDA-deficient cells and CDA-proficient cells (approximately 75%) (Figures 4A and 4B). We observed that the levels of expressed exogenous wild-type and mutated NAMPT proteins (NAMPT-His and the upper NAMPT band) were lower in CDA-depleted HeLa cells than in control cells, and that transfection of any NAMPT-expressing construct reduced PARP-1 protein levels (Figure 4C). However, importantly, the overexpression of either exogenous wild-type or mutated NAMPT did not affect the frequencies of PAR foci or UFBs in CDA-proficient HeLa cells, whereas the overexpression of wild-type NAMPT, but not of the mutated NAMPT, fully restored the frequency of PAR foci and, consequently, UFB frequency to normal levels in CDA-deficient cells (Figures 4D and 4E). We cannot exclude that the reduction of PARP-1 protein levels after transfection of NAMPT-expressing constructs (Figure 4C) did not influence the frequencies of PAR foci in HeLa control cell (Figure 4D), reflecting an excess of PARP-1 in the cells. Altogether these results show that basal PARP-1 activity is fully restored in CDA-deficient cells by the overexpression of an exogenous wild-type NAMPT, whereas the inactive NAMPT mutant has no effect in these cells. These data indicate that the lower basal PARP-1 activity in CDA-deficient cells results from the lower levels of NAMPT activity in these cells.

DISCUSSION

In this study, we identified, for the first time, an unexpected link between CDA and NAMPT, with CDA deficiency leading to a decrease in NAMPT activity. We also found that NAMPT inhibition or depletion in CDA-expressing cells reproduced the main feature of CDA deficiency: a decrease in basal PARP-1 activity leading to an increase in UFB frequency.

We previously showed that the pyrimidine pool disequilibrium resulting from CDA deficiency decreases basal PARP-1 activity, leading to UFB formation. Increasing the size of the intracellular dC and dCTP pools by culturing CDA-expressing cells in the presence of dC was

sufficient to decrease PARP-1 activity. However, our *in vitro* data were not consistent with the direct inhibition of PARP-1 by dC or dCTP (Gemble, Ahuja et al. 2015). Here we report that culturing CDA-expressing cells in the presence of dC was sufficient to decrease NAMPT activity, and that the inhibition or depletion of NAMPT activity lowered PARP-1 activity. These findings strongly suggest that the lower levels of basal PARP-1 activity in CDA-deficient cells result from the partial inhibition of NAMPT activity by the excess dC and dCTP. We tested this hypothesis by performing several experiments with the recombinant NAMPT protein in the presence of various concentrations of dC or dCTP, with an assay measuring the conversion of $^{14}C^{-}$NAM to ^{14}C-NMN, or an approach combining HPLC and high-resolution mass spectrometry for the selective detection of NAM and NMN. Neither of these experimental approaches yielded reproducible results and, despite our best efforts, we were unable to identify the cause of this lack of reproducibility. We cannot, therefore, exclude the possibility that the decrease in NAMPT activity was an indirect consequence of the excess of cellular dC and/or dCTP.

Our results, also show that NAMPT inhibition leads to a decrease in basal PARP-1 activity independently of total NAD^+ levels. However, these results need to be confirmed by measuring the amount of nuclear NAD^+ available for PARP-1. If confirmed in the nucleus, these results raise questions about the mechanism by which the decrease in NAMPT activity lowers PARP-1 activity. NAM is known to be a PARP-1 inhibitor (Rankin, Jacobson et al. 1989). We propose a hypothetical model in which the excess of NAM or of other unknown factors resulting from the decrease in NAMPT activity, decrease PARP-1 dependent PAR foci formation and lead to excess of UFBs (Figure 4F). However, UFB frequency was not affected when NAMPT was inhibited or depleted in CDA-deficient cells. This is in agreement with the data reported by Gemble *et al*. (2015) showing that a decrease in basal PARP-1 activity of about 30% led to the formation of a number of supernumerary UFBs similar to that observed following total PARP-1 inhibition or depletion, suggesting that there is a critical basal threshold of PARP-1 activity for full replication of the genome before the cells entering mitosis (Gemble, Ahuja et al. 2015). Here we observed that a decrease in basal NAMPT inhibition of 23% in CDA-deficient cells is sufficient to reduced PARP-1 activity, indicating a critical basal threshold of NAMPT activity is necessary for an optimal PARP-1 activity. Our study shed light on a new mechanism where decrease in NAMPT activity impairs PARP-1 activity.

Our data also reveal that total NAD^+ quantities were unchanged or increased in CDA-deficient cells, and that the decrease in PARP-1 activity resulting from the inhibition of NAMPT activity

was independent of cellular NMN and NAD^+ levels. These results indicate a cellular maintenance of NAD^+ levels, despite NAMPT inhibition. This point is of particular interest since cancer cells require high levels of NAD^+, and such mechanism might be involved in the resistance to anti-cancer treatments, in particular those targeting NAMPT (Guo, Lam et al. 2017). The activation of the *de novo* NAD^+ biosynthesis pathway via upregulation of quinolinate phosphoribosyl transferase (QPRT) has been reported as a resistance mechanism to NAMPT inhibition (Guo, Lam et al. 2017). Our metabolomic study did not detect metabolites from the *de novo* NAD^+ biosynthesis pathway, except tryptophan, the levels of which were found increased in CDA-depleted HeLa cells, but not in BS cells (Figure S3B) indicating that this mechanism is unlikely to be involved in the maintenance of NAD^+ levels in CDA-deficient cells. Another possibility is that the decrease in NMN levels we observed in both cellular models of CDA-deficiency reflect the partial inhibition of NAMPT activity that we reported and, in addition, an increase in the conversion of NMN to NAD^+ by NMNAT. Finally, since PARP-1 activity is partially inhibited in CDA-deficient cells, it is expected to consume less NAD^+ that might contribute to maintain cellular NAD^+ levels.

In conclusion, our results demonstrate that, in CDA-deficient cells, NAMPT activity is reduced, thereby lowering basal PARP-1 activity. In several preclinical models, combination of NAMPT and PARP-1 inhibitors resulted in cancer cell death and tumor regression, revealing a synthetic lethal interaction between NAMPT and PARP-1 deficiencies (Bajrami, Kigozi et al. 2012, Heske, Davis et al. 2017). Our study suggests that the concomitant reduction of NAMPT and PARP-1 activities in CDA-deficient tumor cells might be associated with a better prognosis.

Methods

Cell lines and drug treatments. BS GM08505B and HeLa cells were purchased from the Coriell Institute and ATCC, respectively (Chabosseau, Buhagiar-Labarchède et al. 2011, Gemble, Ahuja et al. 2015). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Eurobio) supplemented with 10% FCS. BS-$Ctrl_{(BLM)}$ and BS-BLM cells were obtained by transfecting BS GM08505B cells with the EGFP-C1 vector alone (Clontech) or with the same vector containing the full-length BLM cDNA, respectively, in the presence of JetPEI reagent (Ozyme). After 48 h, transfectants were selected on 800–1600 $\mu g.ml^{-1}$ G418 (PAA). Individual colonies were isolated and cultured in medium containing 500.$\mu g\ ml^{-1}$ G418. HeLa-$Ctrl_{(CDA)}$ and HeLa-shCDA cells were obtained by transfecting cells with an empty pGIPZ vector or with the same vector encoding a short hairpin RNA sequence directed against

CDA (Open Biosystems, clone V3LHS_369299), respectively, in the presence of JetPEI reagent. After 48 h, transfectants were selected on 1–5 μg ml^{-1} puromycin (Invivogen). Individual colonies were isolated and cultured in medium containing 1 μg ml^{-1} puromycin. All cell lines were routinely checked for mycoplasma infection. Authenticity was assessed by comparing the short tandem repeat profile generated with the profiles present in the Deutsche Sammlung von Mikroorganismen und Zellkulturen.

Deoxycytidine (dC, # D0776), nicotinamide mononucleotide (NMN #N3501), and FK866 were purchased from Calbiochem (#481908). Drugs were added to the cell culture medium at the following concentrations and for the following amounts of time: FK866, 1μM for 10 h; dC, 1 mM for 10 h; and NMN, 500 μM for 24 h.

Large-scale metabolomic analysis. The metabolomics analysis was performed by Metabolon, Inc (Durham, NC, www.metabolon.com). The cells were cultured and collected according to the sample preparation guidelines provided by Metabolon. Cell pellets were flash-frozen and sent to Metabolon for standard solvent extraction and metabolomic analysis.

Transfection with siRNA. Cells were transfected with a pool of four siRNAs specific for NAMPT (ON-TARGETplus SMART-pool, Dharmacon) or negative control siRNAs (ON-TARGETplus siCONTROL Non Targeting Pool, Dharmacon), in the presence of DharmaFECT 1 (Dharmacon). We used a standard siRNA concentration of 100 nM. The sequences of the siRNAs are provided in Supplementary Table 1.

NAD^+ quantification. NAD^+ levels were quantified with a luciferase assay provided in the NAD^+/NADH Glo Assay kit (#G9071, Promega), used according to the manufacturer's instructions. NAD^+ levels were normalized against cell viability in a CellTiter-Glo® Luminescent Cell Viability Assay (#G7570, Promega), according to the manufacturer's instructions. Luminescence was read on a Tristar2 multimode microplate reader (Berthold Technologies).

Immunoblotting. Proteins were isolated in lysis buffer (8 M urea, 50 mM Tris-HCl, pH 7.5, and 150 mM β-mercaptoethanol), separated by electrophoresis in 4–12% Bis-Tris pre-cast gels (NuPAGE Novex, Life Technologies), and blotted onto polyvinylidene fluoride (PVDF) membranes, which were then incubated with appropriate primary and secondary antibodies.

The following antibodies were used for detection: rabbit anti-NAMPT (#A300-372A, Bethyl Laboratories, Inc., dilution 1:20000), rabbit anti-PARP-1 (#ALX-210-302-R100, Enzo Life Sciences, 1:4000), rabbit anti-CDA (#ab56053, Abcam, dilution 1:500), rabbit anti-BLM (#ab2179, Abcam, dilution 1:5000), rabbit anti-β-actin (#A2066, Sigma-Aldrich, dilution 1:5000), rabbit anti-HSP90 (#ab2928, Abcam, dilution 1:5000), mouse anti-GAPDH (#G8796, Sigma-Aldrich, dilution 1:5000), rabbit anti-ERK42/44 (#4377, Cell Signaling, dilution 1:1000), mouse anti-His-tag (#66005-I, Proteintech, dilution 1:1000), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (#A9169, Sigma-Aldrich, dilution 1:5000), and HRP-conjugated goat anti-mouse IgG (#A3682, Sigma-Aldrich, 1:5000). Bands were visualized by chemiluminescence (Clarity Western ECL Substrate, Bio-Rad), with a ChemiDoc XRS+Molecular Imager and Image Lab Software (Bio-Rad).

Immunofluorescence microscopy. Immunofluorescence staining and analysis were performed as previously described (Rouzeau, Cordelieres et al. 2012). Primary and secondary antibodies were used at the following concentrations: rabbit anti-PICH antibody (H00054821-D01P, Abnova, dilution 1:150), and goat anti-rabbit Alexa Fluor 555 (#A21429, Life Technologies, dilution 1:500). Cell images were acquired with a 3-D deconvolution imaging system consisting of a Leica DM RXA microscope equipped with a piezoelectric translator (PIFOC; PI) placed at the base of a 63x PlanApo N.A. 1.4 objective, and a CoolSNAP HQ interline CCD camera (Photometrics). Stacks of conventional fluorescence images were collected automatically at a *Z*-distance of 0.2 mm (Metamorph software; Molecular Devices). NH_4Cl incubation was not performed for His-tag staining. The primary and secondary antibodies for His-tag staining were used at the following concentrations: rabbit anti-His-tag (#66005-I, Proteintech, dilution 1:500), and goat anti-mouse Alexa Fluor 555 (#A21050, Life Technologies, dilution 1:500).

Poly(ADP)-ribose immunofluorescence. Poly(ADP)-ribose immunofluorescence was analyzed as previously described (Gemble, Ahuja et al. 2015). Briefly, we used 4×10^5 HeLa cells or 8×10^5 BS-Ctrl$_{(BLM)}$ or BS-BLM cells to seed the wells of a six-well plate. The cells were washed in cold PBS on ice and fixed by incubation in a 1:1 (vol/vol) mixture of methanol and acetone for 10 minutes on ice. After three washes in PBS-Tween (0.05%), cells were incubated overnight at 4°C with a mouse anti-PAR antibody (dilution 1:500; generously provided by Valérie Schreiber (Illuzzi, Fouquerel et al. 2014)). The cells were washed three times with PBS-Tween (0.05%) and incubated with a goat anti-mouse Alexa Fluor 555-conjugated antibody (A21050, Life Technologies, dilution 1:500) for 2 h. The cells were

washed twice in PBS-Tween (0.05%), and mounted on slides with Prolong Gold with DAPI (P36931 from Life Technologies). Cell images were acquired with a 3-D deconvolution imaging system consisting of a Leica DM RXA microscope equipped with a piezoelectric translator (PIFOC; PI) placed at the base of a 63x PlanApo N.A. 1.4 objective, and a CoolSNAP HQ interline CCD camera (Photometrics). Stacks of conventional fluorescence images were collected automatically at a *Z*-distance of 0.2 mm (Metamorph software; Molecular Devices). The images shown are the maximum-intensity projections generated with Image J software, from stacks deconvolved with an extension of Metamorph software (Savino, Gebrane-Younes et al. 2001). The number of PAR foci per nucleus was determined by semi-automated counting with a customized macro, as follows: the nucleus stack was first smoothed with a median filter (radius 5), with a user-defined intensity value as a threshold (one value for all experiments); a mask was then generated and transferred onto the stack of foci, to ensure that only foci in nuclei were analyzed. A TopHat filter was applied to the result to eliminate local background and to facilitate the segmentation process, based on a simple threshold (user-defined value). Finally, the macro counted and characterized the foci.

Colorimetric PARP assay kit candidate inhibitor screening: PARP-1 inhibition by FK866 was assessed with the HT universal colorimetric PARP assay kit, with histone-coated strip wells (4677-096-K from Trevigen), according to the manufacturer's instructions.

Site-directed mutagenesis. Site-directed mutagenesis was performed with the pPM-C-His vector carrying the full-length cDNA for human NAMPT (GenBank accession no. BC072439). The His codon in position 247 of NAMPT was mutated into an Ala (H27A) codon with the QuikChange XL site-directed mutagenesis kit (#200251, Agilent), according to the manufacturer's instructions. The primer sequences are provided in Supplementary Table 2.

Vector transfection. Cells were transfected by incubation with pPM-C-His vector empty vector, NAMPT WT, NAMPT H247A in the presence of JetPrime (Polyplus Transfection) for 48 h.

Protein production and purification. A pPM-C-His plasmid carrying the full-length cDNA for human NAMPT was purchased from Applied Biological Materials (abmgood). The NAMPT cDNA was amplified by PCR with primers containing *Nhe*I and *Xho*I sites. The amplified DNA fragment was inserted between the *Nhe*I and *Xho*I sites of pET24a(+)(#69749-

3, Novagen), resulting in the attachment of a his_6 tag at the C-terminus of the protein. A site-directed mutation (H247A) was generated with the QuikChange XL site-directed mutagenesis kit (#200251, Agilent) according to the manufacturer's instructions. The primer sequences are provided in Supplementary table 2. Both constructs were overexpressed in BL21 Dsbc plSO in 1 liter of Terrific Broth. Expression was induced with 0.5% arabinose and 1 mM IPTG at 20°C overnight, with shaking at 160 rpm. The cells were harvested by centrifugation at 4000 x *g* for 15 min at 4°C.

The cell pellets were suspended in lysis buffer (20 mM Tris-HCl pH 8, 500 mM NaCl,10% glycerol, 1 mM TCEP and 1 x Complete EDTA-free protease inhibitor cocktail (Roche)). The cell suspension was disrupted by passage through a T75 cell disruptor (Constant Systems). The resulting cell lysate was centrifuged at 43000 x *g* for 1 hour at 4°C. The supernatant was applied to a HisTrap HP column (GE Healthcare), washed thoroughly and the proteins were eluted in elution buffer (20 mM Tris-HCl pH 8, 500 mM NaCl,10% glycerol, 0.5 mM TCEP, 400 mM imidazole). After overnight dialysis against 20 mM Tris-HCl pH 8, 100 mM NaCl,10% glycerol, 0.5 mM TCEP, the eluate was loaded onto a Capto Q ImpRes ion exchange column (GE Healthcare) for elution with a continuous gradient NaCl (0.1 -1 M) in the same buffer. Fractions containing NAMPT were dialyzed against 20 mM Tris-HCl pH 8, 500 mM NaCl,10% glycerol, 0.5 mM TCEP, and loaded on a HisTrap HP column. NAMPT was eluted with an imidazole gradient. NAMPT-containing fractions were pooled and dialyzed against 20 mM Tris-HCl pH 8, 100 mM NaCl,10% glycerol, 0.5 mM TCEP. The protein was visualized by SDS-PAGE in a 4-20% acrylamide gel and protein concentration was determined by measuring absorbance at 280 nm. The mutant protein was purified with the same protocol as the wild-type protein.

NAMPT enzyme assay. NAMPT activity was assessed by measuring the conversion of ^{14}C-labelled NAM into ^{14}C-NMN. We added 50 µL of NAMPT reaction mixture [50 mM Tris-HCl, pH 7.4, 2 mM ATP, 5 mM $MgCl_2$, 0.5 mM PRPP and 6.2 µM ^{14}C-NAM (American Radiolabeled Chemicals, USA)] to the NAMPT nuclear lysates (50 µg), which were then incubated at 37°C for 1 h. The reaction was stopped by adding 1.6 mL acetone. The mixture was then transferred onto glass microfiber filters (GF/A Ø 24 mm, Whatman) soaked in acetone. The filters were rinsed twice with 1 mL acetone each, dried, and transferred to vials containing 4 mL scintillation cocktail (Perkin Elmer), and the radioactivity of ^{14}C-NMN was quantified in a liquid scintillation counter, in disintegrations per minute (dpm), with a Tri-Carb 2910 TR machine (PerkinElmer). NAMPT activity was normalized against total protein concentration,

as determined in the Qubit protein assay. The specific NAMPT inhibitor FK866 was added as a control, to evaluate the validity of the assay. The same protocol was performed for the NAMPT recombinant (1µg).

Cellular fractionation. Cells were harvested, resuspended in 0.01 M sodium phosphate buffer, pH 7.4 (15 µL/10^6 cells), and frozen overnight at -80°C. The lysates were then thawed at room temperature and the cell debris was removed by centrifugation at 23,000 x *g*, for 90 min at 2°C. The supernatant was collected and stored at -80°C (cytoplasmic fraction). The pellet was rinsed with sodium phosphatase buffer and centrifuged at 16,000 x *g* for 10 min at 4°C. The pellets were then resuspended (12.5 µL/10^6 cells) in a nuclear fraction mixture (10 mM HEPES pH 7.8, 1.5 mM $MgCl_2$, 25% glycerol, 400 mM KCl, 0.2 mM EDTA, PMSF, DTT and 1x anti-protease cocktail (#11697498001, Roche) and incubated for 20 min on a bed of ice. The lysates were frozen in liquid nitrogen for 30 seconds and allowed to thaw for 7 min at room temperature. Finally, the mixture was centrifuged at 16,000 x *g*, for 10 min at 4°C and the supernatant was collected (nuclear fraction) and stored at -80°C. Protein concentration was determined in the Qubit protein assay (Life Technologies). Fractionation was checked by the immunoblotting of 25 µg protein: BLM was used as a control for the nuclear fraction.

Statistical analysis. At least three independent experiments were carried out to generate each data set. The statistical significance of differences was calculated with two-tailed unpaired Student's t-tests.

Supplementary methods

Supplementary Table 1

siRNA	Sequence 5’ to 3’	description	Reference
Non-targeting siRNA pool	UGGUUUACAUGUCGACUAA	ONTARGETplus SMART-pool, Dharmacon Ref : L-004581-00-0005	(Chabosseau, Buhagiar-Labarchède et al. 2011)
	UGGUUUACAUGUUGUGUGA		
	UGGUUUACAUGUUUUCUGA		
	UGGUUUACAUGUUUUCCUA		
siNAMPT pool	GGUAAGAGUUUCCUGUUA		This work
	CAAAUUGGAUUGAGACUAU		
	UAACUUAGAUGGUCUGGAA		
	CAAGCAAAGUUUAUUCCUA		

Supplementary table 2

PCR primers		Sequence 5’ to 3’	
NAMPT – H247A	**F**	GTTCCAGCAGCAGAAGCCAGTACCATAACAGCT	This work
NAMPT – H247A	**R**	AGCTGTTATGGTACTGGCTTCTGCTGCTGGAAC	This work
NheI	**F**	TTTGTTTAACTTTAAGAAGGAGATATACAT ATGAATCCTGCGGCAGAAGCCGA	This work
XhoI	**R**	ATCTCAATGGTGATGGTGATGGTGCTCGAG ATGATGTGCTGCTTCCAGTC	This work

Acknowledgments

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Figure 1

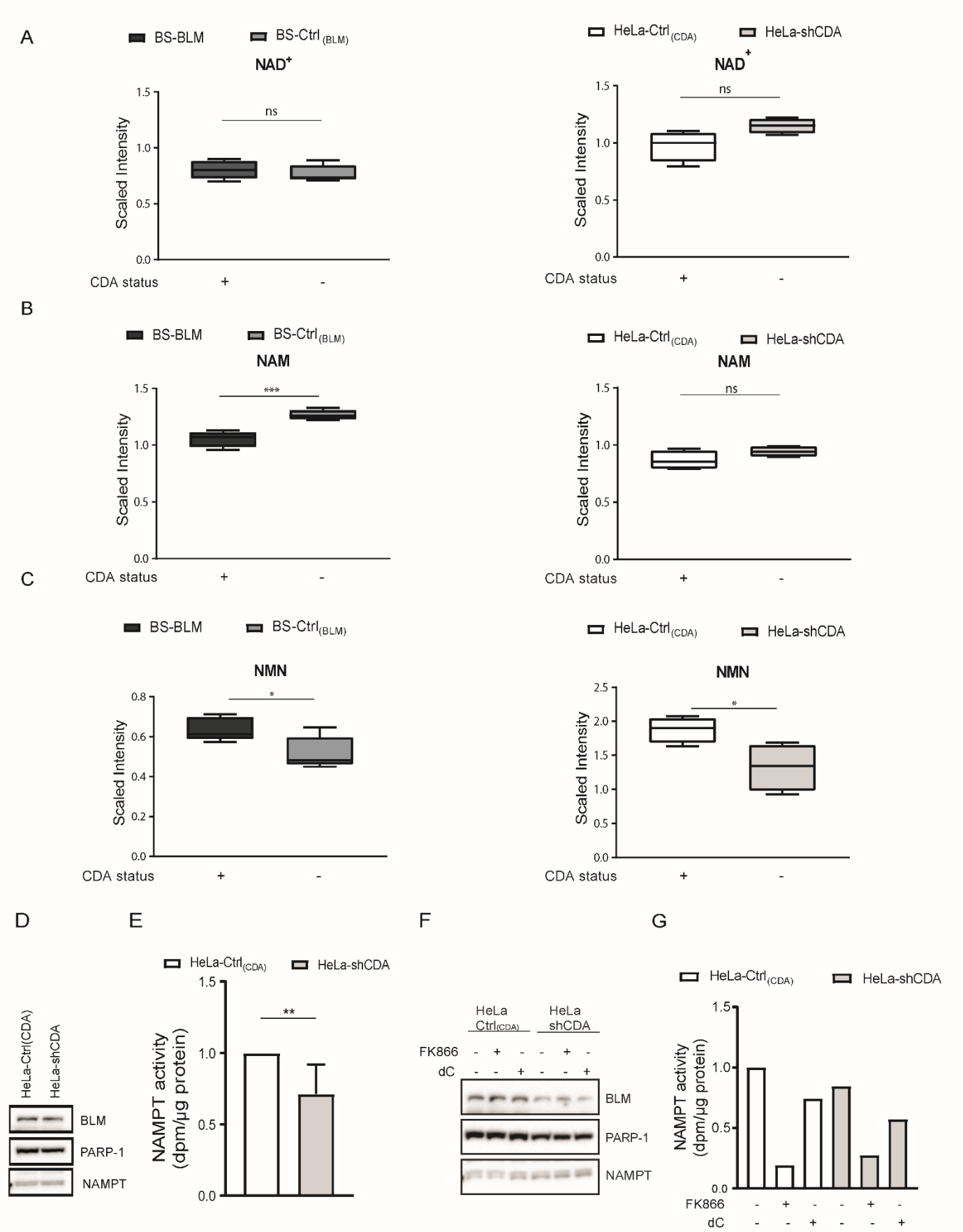

A
BS-BLM
BS-Ctrl (BLM)
NAD+
ns
Scaled Intensity
CDA status
HeLa-Ctrl (CDA)
HeLa-shCDA
B
NAM
***
C
NMN
*
D
HeLa-Ctrl(CDA)
HeLa-shCDA
BLM
PARP-1
NAMPT
E
**
NAMPT activity (dpm/µg protein)
F
HeLa Ctrl(CDA)
HeLa shCDA
FK866
dC
G

Figure 2

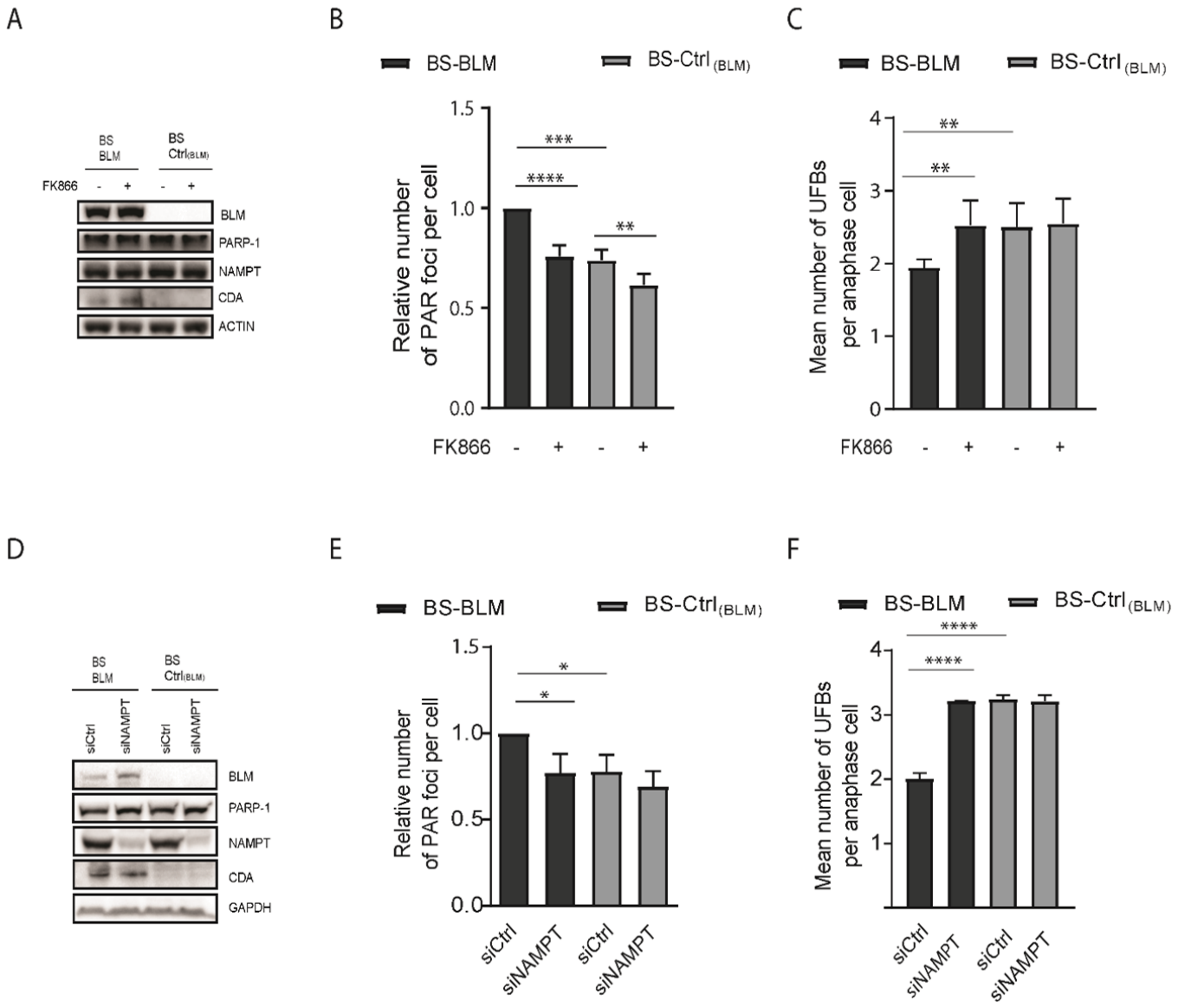

A
BS BLM
BS Ctrl(BLM)
FK866 - + - +
BLM
PARP-1
NAMPT
CDA
ACTIN
B
BS-BLM
BS-Ctrl (BLM)
Relative number of PAR foci per cell
1.5
1.0
0.5
0.0
***
****
**
FK866 - + - +
C
BS-BLM
BS-Ctrl (BLM)
Mean number of UFBs per anaphase cell
4
3
2
1
0
**
**
FK866 - + - +
D
BS BLM
BS Ctrl(BLM)
siCtrl
siNAMPT
siCtrl
siNAMPT
BLM
PARP-1
NAMPT
CDA
GAPDH
E
BS-BLM
BS-Ctrl(BLM)
Relative number of PAR foci per cell
1.5
1.0
0.5
0.0
*
*
siCtrl
siNAMPT
siCtrl
siNAMPT
F
BS-BLM
BS-Ctrl (BLM)
Mean number of UFBs per anaphase cell
4
3
2
1
****
****
siCtrl
siNAMPT
siCtrl
siNAMPT

Figure 3

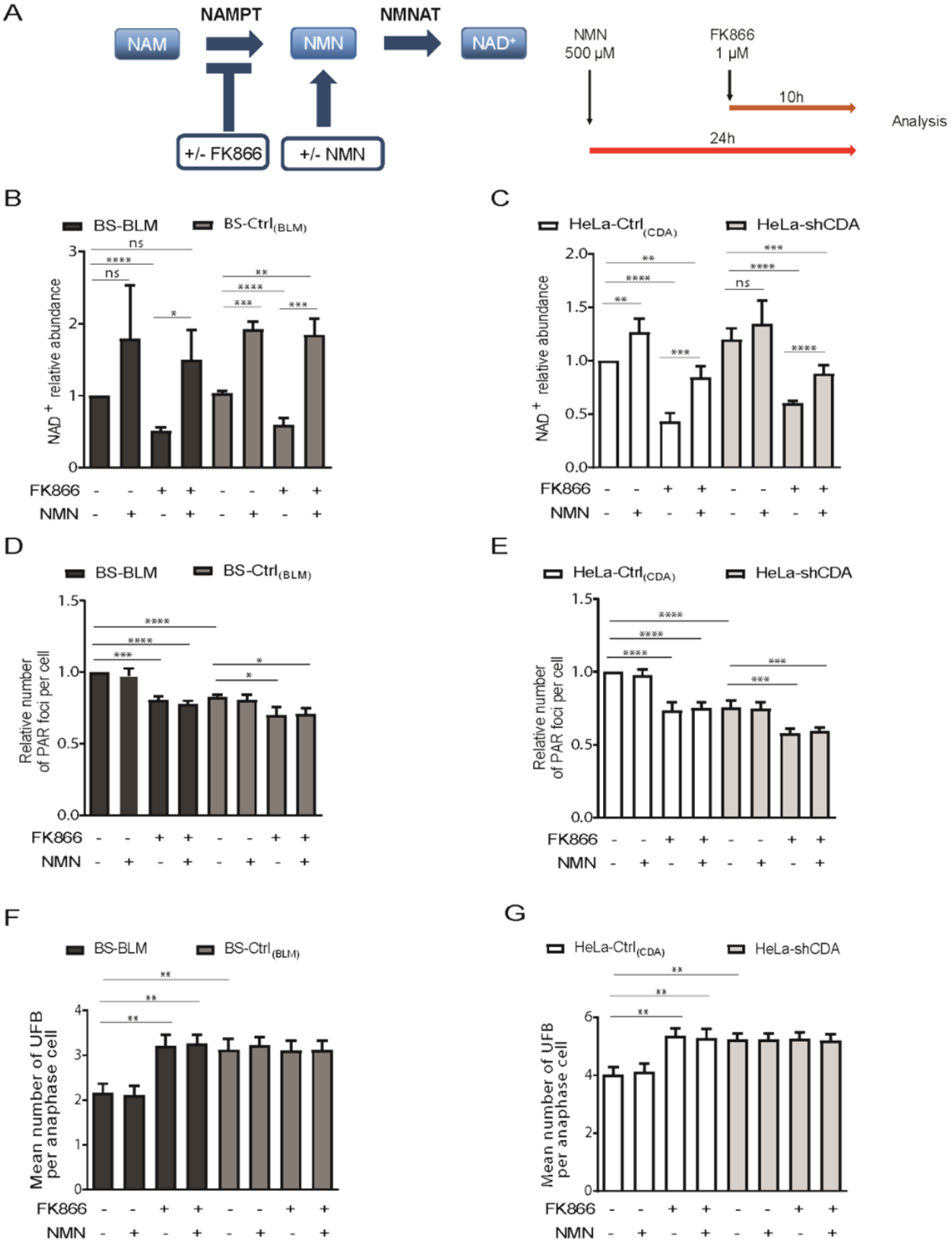

A
NAMPT
NMNAT
NAM
NMN
NAD+
+/- FK866
+/- NMN
NMN
500 µM
FK866
1 µM
10h
24h
Analysis
B
BS-BLM
BS-Ctrl(BLM)
NAD+ relative abundance
FK866
NMN
C
HeLa-Ctrl(CDA)
HeLa-shCDA
NAD+ relative abundance
FK866
NMN
D
BS-BLM
BS-Ctrl(BLM)
Relative number of PAR foci per cell
FK866
NMN
E
HeLa-Ctrl(CDA)
HeLa-shCDA
Relative number of PAR foci per cell
FK866
NMN
F
BS-BLM
BS-Ctrl(BLM)
Mean number of UFB per anaphase cell
FK866
NMN
G
HeLa-Ctrl(CDA)
HeLa-shCDA
Mean number of UFB per anaphase cell
FK866
NMN

Figure 4

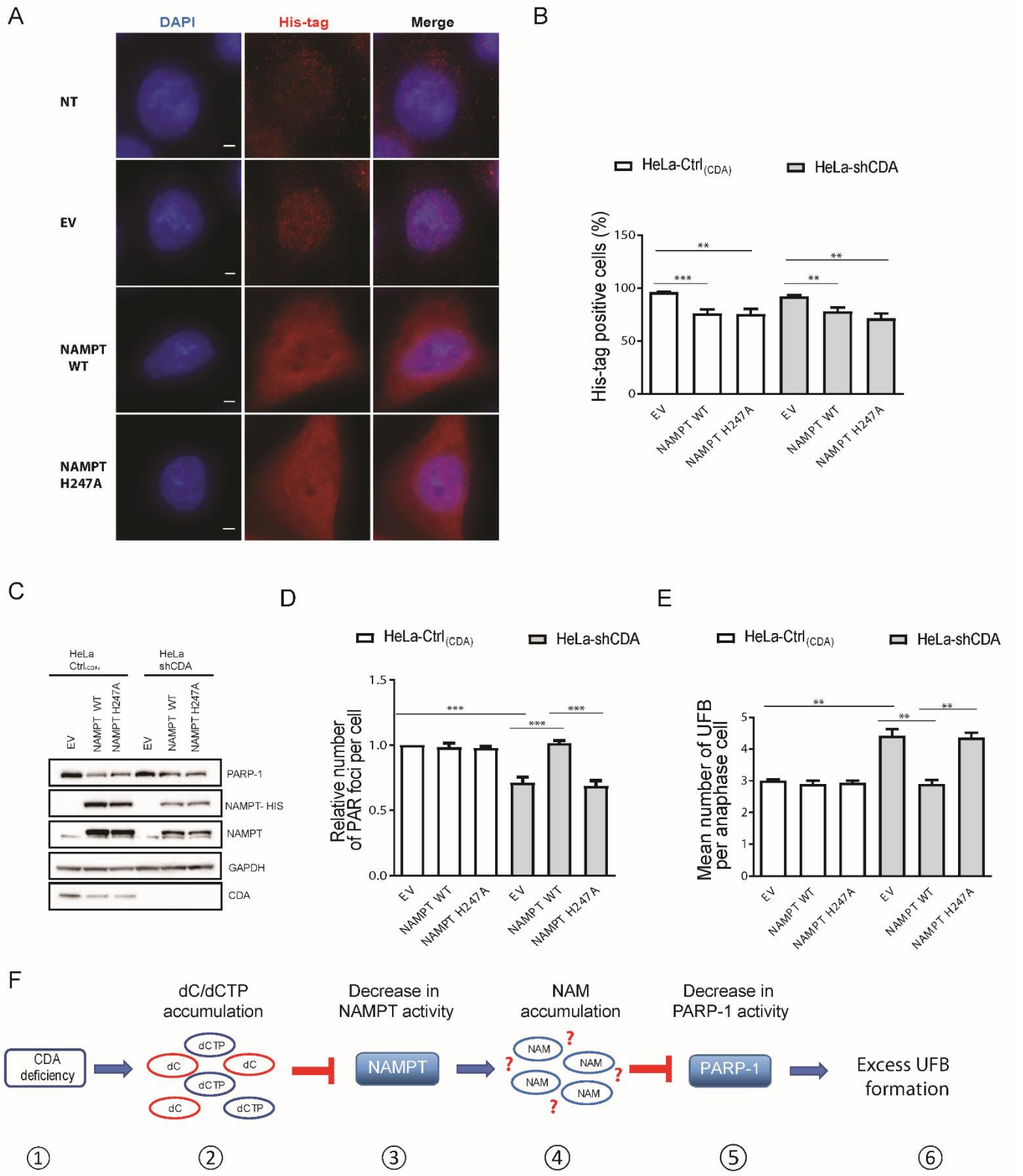

A
DAPI
His-tag
Merge
NT
EV
NAMPT WT
NAMPT H247A
B
HeLa-Ctrl(CDA)
HeLa-shCDA
His-tag positive cells (%)
150
100
50
0
**
***
**
**
EV
NAMPT WT
NAMPT H247A
EV
NAMPT WT
NAMPT H247A
C
HeLa Ctrl(CDA)
HeLa shCDA
EV
NAMPT WT
NAMPT H247A
EV
NAMPT WT
NAMPT H247A
PARP-1
NAMPT- HIS
NAMPT
GAPDH
CDA
D
HeLa-Ctrl(CDA)
HeLa-shCDA
Relative number of PAR foci per cell
1.5
1.0
0.5
0.0
***
***
***
EV
NAMPT WT
NAMPT H247A
EV
NAMPT WT
NAMPT H247A
E
HeLa-Ctrl(CDA)
HeLa-shCDA
Mean number of UFB per anaphase cell
5
4
3
2
1
0
**
**
**
EV
NAMPT WT
NAMPT H247A
EV
NAMPT WT
NAMPT H247A
F
CDA deficiency
dC/dCTP accumulation
dCTP
dC
dC
dCTP
dC
dCTP
Decrease in NAMPT activity
NAMPT
NAM accumulation
NAM
NAM
NAM
NAM
Decrease in PARP-1 activity
PARP-1
Excess UFB formation
①
②
③
④
⑤
⑥

Figure legends

Figure 1. NAD metabolism is altered in CDA-deficient cells. (A-C) Box plots were used to represent the three key metabolites (NAD^+, NAM and NMN) in BS-BLM (in grey), BS-$Ctrl_{(BLM)}$ (in medium grey), HeLa-$Ctrl_{(CDA)}$ (in white) and HeLa-shCDA (in light grey). The central 50% of the data is represented by the shaded boxes and the range of the data is indicated by the whiskers. The solid horizontal bar indicates the median value of the measured values. Data were scaled such that the median value across all samples was set to 1. The data presented are means $\pm$ SD from at least four independent experiments. All the values were generated by Metabolon Inc. (USA). **(D)** Nuclear BLM, PARP-1 and NAMPT protein levels, assessed by immunoblotting on HeLa-$Ctrl_{(CDA)}$ and HeLa-shCDA cells. **(E)** Measurement of basal levels of nuclear NAMPT activity in both HeLa-$Ctrl_{(CDA)}$ (in white) and in HeLa-shCDA (in light grey) cells. The data shown are the mean $\pm$ SD from five independent experiments. **(F)** Nuclear PARP-1 and NAMPT levels assessed by immunoblotting in HeLa-$Ctrl_{(CDA)}$ and HeLa-shCDA cells left untreated or treated with 1 μM FK866 or 1 mM dC for 10 h. The significance of differences was assessed with Student's *t*-test (ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). **(G)** Nuclear NAMPT activity in HeLa-Ctrl $_{(CDA)}$ (in white) and HeLa-shCDA (in light grey) cells left untreated or treated with 1 μM FK866 or 1 mM dC for 10h (n=2).

Figure 2. NAMPT inhibition or depletion impairs PARP-1 activity. (A) BLM, PARP-1, NAMPT and CDA protein levels assessed by immunoblotting in BS-BLM and BS-$Ctrl_{(BLM)}$ cell lines left untreated or treated with 1 μM FK866 for 10 h. **(B)** Relative number of PAR foci in BS-BLM (in grey) and BS-$Ctrl_{(BLM)}$ (in medium grey) cell lines left untreated or treated with 1 μM FK866 for 10 h. The data shown are the means $\pm$ SD from four independent experiments (> 500 cells per condition). **(C)** Mean number of UFBs per anaphase cell, for BS-BLM (in grey) and BS-$Ctrl_{(BLM)}$ (in medium grey) cell lines left untreated or treated with 1 μM FK866 for 10 h. The data shown are the means $\pm$ SD from three independent experiments (> 80 anaphase cells per condition). **(D)** BLM, PARP-1 and NAMPT protein levels assessed by immunoblotting in BS-BLM and BS-$Ctrl_{(BLM)}$ cell lines transiently transfected with the indicated siRNAs. **(E)** Relative number of PAR foci in BS-BLM (in grey) and BS-$Ctrl_{(BLM)}$ (in medium grey) cell lines transiently transfected with the indicated siRNAs. The data shown are

the means ± SD from four independent experiments (> 350 cells per condition). **(F)** Mean number of UFBs per anaphase cell, for BS-BLM (in grey) and BS-Ctrl$_{(BLM)}$ (in medium grey) cell lines transiently transfected with the indicated siRNAs. The data shown are means ± SD from three independent experiments (> 120 anaphase cells per condition). The significance of differences was assessed with Student's *t*-test (ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

Figure 3. The decrease in PARP-1 activity resulting from NAMPT inhibition is independent of NAD$^+$ levels. (A) Schematic representation of FK866 and NMN treatments in both CDA-proficient and CDA-deficient cells (left panel). Concentrations and time of NMN and FK866 treatments in CDA-proficient and in CDA-deficient cells (right panel). MNN treatment was first added to cell culture medium for a duration of 24h and FK866 treatment was added to the medium 14h later for a duration of 10h prior analysis of NAD$^+$ levels, PAR foci and UFB frequency. **(B)** Measurement of intracellular NAD$^+$ levels in BS-BLM (in grey) and BS-Ctrl$_{(BLM)}$ (in medium grey) cell lines left untreated or treated with 1 μM FK866 for 10 h and/or with 500 μM NMN for 24 h. The data shown are means ± SD from three independent experiments. **(C)** Measurement of intracellular NAD$^+$ levels in HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA (in light grey) cell lines left untreated or treated with 1 μM FK866 for 10h and/or with 500 μM NMN for 24 h. The data shown are means ± SD from five independent experiments. **(D)** Relative number of PAR foci in BS-BLM (in grey) and BS-Ctrl$_{(BLM)}$ (in medium grey) cell lines left untreated or treated with 1 μM FK866 for 10 h and/or with 500 μM NMN for 24 h. The data shown are means ± SD from three independent experiments (> 400 cells per condition). **(E)** Relative number of PAR foci in HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA (in light grey) cell lines left untreated or treated with 1 μM FK866 for 10 h and/or with 500 μM NMN for 24 h. The data shown are means ± SD from four independent experiments (> 400 cells per condition). **(F)** Mean number of UFBs per anaphase cell, for BS-BLM (in grey) and BS-Ctrl$_{(BLM)}$ (in medium grey) cell lines left untreated or treated with 1 μM FK866 for 10 h and/or with 500 μM NMN for 24 h. The data shown are means ± SD from three independent experiments (> 120 anaphase cells per condition). **(G)** Mean number of UFBs per anaphase cell, for HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA (in light grey) cell lines left untreated or treated with 1 μM FK866 for 10 h and/or with 500 μM NMN for 24 h. The data shown are means ± SD from three independent experiments (> 100 anaphase cells per condition). The

significance of differences was assessed with Student's *t*-test (ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

Figure 4. The low levels of PARP-1 activity in CDA-deficient cells are rescued by the overexpression of wild-type NAMPT (A) Representative immunofluorescence deconvoluted *z*-projection images showing DAPI and His-tag staining in HeLa-Ctrl$_{(CDA)}$ cell line not transfected (NT) or transiently transfected with pPM-C-His empty vector (EV), or with a pPM-C-His construct expressing wild-type NAMPT (NAMPT WT) or mutated NAMPT (NAMPT H247A). Nuclei were visualized by DAPI staining (blue) and His-tag was visualized with Alexa Fluor 555 (red). Scale bar: 5 µm **(B)** Percentage of His-tag-positive cells in HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA (in light grey) cell lines transiently transfected with EV, NAMPT WT or NAMPT H247A. The data shown are means ± SD from four independent experiments. **(C)** Levels of the PARP-1, NAMPT-HIS, NAMPT and CDA proteins assessed by immunoblotting in HeLa-Ctrl$_{(CDA)}$ and HeLa-shCDA cells transiently transfected with EV, NAMPT WT or NAMPT H247A. **(D)** Relative number of PAR foci in HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA) (in light grey) cell lines transiently transfected with EV, NAMPT WT or NAMPT H247A. The data shown are means ± SD from four independent experiments (> 800 cells per condition). **(E)** Mean number of UFBs per anaphase cell, for HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA (in light grey) cell lines transiently transfected with EV, NAMPT WT or NAMPT H247A. Errors bars represent means ± SD from three independent experiments (> 120 anaphase cells per condition). The significance of differences was assessed in Student's *t*-tests (ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). **(F)** (1) CDA deficiency leads to (2) intracellular dC/dCTP accumulation that (3) decreases NAMPT activity, leading to the (4) intracellular accumulation of NAM, an inhibitor of PARP-1 or other unknown factors. (5) Excess NAM results in lower levels of PARP-1 activity, causing (6) excess UFB formation.

Figure S1

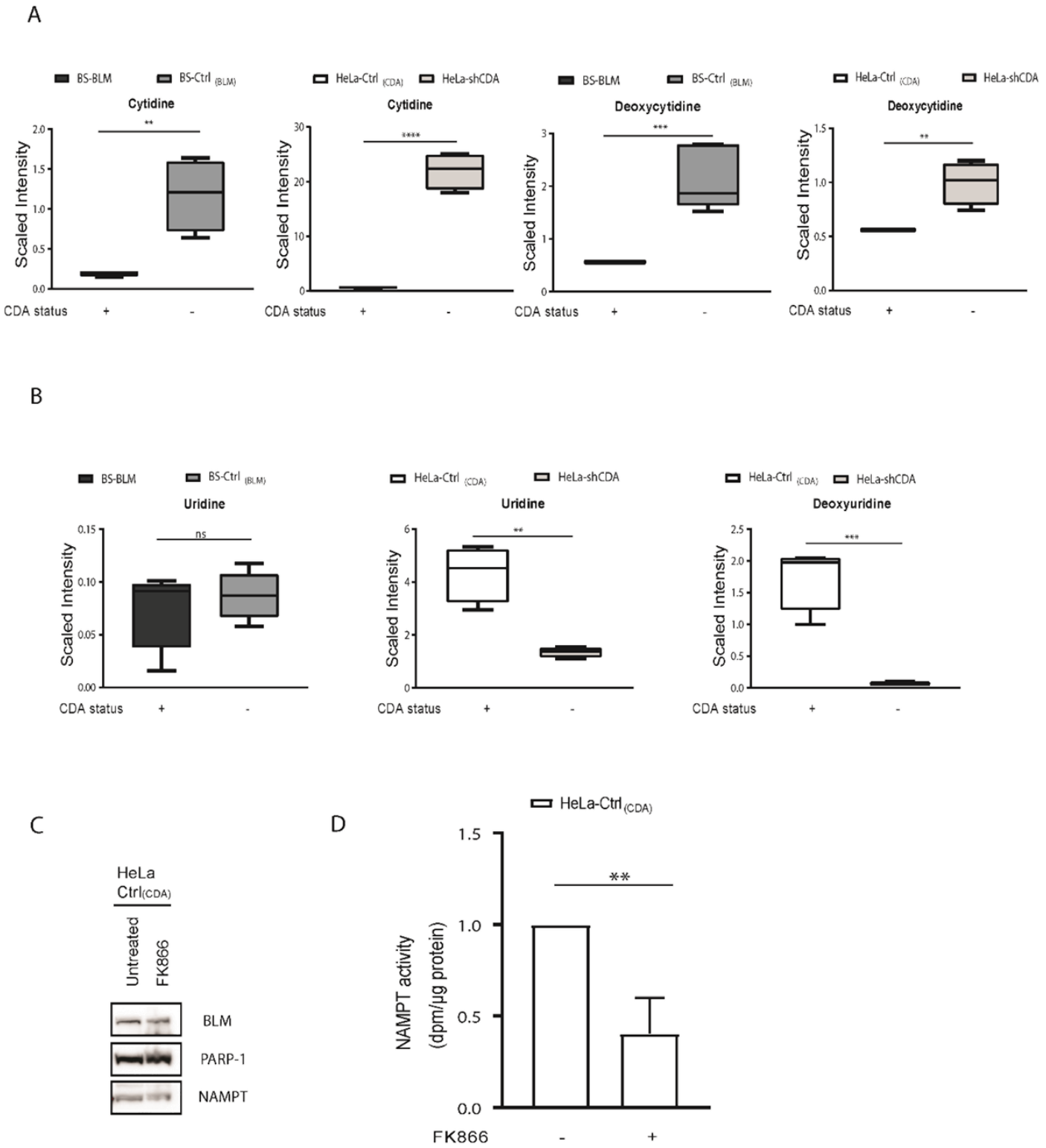

A
BS-BLM
BS-Ctrl (BLM)
HeLa-Ctrl (CDA)
HeLa-shCDA
Cytidine
Deoxycytidine
Scaled Intensity
CDA status
+
-
B
Uridine
Deoxyuridine
ns
C
HeLa Ctrl(CDA)
Untreated
FK866
BLM
PARP-1
NAMPT
D
HeLa-Ctrl(CDA)
NAMPT activity (dpm/µg protein)
FK866

Figure S2

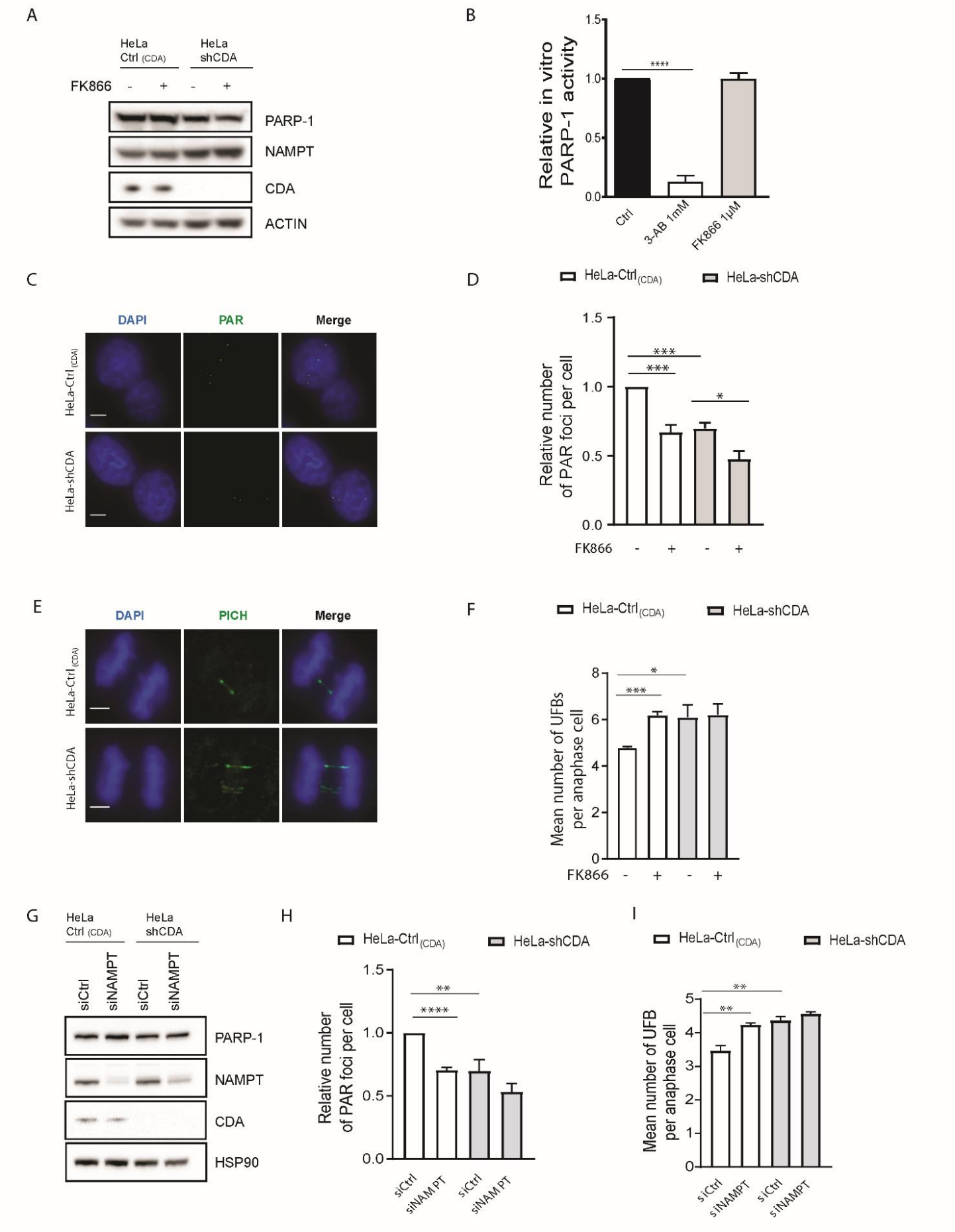
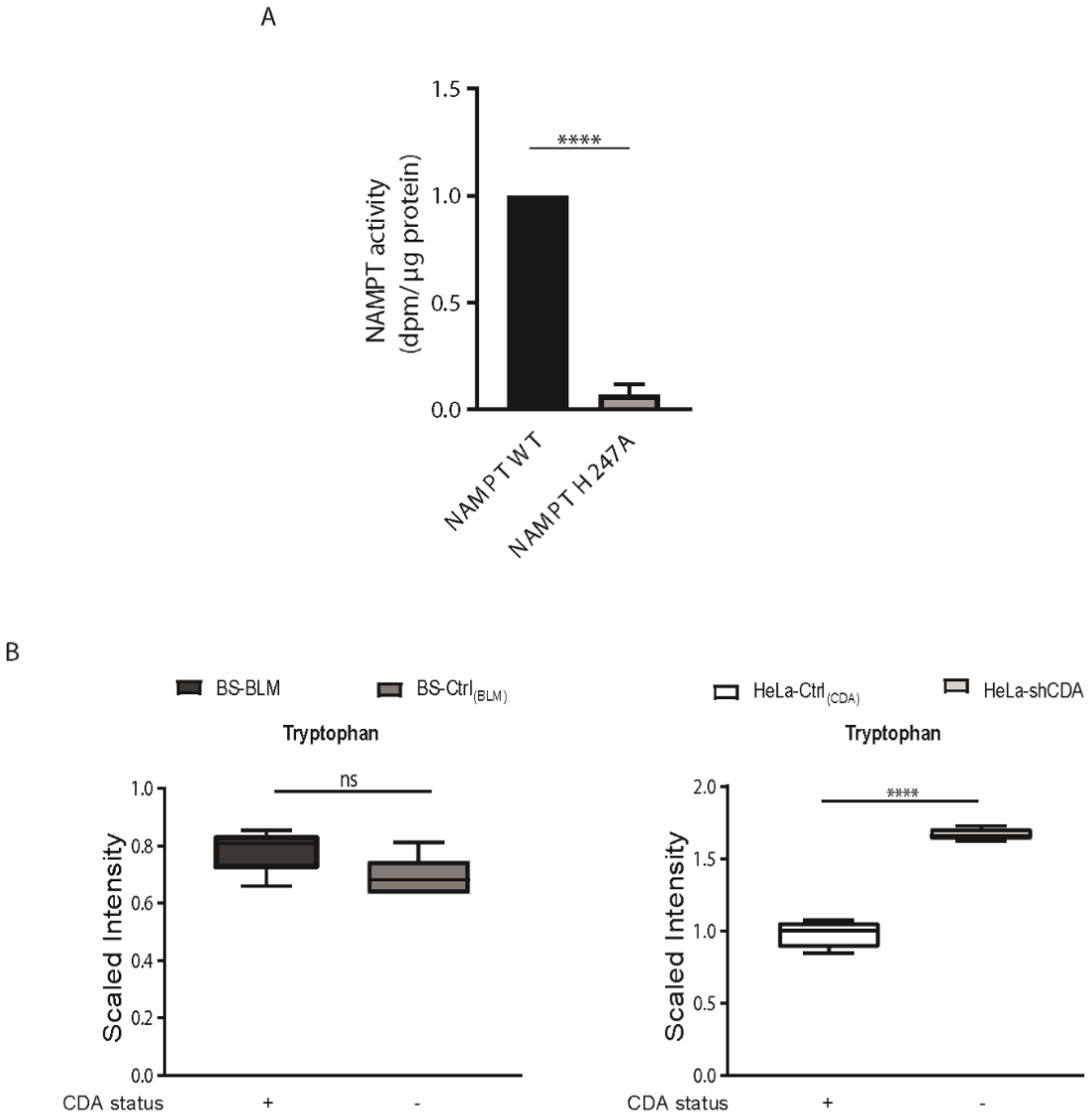

A
HeLa Ctrl (CDA)
HeLa shCDA
FK866 - + - +
PARP-1
NAMPT
CDA
ACTIN
B
Relative in vitro PARP-1 activity
1.5
1.0
0.5
0.0
****
Ctrl
3-AB 1mM
FK866 1μM
C
DAPI
PAR
Merge
HeLa-Ctrl (CDA)
HeLa-shCDA
D
HeLa-Ctrl (CDA)
HeLa-shCDA
Relative number of PAR foci per cell
1.5
1.0
0.5
0.0
***
***
*
FK866 - + - +
E
DAPI
PICH
Merge
HeLa-Ctrl (CDA)
HeLa-shCDA
F
HeLa-Ctrl (CDA)
HeLa-shCDA
Mean number of UFBs per anaphase cell
8
6
4
2
0
*
***
FK866 - + - +
G
HeLa Ctrl (CDA)
HeLa shCDA
siCtrl
siNAMPT
siCtrl
siNAMPT
PARP-1
NAMPT
CDA
HSP90
H
HeLa-Ctrl (CDA)
HeLa-shCDA
Relative number of PAR foci per cell
1.5
1.0
0.5
0.0
**
****
siCtrl
siNAMPT
siCtrl
siNAMPT
I
HeLa-Ctrl (CDA)
HeLa-shCDA
Mean number of UFB per anaphase cell
5
4
3
2
1
0
**
**
siCtrl
siNAMPT
siCtrl
siNAMPT

Figure S3


A
NAMPT activity
(dpm/µg protein)
1.5
1.0
0.5
0.0
****
NAMPT WT
NAMPT H247A
B
BS-BLM
BS-Ctrl(BLM)
Tryptophan
ns
Scaled Intensity
1.0
0.8
0.6
0.4
0.2
0.0
CDA status
+
-
HeLa-Ctrl(CDA)
HeLa-shCDA
Tryptophan
****
Scaled Intensity
2.0
1.5
1.0
0.5
0.0
CDA status
+
-

Supplementary figure legends

Figure S1. Nucleotide metabolism is altered in CDA-deficient cells and nuclear NAMPT is inhibited by FK866. (A-B) Box plots were used to represent four key metabolites (cytidine and deoxycytidine (A) and uridine and deoxycytidine (B)) in BS-BLM (in grey), BS-Ctrl$_{(BLM)}$ (in medium grey), HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA (in light grey) cells. The central 50% of the data is depicted by a shaded box and the range of the data is represented by the whiskers. An outlier following the trend towards higher levels of cytidine was excluded from the statistical analysis. The solid horizontal bar indicates the median of the measured values. Data were scaled such that the median value across all samples was set to 1. The data presented are the represents means $\pm$ SD from at least four independent experiments. All the values were generated by Metabolon Inc. (USA). **(C)** Nuclear PARP-1 and NAMPT protein levels were assessed by immunoblotting in HeLa-Ctrl $_{(CDA)}$ cells left untreated or treated with 1 μM FK866 for 10 h. **(D)** Measurement of basal NAMPT activity in HeLa-Ctrl$_{(CDA)}$ cells left untreated or treated with 1 μM FK866 for 10 h. The data shown are means $\pm$ SD from three independent experiments. The significance of differences was assessed in Student's *t*-tests (ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

Figure S2. NAMPT inhibition decreases basal PARP-1 activity. (A) PARP-1, NAMPT and CDA protein levels were assessed by immunoblotting with HeLa-Ctrl$_{(CDA)}$ and HeLa-shCDA cell lines left untreated or treated with 1 μM FK866 for 10 h. **(B)** *In vitro* analysis of PARP-1 activity in the presence of 1 mM 3-AB or 1 μM FK866. The data shown are the mean $\pm$ SD from four independent experiments. **(C)** Representative immunofluorescence deconvoluted *z*-projection images of HeLa-Ctrl$_{(CDA)}$ and HeLa-shCDA cells showing PAR foci in interphase cells. Nuclei were visualized by DAPI staining (blue). PAR foci were stained with PAR antibody (in green, Alexa Fluor 555). Scale bar: 5 μm. **(D)** Relative number of PAR foci in HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA (in slight gray) cell lines left untreated or treated with 1 μM FK866 for 10 h. The data shown are means $\pm$ SD from three independent experiments (> 350 cells per condition). **(E)** Representative immunofluorescence deconvoluted *z*-projection images of PICH-positive UFBs in HeLa-Ctrl$_{(CDA)}$ and HeLa-shCDA anaphase cells. DNA was visualized by DAPI staining (blue). UFBs were stained with PICH antibody (in green, Alexa Fluor 555). Scale bar: 5 μm. **(F)** Mean number of UFBs per anaphase cell, for HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA (in slight gray) cell lines left untreated or treated

with 1 μM FK866 for 10 h. The data shown are means ± SD from three independent experiments (> 80 anaphase cells per condition). **(G)** PARP-1, NAMPT and CDA protein levels assessed by immunoblotting in HeLa-Ctrl$_{(CDA)}$ and HeLa-shCDA cell lines transiently transfected with the indicated siRNAs. **(H)** Relative number of PAR foci in HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA (in slight gray) cell lines transiently transfected with the indicated siRNAs. The data shown are means ± SD from four independent experiments (> 350 cells per condition). **(I)** Mean number of UFBs per anaphase cell, for HeLa-Ctrl $_{(CDA)}$ (in white) and HeLa-shCDA (in slight grey) cell lines transiently transfected with the indicated siRNAs. The data shown are means ± SD from three independent experiments (> 120 anaphase cells per condition). The significance of differences was assessed in Student's *t*-tests (ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

Figure S3. **NAMPT activity is decreased by mutation of its active site and NAD metabolism is altered in CDA-deficient cells. (A)** Measurement of the activity of a wild-type recombinant NAMPT protein (NAMPT WT) and a mutated recombinant NAMPT protein (NAMPT H247A). The data shown are means ± SD from three independent experiments. **(B)** Box plots were used to represent the tryptophan metabolite in BS-BLM (in grey), BS-Ctrl $_{(BLM)}$ (in medium grey), HeLa-Ctrl$_{(CDA)}$ (in white) and HeLa-shCDA (in light grey). The central 50% of the data is represented by the shaded boxes and the range of the data is indicated by the whiskers. The solid horizontal bar indicates the median value of the measured values. Data were scaled such that the median value across all samples was set to 1. The data presented are means ± SD from at least four independent experiments. All the values were generated by Metabolon Inc. (USA). The significance of differences was assessed in Student's *t*-tests (ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

Discussion

From the results obtained in this project, we showed that the nucleotide pool imbalance resulting from CDA deficiency decreases NAMPT activity. Chemical inhibition of NAMPT or siRNA-mediated depletion leads to PARP-1 inhibition, regardless of NAD^+ levels. The expression of exogenous wild-type NAMPT, but not of NAMPT mutated in its active site, is sufficient to fully restore basal PARP-1 activity in CDA-deficient cells, revealing an unexpected alliance between pyrimidine pool and NAD salvage pathways in maintaining genome stability.

As already mentioned in the project presentation section, the objective my PhD was to decipher how CDA deficiency leads to a decrease in PARP-1 activity. The important question arose from the work of Gemble *et al.* (2015) showing that in cells lacking CDA, PARylation activity was decreased (Gemble, Ahuja et al. 2015). Basal PARylation levels were assayed by immunofluorescence microscopy and customized software for automatic counting of PAR foci. Although several members of the PARP family have been identified to catalyze PARylation activity, in particular PARP-1 and PARP-2 (Vyas, Matic et al. 2014), it is recognized that PARP-1 accounts for 80% of the total PARylation activity (Amé, Rolli et al. 1999). Thus, it was considered by Gemble *et al.* (2015) that the decrease in basal PARylation levels observed in CDA-deficient cells was mainly reflecting a decrease in basal PARP-1 activity. Indeed, this study, the decrease in PARP-1 activity in CDA-deficient cells, evaluated by counting the basal PAR foci, was shown to compromise the completion of DNA replication, as detected by the higher frequency of unreplicated centromeres in these cells, and to lead to excess UFB formation. These cellular features associated with CDA deficiency were abolished by treating CDA-deficient cells with the PARP-1 activators H2O2 or campthotecin and, importantly, were fully reproduced by the depletion of PARP-1 (Gemble, Ahuja et al. 2015). These results demonstrated that the decrease in the frequency of PAR foci in CDA-deficient cells was mainly reflecting a significant reduction of the basal PARP-1 catalytic activity (Gemble, Ahuja et al. 2015).

It has recently been demonstrated by Hanzlikova *et al.* (2018) that most, if not all, PAR synthesis detected in unperturbed cell reflect PARP-1 activity at sites of unligated Okazaki fragments during DNA replication. The authors propose that PARP-1 recruits the SSBR machinery to complete Okazaki fragment ligation, constituting a novel “backup” pathway for

processing Okazaki fragments (Hanzlikova, Kalasova et al. 2018). These results claiming that the only PAR foci detected in unperturbed cells correspond to unligated Okazaki fragments raise the question of the interpretation of the data we obtained in CDA-deficient cells. Indeed, these data led us to ask whether the decrease in basal PARP-1 activity in CDA-deficient unperturbed cells could reflect a decreased formation of unligated Okazaki fragments during S phase.

We should first stress that the experimental approach used by Hanzlikova *et al.* (2018) to detect PARylation activity is very different from the one we use in the team. Indeed, to detect endogenous PAR, the authors incubate the cells for a short time with a potent PARG inhibitor, whereas we do not treat our cells before PAR analysis by immunofluorescence. Thus, investigations are necessary to ensure that we detect similar PAR foci with both experimental approaches. Another important point is that BS cells present a constitutive activation of γH2AX, a well-known marker of DSBs (Rao, Conti et al. 2007). Our team observed and reported a constitutive activation of both γH2AX and ChK2 in unperturbed CDA-depleted HeLa cells (Gemble, Ahuja et al. 2015), indicating that CDA-deficient cells present constitutive endogenous DNA breaks. Moreover, our team also reported in CDA-deficient cells a marked accumulation of ssDNA gaps at replication forks, and the associated increase in fork reversal, visualized by electron microscopy (Gemble, Ahuja et al. 2015). Since PARP-1 is activated in the presence of SSBs and DSBs (Langelier, Planck et al. 2011), it was expected an increase in basal PARylation levels in CDA-deficient cells, instead of a decrease. These results suggest that either the decrease in the frequency of PAR foci and, thus, in basal PARP-1 activity in CDA-deficient cells, is independent of the DNA lesion that commonly activates PARP-1 activity, or that the accumulation of unrepaired DNA lesions in these cells, likely including unligated Okazaki fragments, is the consequence of reduced PARP-1 activity. In conclusion, several important experiments need to be designed and performed to answer all these important questions.

1. NAD metabolism is altered in CDA-deficient cells

A large-scale metabolomics study was performed (Metabolon, Inc., USA) in two cell lines expressing or not CDA – BS-BLM/BS-Ctrl$_{(BLM)}$, and HeLa-Ctrl$_{(CDA)}$/HeLa-shCDA - to identify metabolomic pathways altered in the absence of CDA. Since PARylation activity of PARP-1 depends on NAD metabolism, we examined with particular attention the NAD biosynthesis pathways. The metabolomics study showed that NAD^+ levels were unchanged in both CDA-deficient cell lines (results section, Figure 1A). However, by measuring the NAD^+ levels using a NAD^+ assay kit, we confirm that NAD^+ levels were in fact unchanged in the BS cells but we found that they were significantly increased in CDA-depleted HeLa cells. As PARP-1 is partially inhibited in both cell lines, an increase in NAD^+ was expected in both CDA deficient cells lines, which was not the case. These results suggested that the decrease in PARP-1 activity was independent of the NAD^+ levels.

NAD^+ biosynthesis in mammals is performed by two major pathways: the *de novo* pathway and the salvage pathway (Mattevi 2006, Nikiforov, Kulikova et al. 2015). In the *de novo* pathway, Trp is used as a substrate to initiate NAD^+ production which requires a chain of nine enzymatic reactions (Mattevi 2006). The Trp levels were increased in CDA-depleted HeLa cells and unchanged in BS cells (results section, Figure S3B). Other important metabolites such as ACMS and QA that are present in the *de novo* pathway were not detected in this study, rending difficult to explore whether CDA depletion could alter the *de novo* pathway. Thus, the results observed for the Trp levels, although they followed the same tendency than the NAD^+ levels (if we considered the result from the NAD^+ assay kit), did not help us to understand how CDA deficiency leads to reduced PARP-1 activity.

The *de novo* pathway is not sufficient to maintain the physiological NAD concentration on the cell. Indeed, the salvage pathway is manly used to produce the NAD^+ levels necessary for the biological processes (Nikiforov, Dölle et al. 2011), recycling NAM from the NAD^+-dependent proteins such as PARPs and SIRTs, into NMN through NAMPT enzyme and, then, into NAD^+ by NMNAT enzymes (Burgos, Vetticatt et al. 2013). This pathway is the most economic and faster pathway to produce NAD^+ (Chiarugi, Dölle et al. 2012). Our metabolomics study revealed that NAM levels were increase in BS cells and unchanged in HeLa-depleted cells (results section, Figure 1B). However, NMN levels were significantly decreased in both cell lines (results

section, Figure 1C). Since NMN levels were decreased in both CDA-deficient BS and HeLa cell lines we hypothesized that NAMPT was impaired in these cells. Indeed, a reduced NAMPT activity might lead to an excess of NAM intracellular accumulation, a PARP-1 inhibitor, that consequently could lead to its inhibition (Rankin, Jacobson et al. 1989).

2. NAMPT activity is partially inhibited in CDA-deficient cells

Our metabolomic study showing that NAM levels were increase in BS cells and had the same tendency in CDA-depleted HeLa cells, and that NMN levels were significantly reduced in both CDA-deficient cell lines prompted us to explore the possibility that NAMPT activity is impaired in CDA deficient cells. We used an assay based on the conversion of ^{14C}NAM into ^{14C}NMN (Elliott, Ajioka et al. 1980) to measured NAMPT activity in CDA-depleted HeLa cells and found a slight but significant decrease in NAMPT activity in these cells (results section, Figure 1E). Another approach combining HPLC coupled to High Resolution Mass Spectrometry (HPLC-HRMS), to detect and measured selectively NAM and NMN in our cells under different conditions is also in progress in collaboration with Jérôme Guitton and Christelle Machon (Centre hospitalier Lyon-Sud – HCL). Importantly, our experiments to measure NAMPT activity are performed using nuclear extracts because all the experiments we did to measure basal PARP-1 activity were performed by counting PAR foci within the nucleus. Indeed, since NAMPT is located in the nucleus and in the cytoplasm and, possibility in the mitochondria (Shackelford, Mayhall et al. 2013, Dalamaga, Christodoulatos et al. 2018), and that the functions of NAMPT in these different cellular compartments are not clear, we decided to limit our studies to the nuclear NAMPT .

We next asked how CDA deficiency leads to a decrease in nuclear NAMPT activity. CDA deficiency is known to cause a pyrimidine pool disequilibrium with an intracellular accumulation of dC and dCTP (Chabosseau, Buhagiar-Labarchède et al. 2011). Moreover, culturing cells in the presence of dC, that consequently leads to dCTP accumulation in cells, impairs PARP-1 activity; in vitro experiments showed that dCTP does not inhibit directly PARP-1 (Gemble, Ahuja et al. 2015). Thus, we explored whether the decrease in NAMPT activity in CDA-deficient cells was due to dC/dCTP accumulation. We found that culturing CDA-proficient cells in the presence of dC was sufficient to decrease nuclear NAMPT activity (results section,

Figure 1G). We also constructed and produced a NAMPT recombinant protein in collaboration with Patricia Duchambon (Plateforme production protéines recombinantes, U1196 – CNRS UR 9187, Institut Curie, Orsay). Using recombinant NAMPT, we obtained several times results showing that both dC and dCTP inhibit NAMPT at a concentration that reflects physiological conditions, suggesting that dC and dCTP might inhibit directly NAMPT activity. Unfortunately, with both the ^{14C}NAM assay and the HPLC-HRMS method, problems of reproducibility and, despite our efforts, we did not solve them yet and, thus, did not confirm this inhibition. This work it still in progress, using the two experimental approaches.

In conclusion, at this point, we cannot formally exclude that the decrease in NAMPT activity is an indirect consequence of the intracellular accumulation of dC and/or dCTP in CDA-deficient cells.

3. NAMPT inhibition impairs basal PARP-1 activity, regardless of NAD^+ levels

NAMPT is the rate-limiting enzyme in the salvage pathway, and changes in this enzyme, affects both NAD^+ levels and cellular processes dependent from NAD^+ such PARylation performed by PARPs and deacetylation by SIRTs (Revollo, Grimm et al. 2004). On the basis of our results, we thus asked whether NAMPT inhibition could impair PARP-1 activity, independently of NAD^+ levels.

To test this hypothesis, we first chemically inhibited NAMPT in CDA-proficient cells with the specific NAMPT inhibitor, FK866 (Hasmann and Schemainda 2003), and found that PARP-1 activity was significantly impaired, mimicking CDA deficiency (results section, Figure 2B and S2D). We confirmed that the decrease in PARP-1 activity was the direct consequence of NAMPT inhibition since (1) the recombinant PARP-1 protein was not inhibited in vitro by FK866 (results section, Figure S2B), excluding the possibility of a direct PARP-1 inhibition by FK866, and (2) the siRNA-mediated NAMPT knockdown, also impaired PARP-1 activity in CDA proficient cells (result section, Figures 2E and S2H). As reported by Gemble *et al*. (2015), a reduced PARP-1 activity results in the increase in UFB formation during mitosis. NAMPT inhibition or depletion also led to a significant increase in UFB frequency in CDA-proficient cells, to levels similar to those in CDA-deficient cells (results section, Figures 2C, 2F, S2F and S2I). However, UFB frequency was not affected when NAMPT was inhibited or depleted in CDA-deficient cells. This is in agreement with the data reported by Gemble *et al*. (2015)

showing that a decrease in basal PARP-1 activity of about 30% led to the formation of a number of supernumerary UFBs similar to that observed following total PARP-1 inhibition or depletion, suggesting that there is a critical basal threshold of PARP-1 activity for full replication of the genome before the cells entering mitosis (Gemble, Ahuja et al. 2015). Here we observed that a decrease in basal NAMPT inhibition of 23% in CDA-deficient cells is sufficient to reduced PARP-1 activity, indicating a critical basal threshold of NAMPT activity is necessary for an optimal PARP-1 activity. Our study shed light on a new mechanism where decrease in NAMPT activity impairs PARP-1 activity.

We did not observe any correlation between the levels of NAMPT expression and activity. Despite decrease in basal NAMPT activity in CDA-deficient cells, the expression levels of NAMPT were the same. NAMPT inhibition by FK866 treatment also does not affected its expression levels. This indicates that the decrease in the basal NAMPT activity in CDA-deficient cells likely results from a partial inhibition, through its active site, by dC/dCTP or other metabolites. This observation means that in the context of cancer, correlation between NAMPT and PARP-1 can only be detected at the expression levels, that do not reflect their activities (Shackelford, Mayhall et al. 2013). For example, one study reported that multiple myeloma cells overexpressing PARP-1 can survive only through NAMPT overexpression, that in turn restores NAD^+ levels that are consumed by PARP-1 (Venkateshaiah, Khan et al. 2013). Another study reported in triple-negative breast cancer cells that inhibition of both NAMPT and PARP-1 leads to cancer cell death and tumor regression due to decrease availability of NAD^+ (Bajrami, Kigozi et al. 2012). Indeed, inhibiting NAMPT by either RNAi or FK866 treatment reduces NAD^+ levels which, in turn reduces the competition between NAD^+ and PARP inhibitor for the PARP catalytic domain, increasing, thus, the deleterious effect of PARP-1 inhibitor on cells (Bajrami, Kigozi et al. 2012).

Our results demonstrate that PARP-1 inhibition induced by the decrease in NAMPT activity in CDA-deficient cells is independent of NAD^+ levels. Indeed, since NMN treatment of cell restore NAD^+ levels (Revollo, Grimm et al. 2007, Stromsdorfer, Yamaguchi et al. 2016, Elhassan, Philp et al. 2017), we left untreated or treated CDA-proficient cells and CDA-deficient cells with NMN, in the presence or not of FK866, and analyzed both PARylation levels and UFB frequency. We found that although NMN treatment increased NAD^+ levels, it did not rescue PAR foci and UFB frequencies in cell treated with FK866 (results section, Figures 3D, 3E, 3F and

3G), demonstrating that the decrease in PARP-1 activity induced by NAMPT inhibition is independent of total NAD^+ levels. However, these results need to be confirmed by measuring the amount of nuclear NAD^+ available for PARP-1. If confirmed, these results are really intriguing since it has been reported that treatment of cell with NMN enhances PARP-1 activity by increasing the availability of NAD^+ levels (Mendelsohn and Larrick 2017): we did not observed an increase in basal PARylation levels, neither in CDA-deficient cells nor in CDA-proficient cells. Moreover, as indicated above, the decrease in PARylation activity in CDA-deficient cells or in cells expressing or not CDA treated with FK866 is not rescued by the increased in total NAD^+ levels induced by NMN treatment (results section, Figures 3B and 3C). Thus, the partial inhibition of PARP-1 activity induced by the decrease in NAMPT activity is due to another mechanism than the reduced NAD^+ availability.

These results raise the question of how NAD^+ levels are maintained in cells presenting a defect in NAMPT. Since NMN levels are decrease in both BS and CDA-depleted HeLa cells, relative to controls, we excluded an NAD production through the conversion of NR (nicotinamide ribose) in NMN by NRKs (nicotinamide ribose kinases). The *de novo* pathway might contribute to maintain NAD^+ levels in CDA-depleted HeLa cells, as suggested by the results showing an increase in the Trp levels in these cells, relative to control cells (results section, Figure S3B). However, we did not observed an increase in Trp levels in BS cells (results section, Figure S3B), suggesting that another mechanism might contribute to maintain NAD^+ levels, at least in these cells. Another possibility is that decrease in basal PARP-1 activity, likely through inhibition by a metabolite such NAM, leads to a decrease in NAD consumption by PARP-1 at the basal level, preserving thus the NAD pool.

Since NAMPT uses NAM as a substrate, and this metabolite is a well-known natural inhibitor of PARP-1 (Rankin, Jacobson et al. 1989) we proposed in conclusion that NAMPT defect in CDA-deficient might lead to intracellular accumulation of NAM (although not detected in CDA-depleted HeLa cells) or another unidentified metabolite that, in turn, inhibits PARP-1 (results section, Figure 4F).

Based on the results obtained during my thesis, we proposed the following hypothetical model:

CDA deficiency leads to an intracellular accumulation of dC/dCTP, which consequently impairs NAMPT activity. The weakened NAMPT activity is responsible for the accumulation of NAM or

other yet unknown factors in the cells, leading to a decreased in PARP-1 activity and the subsequent excess UFB formation (Figure 43).

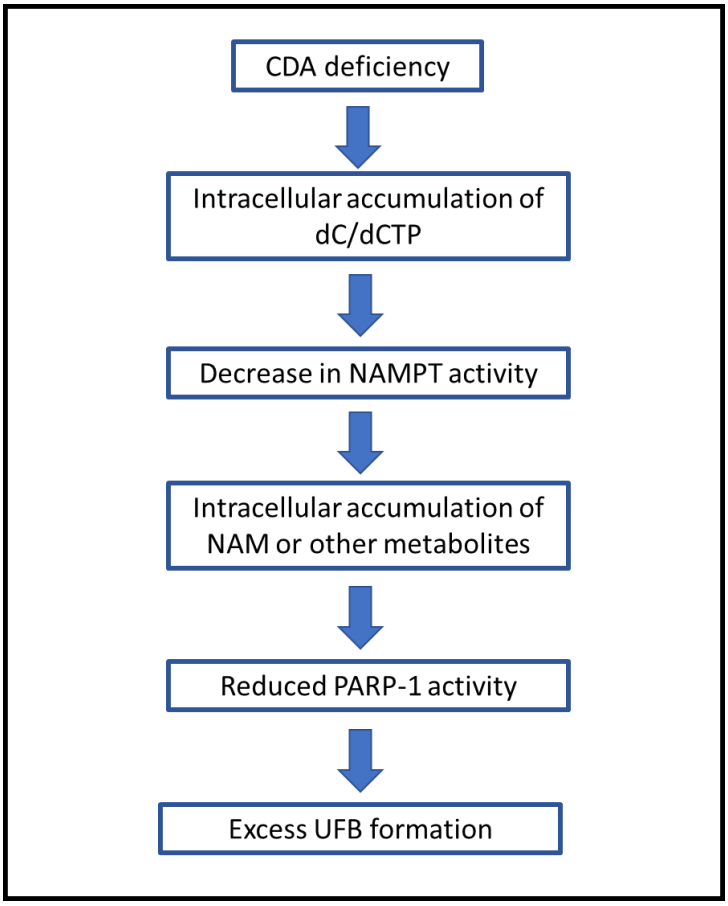


Figure 43. Proposed model for explaining how CDA deficiency may lead to reduced PARP-1 activity and subsequent UFB formation.

Conclusion and future perspectives

Conclusion and future perspectives

The results obtained during my phD thesis allowed us to identify a new relationship between CDA deficiency and the NAMPT salvage pathway, an important pathway in the NAD metabolism. CDA deficiency leads to the intracellular accumulation of dC/dCTP (Chabosseau, Buhagiar-Labarchède et al. 2011, Gemble, Ahuja et al. 2015). Our findings indicated that the excess dC/dCTP decreases NAMPT activity that, in turn, impairs PARP-1 activity leading to an increase in UFB frequency. We proposed that the reduction of PARP-1 activity might be due to an excess of NAM, the NAMPT substrate that is also a natural inhibitor of PARP-1. We also demonstrated that the reduction of PARP-1 resulting from NAMPT inhibition is independent of NAD^+ levels, highlighting a new link between NAMPT and PARP-1 and an indirect role of NAMPT in maintaining genome instability.

As already mentioned, our team demonstrated that CDA is downregulated in about 60% of tumor cells and tissues (Mameri, Bièche et al. 2017). Inversely, NAMPT is reported to be overexpressed in several cancers such as ovarian (Shackelford, Bui et al. 2010), breast (Zhou, Bi et al. 2018), prostate (Wang, Hasan et al. 2010) and melanoma (Elena, Cristina et al. 2013), but NAMPT activity was not measured in these tumors. Our data indicate that NAMPT activity could be independent from its expression since in at least CDA-deficient cells and FK866-treated cells, NAMPT activity is decrease or abolished, whereas the protein levels remain unchanged. Nevertheless, it could be interesting to investigate a possible correlation between CDA loss and NAMPT under-expression. Through *in-silico* analyses of *CDA* and *NAMPT* expression levels in several cohorts of cancer cell lines (cancer cell line encyclopedia - CCLE), we observed a possible correlation between *CDA* and *NAMPT* expression levels in some cancer cell lines, as illustrated in Figure 44. In the three cohorts of tumor cell lines (overall cancer cell lines (A), breast cancer (B) and lung cancer cell lines (C)), we observed a positive correlation between *CDA* and *NAMPT* expression levels, although in breast cancer cell lines did not achieved statistical significance, probably due to low number of samples. In several cancer cell lines, *NAMPT* expression follows the same tendency as *CDA* expression, where NAMPT expression is decreased when CDA expression is decreased and vice-versa.

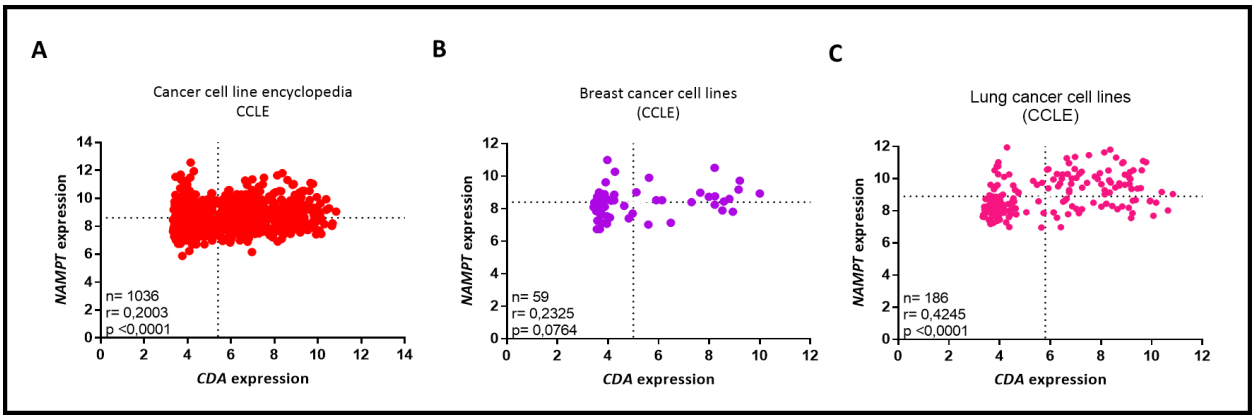


Figure 44. CDA and NAMPT expression levels are positively correlated in cancer. *CDA* and *NAMPT* levels in **A)** overall cancer cell lines, **B)** breast cancer cell lines, **C)** lung cancer cell lines (from cancer cell line encyclopedia). Scatterplots show the Pearson correlation between mRNA microarray data and dashed vertical lines correspond to the mean *CDA* and *NAMPT* expression. $P < 0.05$ were considered statistically significant.

It will be interesting to study this subtype of tumors, associating low levels of both CDA and NAMPT, compared to the tumors with other profiles of CDA and NAMPT expression levels to determine whether they present a different outcome in terms of evolution and aggressiveness of the disease. Mameri *et al.* (2017) reported that CDA deficient cells are sensitive to particular drugs such as aminoflavone (Mameri, Bièche et al. 2017), an anti-cancer drug that lead to cancer cell death in triple-negative breast cancer (Brinkman, Chen et al. 2016). Moreover, in several preclinical models, combination of NAMPT and PARP-1 inhibitors resulted in cancer cell death and tumor regression, revealing a synthetic lethality between NAMPT and PARP-1 deficiencies (Bajrami, Kigozi et al. 2012, Heske, Davis et al. 2017). Thus, it will be interesting to test the drugs that target CDA-deficient tumors, in association with NAMPT and PARP-1 inhibitors. Determining whether the synthetic lethal interaction between NAMPT and PARP-1 deficiencies are dependent on the drugs that target CDA expression, will also be of main interest.

In conclusion, our study revealed an unexpected link between CDA, NAMPT and PARP-1, that opens new opportunities of research at the interface between DNA repair and NAD biology, an emerging field of great interest (Sobol 2014).

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Résumé

Le syndrome de Bloom (SB) est une maladie humaine autosomique récessive rare résultant d'une mutation sur les deux copies du gène BLM, qui code pour la protéine BLM, une 3'-5' ADN hélicase de la sous-famille recQ. Les cellules SB présentent une forte instabilité génétique et les patients atteints du SB sont prédisposés au développement de tous les types de cancers affectant la population générale. La déplétion en BLM conduit à une chute drastique de l'expression de la cytidine désaminase (CDA), une enzyme de la voie de sauvetage des pyrimidines qui catalyse la désamination hydrolytique de la cytidine (C) et de la désoxycytidine (dC) en uridine (U) et désoxyuridine (dU). La déficience en CDA conduit à un excès de dC et de dCTP (désoxycytidine triphosphate) dans les cellules SB, mais également dans les cellules qui expriment BLM, ce qui entraîne une instabilité génétique. Effectivement, ce déséquilibre du pool de pyrimidines conduit à une diminution significative de l'activité basale de la poly (ADP-ribose) polymérase 1 (PARP-1) qui, elle-même, entraîne une réduction de l'activation de Chk1. Ceci affaiblit l'efficacité des points de contrôle du cycle cellulaire en aval, favorisant l'accumulation en mitose de séquences d'ADN non répliquées qui conduisent à une formation excessive de ponts anaphasiques ultrafins (UFB). Ces séquences concernent essentiellement des régions du génome « difficiles à répliquer », comme les centromères et les sites fragiles. L'objectif de mon projet thèse était de décrypter le mécanisme conduisant à la réduction de l'activité basale de PARP-1 dans les cellules déficientes en CDA. Nous avons effectué une étude comparative des métabolomes de deux couples de lignées isogéniques exprimant ou non CDA qui a révélé une augmentation du niveau de nicotinamide (NAM), substrat de la nicotinamide phosphoribosyltransférase (NAMPT), et une diminution du niveau de nicotinamide mononucléotide (NMN), produit de la NAMPT, dans les cellules déficientes en CDA. Nous avons confirmé la réduction de l'activité de la NAMPT nucléaire dans les cellules déficientes en CDA. Nous avons également montré que la déplétion en NAMPT par ARN interférence ou l'inhibition chimique de l'activité de la NAMPT reproduit la réduction de l'activité basale de PARP-1 dans les cellules exprimant CDA, et pas dans les cellules déficientes en CDA. De plus, l'expression exogène de la NAMPT sauvage, mais pas celle de la NAMPT mutée dans son site catalytique, restaure complètement l'activité basale de PARP-1 dans les cellules déficientes en CDA, entraînant de fait une normalisation de la fréquence des UFBs dans ces cellules. Ces résultats indiquent que la réduction de l'activité basale de PARP-1 dans les cellules déficientes en CDA résulte de la diminution de l'activité de la NAMPT. Nous proposons un modèle dans lequel l'accumulation intracellulaire de dC /dCTP résultant de la déficience en CDA pourrait entraver l'activité de la NAMPT nucléaire, provoquant une accumulation intracellulaire de NAM, un inhibiteur naturel connu de PARP-1, qui par conséquent réduirait l'activité basale de PARP-1. Nos résultats révèlent pour la première fois, un lien entre la déficience en CDA et le métabolisme du nicotinamide, voie métabolique essentielle pour le maintien de l'intégrité de la cellule.

Mots Clés

CDA, NAMPT, PARP-1, Pool de nucléotides, Instabilité génétique

Abstract

Bloom syndrome (BS) is a rare human autosomal recessive disorder resulting from mutations in both copies of the BLM gene, encoding BLM, a 3'-5' RecQ DNA helicase. BS cells present a strong genetic instability and BS patients are predisposed to a wide range of cancers that commonly affect the general population. BLM depletion leads to the downregulation of cytidine deaminase (CDA), an enzyme of the pyrimidine salvage pathway that catalyzes the hydrolytic deamination of cytidine (C) and deoxycytidine (dC) to uridine (U) and deoxyuridine (U), respectively. CDA defect leads to an excess of cellular dC and deoxycytidine triphosphate (dCTP) in either BS cells or BLM-expressing cells, that jeopardizes genome stability. Indeed, this nucleotide pool disequilibrium leads to a significant reduction of basal Poly (ADP-ribose) polymerase 1 (PARP-1) activity. The resulting low levels of PARP-1 activity disturb Chk1 activation and decrease the efficiency of downstream checkpoints, leading to the accumulation, during mitosis, of unreplicated DNA at some "difficult-to-replicate" loci in the genome, such as centromeres, fragile sites, leading to excess ultrafine anaphase bridge (UFB) formation. The objective of my PhD project was to decipher the mechanism leading to the reduction of basal PARP-1 activity in the absence of CDA. We performed a metabolomic study that revealed an increase in nicotinamide (NAM) levels, the substrate of nicotinamide phosphoribosyltransferase (NAMPT), and a decrease in nicotinamide mononucleotide (NMN) levels, the product of NAMPT. We confirmed the reduction of the nuclear NAMPT activity in CDA-deficient cells. We found that the siRNA-mediated NAMPT knockdown or the chemical NAMPT inhibition reproduce the reduction of basal PARP-1 activity in CDA-proficient cells, but not in CDA-deficient cells. Moreover, expression of exogenous wild type NAMPT, but not of the NAMPT catalytic mutant, fully rescued the reduction of basal PARP1 activity, and the subsequent increase in UFB frequency in CDA-deficient cells. These results indicate that the reduced basal PARP-1 activity in CDA-deficient cells is due to a reduced NAMPT activity. We propose a model in which the intracellular accumulation of dC/dCTP resulting from CDA deficiency might impair the nuclear NAMPT activity, resulting in an intracellular accumulation of NAM, a known natural inhibitor of PARP-1, that consequently reduces PARP-1 activity. Our results highlight for the first time a link between cytidine deaminase deficiency and nicotinamide metabolism, a pathway essential for the maintenance of cell integrity.

Keywords

CDA, NAMPT, PARP-1, Nucleotide pool, Genetic instability