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Study of two epigenetic age-associated modifications: DNA methylation of the rRNA gene promoter in humans and histone tail acetylation at the sub-telomeric region in *S. cerevisiae*

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2014

“*The two grand tyrants of the Earth, Time and Chance”*

Johann Gottfried Herder

INDEX

ABBREVIATIONS

5-hmC	5-Hydroxymethylcytosine
5Mc	5-methylcytosine
Å	Angstrom
Adh2	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
aza-dC	5-aza-2_-deoxycytidine
CGI	CpG island
CLS	Chronological lifespan
CPE	Core promoter element
CpG	Cytosine phosphate Guanine
CR	Calorie restriction
DNA	Deoxyribonucleic Acid
DNMT	DNA methyltransferase
dNTPs	Nucleoside triphosphate
DZ	Di-Zygotic
ER	Endoplasmic reticulum
ERCs	Extrachromosomal rDNA circles
ETS	External transcribed spacer
HDACs	Histone deacetylases
HERV-K	Human endogenous retrovirus K
HP1	Heterochromatin protein 1
IRSs	Interspersed ripetitive sequences
ITS	Internal transcribed spacer
Kb	Kilo base
lncRNA	Long non-coding RNA
LSU	Large sub unit
miRNA	MicroRNA
MBPs	Methyl-Binding Proteins
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MZ	Mono-Zygotic

NAD	Nicotinamide adenine dinucleotide
NOR	Nucleolar organizer region
NoRC	Nucleolar remodeling complex
NTS	Non-transcribed spacers
OD	Optical Density
ORF	Open reading frame
p53	Protein 53
Pck1	Phosphoenolpyruvate carboxykinase
qPCR	quantitative PCR
RB	Retinoblastoma protein
RLS	Replicative lifespan
RNA	Ribonucleic acid
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
S	Svedberg
SAM	S-adenosyl-lmethionine
SAP	Shrimp Alkaline Phosphatase
SC	Synthetic complete medium
Sir	Silent information regulator
SL1	Selectivity factor
Sod	Superoxide dismutase
SSU	Small sub unit
TBP	TATA- binding protein
TAFs	Transcription activating factors
TIF	Transcription initiation factor
TPE	Telomere position effect
UBF	Upstream binding factor
UPE	Upstream promoter element
WS	Werner syndrome
YPD	Agar Yeast Extract-Peptone-Dextrose

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SOMMARIO

Il presente lavoro di tesi è stato realizzato in parte presso il laboratorio di genetica dell'Università della Calabria, sotto la supervisione della Professoressa Dina Bellizzi e in parte presso la Davis School of Gerontology, in collaborazione con il Professor Valter Longo direttore del USC Longevity Institute.

L'invecchiamento è un processo complesso. I fenotipi d'invecchiamento variano da organismo a organismo, ma un generale declino fisiologico si realizza con l'avanzare dell'età.

Una correlazione tra modificazioni epigenetiche e invecchiamento è stato proposto molti anni fa, e nel corso degli anni molteplici studi hanno fornito le prove dell'esistenza di tale connessione, suggerendo che si tratta di un fenomeno conservato lungo il processo evolutivo.

In questo lavoro sono stati investigati due dei più importanti meccanismi epigenetici associati all'invecchiamento: la metilazione del DNA e l'acetilazione degli istoni.

È noto che a livello globale la metilazione del DNA tende a diminuire nel corso dell'invecchiamento, con un concomitante aumento, invece, ai promotori di specifici geni. Presso il laboratorio di genetica dell'Università della Calabria è stata investigata la presenza di citosine metilate in siti CpG all'interno della regione del promotore dei geni che codificano per l'RNA ribosomiale umano nonché l'associazione tra tali livelli, l'invecchiamento e la frailty.

L'attenzione è stata focalizzata su questo sito a causa di alcune peculiari caratteristiche che esso presenta: il ruolo cruciale dell'rRNA ribosomiale nelle funzioni cellulari, l'alto livello di conservazione di questa sequenza nucleotidica lungo il processo evolutivo, nonchè l'organizzazione in cluster dei geni che codificano per l'rRNA.

I livelli di metilazione sono stati valutati in campioni di sangue estratti da individui di età differente e con differenti fenotipi d'invecchiamento mediante la piattaforma Sequenom MassARRAY EpiTYPER. Dall'analisi è emersa l'esistenza di una correlazione tra i livelli di metilazione di specifici dinucleotidi CpG, presenti nel promotore genico da noi investigato, l'invecchiamento e i diversi fenotipi ad esso correlati. I risultati di questo studio sono mostrati nel Capitolo 2.

Studiare l'invecchiamento negli esseri umani, tuttavia, comporta numerose difficoltà, per questa ragione sono spesso utilizzati sistemi modello.

Uno dei più importanti organismi modello è il lievito *Saccharomyces cerevisiae* attraverso cui è stato possibile individuare numerosi geni rilevanti nel processo di invecchiamento, tra cui il gene Sir2 che codifica per un'istone deacetilasi.

Sir2 è un gene associato con resistenza cellulare allo stress e con la regolazione della durata della vita sia replicativa che cronologica nel lievito. Presso il laboratorio del Prof. Longo è stato in precedenza dimostrato che Sir2 blocca l'estensione della durata della vita cronologica causata dalla restrizione calorica o da mutazioni nei pathway Tor/Sch9, Ras/cAMP/PKA e promuove, inoltre, la protezione cellulare sia contro lo stress termico che ossidativo.

Evidenze crescenti dimostrano che Sir2 e la sua controparte, Sas2, regolano la durata della vita di S. cerevisiae mediante acetilazione e deacetilazione degli istoni H4K16 nella regione sub-telomerica.

In questo studio, abbiamo identificato un nuovo meccanismo attraverso cui Sir2, agendo sulla regione sub-telomerica, regola la protezione contro il danno ossidativo, senza influenzare l'invecchiamento cronologico, segno che questi eventi sono regolati da pathway differenti. I dati riportati supportano i risultati ottenuti in precedenza e allo stesso tempo forniscono una spiegazione parziale del meccanismo sottostante alla maggiore resistenza mostrata dalle cellule mancanti del gene Sir2, come riportato nel Capitolo 3.

Durante il periodo trascorso presso i laboratori del Longevity Institute sono stata inoltre coinvolta in un secondo progetto, dimostrando che una dieta ipocalorica e non solo il digiuno sono efficaci nel ridurre la progressione tumorale e gli effetti collaterali associati alla chemioterapia, identificando inoltre nel sistema immunitario un attore fondamentale di questo processo. Data la rilevanza di questi dati, la USC si riserva la possibilità di bloccarne la divulgazione prima della pubblicazione. Tuttavia, una breve descrizione di questo lavoro è fornita in appendice A.

Nella prima parte del mio programma di dottorato di ricerca, condotta presso l'Università della Calabria, ho inoltre collaborato ad uno studio volto a risolvere il dibattito circa la possibile presenza di citosine metilate all'interno del DNA mitocondriale (mtDNA). I risultati di questo studio sono illustrati nell'articolo scientifico "The Control Region of Mitochondrial DNA Shows an Unusual CpG and Non-CpG Methylation Pattern", DNA Research, riportato in appendice B.

SUMMARY

The work presented in this thesis has been realized partially at the Calabria University in the Genetics laboratory, under the supervision of Prof. Dina Bellizzi and partially at the Davis School of Gerontology, in collaboration with Prof. Valter Longo, director of the USC Longevity Institute.

Aging is a complex process. The phenotypes of aging vary from organism to organism, but a general physiological decline is recorded with age.

The relationship between epigenetics and aging was proposed many years ago, and today the evidence about this relationship come from several studies, suggesting this as an evolutionarily conserved process.

In this work I investigated two of the most important epigenetic aging-associated features: DNA methylation, and histone tail acetylation.

As is well known, the global DNA methylation tends to decrease during aging, with a coinciding increase localized within the promoters of specific genes. At the Genetics laboratory of the University of Calabria, I investigated the presence of methylated cytosine in CpG sites within the promoter region of the genes coding for ribosomal RNA in association with human aging and frailty.

The attention has been focused on this region because of some unusual characteristics that it presents: ribosomal rRNA's critical role in cellular function, high conservation of this nucleotide sequence along the evolutionary process, as well as organization in cluster of rDNA genes.

The methylation levels were measured in blood samples of people of different ages and with different frailty phenotypes by means of the MassARRAY EpiTYPER Sequenom platform. The analysis demonstrated a correlation between the methylation levels of specific CpG dinucleotides, aging and the different aging phenotypes. The results are shown in Chapter 2.

Studying aging in humans, however, involves several problems, for this reason model systems are often used.

One of the most important model organisms is the budding yeast *Saccharomyces cerevisiae* through which several important genes in the aging process have been identified, including the gene *Sir2*, which encodes for a histone deacetylases

Sir2 is associated with cellular stress resistance and lifespan regulation both replicative and chronological in yeast. The Longo lab has previously demonstrated

that Sir2 blocks the extreme chronological lifespan extension due to calorie restriction or mutations in the Tor/Sch9, and Ras/cAMP/PKA pathways and promotes cellular protection against thermo and oxidative stresses.

Growing evidence demonstrate that Sir2 and its counterparty, Sas2, regulate lifespan in *S. cerevisiae* by means of histone H4K16 acetylation-deacetylation at sub-telomeric region.

In this study, we identified a novel mechanism through which Sir2 regulates the protection against oxidative damage, without affecting the chronological aging, sign that different pathways regulate these events. Data reported here support previous results and at the same time provide a partial explanation of the underlying mechanism to the higher cell protection in the lack of Sir2, as reported in Chapter 3.

During the period I spent at the Longevity Institute laboratories, I was also involved in a second project, showing that a diet and not only fasting is effective in reducing tumor progression and chemotherapy-associated side effects thus identifying in the immune system a crucial player. Given the relevance of these data, the USC reserves the right to prevent the release of them if they are still in press. However a brief description of this work is provided in the appendix A.

Lastly in the first part of my PhD program, performed at the University of Calabria, I collaborated in a study to solve the debate about the possible presence of methylated cytosine within the mitochondrial DNA (mtDNA).

The results of this study are illustrated in the published paper “The Control Region of Mitochondrial DNA Shows an Unusual CpG and Non-CpG Methylation Pattern”, DNA Research, presented in the appendix B.

CHAPTER 1: GENERAL INTRODUCTION

1.1 AGING AND AGING PHENOTYPES

Aging is a highly complex phenomenon, in fact it is not only 'the passage of time', but it is the 'biological process of growing older'. Aging can be considered as the amount of all changes in structure and function which are deleterious for a cell or an organism, and it leads to an unavoidable consequence, the failure of survival and therefore the death. However the lifespan of each organism is determined both by the innate rate of physiological decline and by the importance of the environmental phenomena destabilizing its homeostatic balance *(1)*.

Despite the large amount of data available about aging and age-related changes in a wide range of organisms, and the abundance of knowledge produced, a final definition of aging and its reasons have been the object of countless debates and many theories of aging from evolutionary, molecular, cellular or systemic point of view have been proposed during the years, sometimes with contradictory results, highlighting the need of more investigation in the aging field.

Nonetheless, the majority of bio-gerontologists today seems to agree at least on the fundamental mechanisms of the aging process.

Life is characterized by a continuous series of environmental changes. The aging process depends on the individual´s ability to respond, control, and adapt to them, maintaining a state of dynamic equilibrium, known as *homeodynamics*, which in turn depends on a maintenance and repair system *(2)*.

The individual's ability to respond and to survive the environmental damages can be described as having a *homeodynamic space.* However, because of the imperfections of the maintenance and repair systems, a vulnerability zone is always present. When the failure starts to be severe, the system can break down or even collapse bringing to aging first, and consequently to death (**Figure 1.1**) *(3)*.

Recent evidence points to DNA damage accumulation, due to environmental damage and failure of DNA repair systems, as one of the major drivers of the aging process *(4)*. Aging therefore involves a main feature: fitness decline, with the increase of mortality rate and a parallel reduction in fertility due to a progressive decline in the ability to respond to environmental challenge *(5)*.

Moreover, on a microscopic level, the consequences of age-related changes to the cellular macromolecular components lead to gradual loss of normal structure and function called ‘‘chronological aging’’, due simply by the passage of time. For continuously dividing cells, instead, there is another challenge of ‘‘replicative aging’’. We could say that normal cells are designed for a limited number of successive cell growth-and-division cycles. This limit is known as the Hayflick Limit *(6)*. Cells go on growing until 40-60 divisions, and then they slow down and finally stop. This state is known as senescence, and it is an irreversible state because of the accumulation of cellular damage, such as telomere shortening and replication-associated DNA mutations, that occurs during the process of cell division *(7)*.

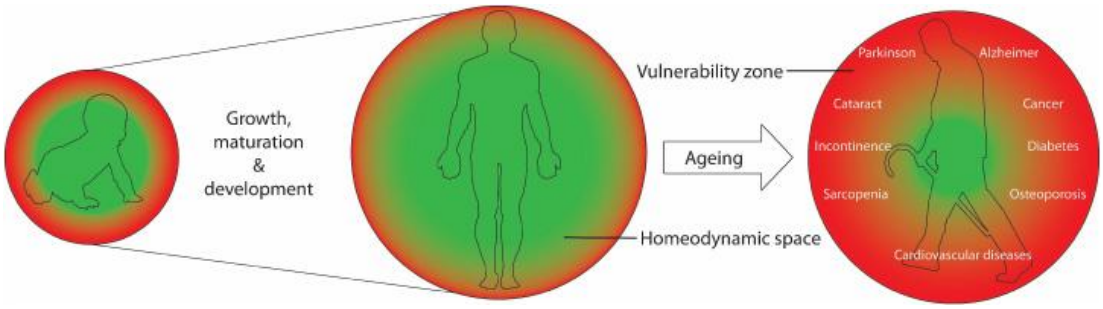

Figure 1.1 Representation the Homeodynamic.

Homeodynamic space is the ability of the living systems to respond and counteract stress, to repair and remove the damage, and to undergo constant remodeling and adaptation. Genetic polymorphism and epigenetic factors including prenatal exposures and lifestyle establish a personalized functional homeodynamic space during growth, development and maturation, within the evolutionary constraints of essential lifespan of the species. Due to the imperfections of the maintenance and repair systems, there is always a small vulnerability zone. Aging is the progressive shrinkage of the homeodynamic space, resulting in an increase in the vulnerability zone and in the probability of emergence of age-related diseases and the eventually death.

Aging in humans is broadly a linear process, characterized by changes in appearance, due to loss of muscle and bone mass, a lower metabolic rate, longer reaction times, declines in certain memory functions, as well as in all body functions. Although no

specific mutation is known to increase human lifespan, several genetic diseases appear to accelerate many features of the normal aging *(8)*.

Aging studies are increasing in biomedical research, especially those attempting to slow this process down. In fact aging itself is the leading risk factor for a variety of widespread diseases, such as selected cancers, cardiovascular disease, and Alzheimer's disease, but also conditions such as frailty which, in principle, was distinct from diagnosed diseases or disability *(9, 10)*. The concept of frailty is suggested to help in understanding the heterogeneity of functional decline observed with chronological aging. The presence of some components identifies a person as being frail, pre-frail or not-frail *(11)*.

In the last years many definitions of frailty have arisen. The most valued has been proposed by Schuurmans and colleagues "frailty is a loss of resources in several domains of functioning, which leads to a declining reserve capacity for dealing with stressors" *(12)*. The main consequence of such vulnerability is an increased risk of multiple adverse health-related outcomes.

Nonetheless, to directly study aging in humans can be very difficult, especially because of the long lifespan and the ethical concerns. However well characterized systems for aging research are available: mice, rats, flies, worms and yeast, are the most popular. In the last century model organisms have acted as engines of genes and mechanisms involved in extension of lifespan discovery *(13)*.

1.2 EPIGENETICS

In the last sixty years, it became evident that the genomic code of DNA is not the only carrier of information within a cell or an organism. With "epigenome" we refer to the whole epigenetic modifications occurring on a genome-wide scale *(14)*. Epigenetic represents heritable information that is not directly encoded by the DNA sequence itself but involves chemical alterations of chromatin. The concept of epigenetics was first proposed by Conrad Waddington in 1942. According to him, environmental inputs could be converted into an internal genetic factor by "canalization of development", explaining how complex phenotypes could form from interaction between genes and environment both internal and external *(15)*.

To explain this concept Waddington uses a rolling ball to symbolize a genotype and a downward sloping hill, upon which the ball rests, to symbolize epigenetics (**Figure 1.2)**.

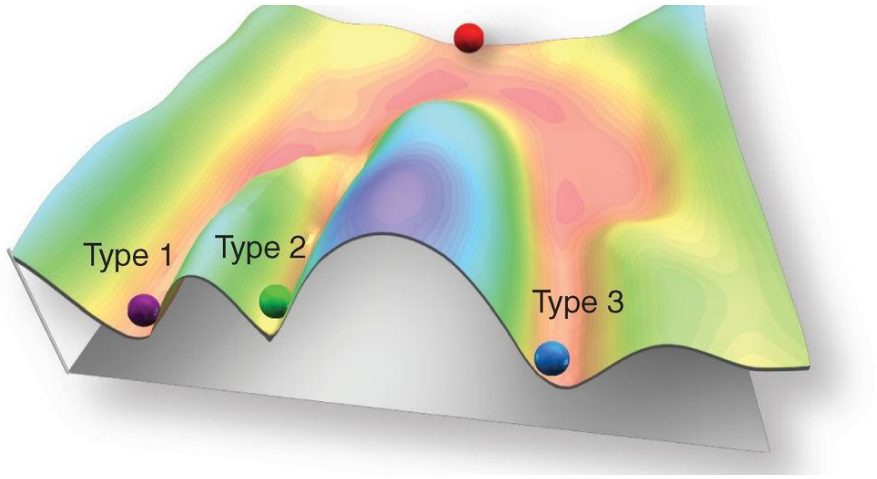

Figure 1.2 Waddington's Epigenetic Landscape.
The development of a cellular state is represented by a ball rolling down a landscape of bifurcating valleys. At various points in this dynamic visual metaphor, the ball that represents a stem cell can take specific permitted trajectories, each representing different cell types. Source *(16).*

As the ball travels down the hill it encounters a number of paths, each one bringing the ball in a variety of different directions and leading to a variety of different outcomes. The idea is that a same genotype encounters a certain number of different signals as it progresses through development. The path each gene takes will confer ultimate phenotypic expression *(17)*.

More recently, Berger and colleagues established a new definition of epigenetics. From this point of view, "An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" *(18)*.

Epigenetic changes are stables, however at the same time they can be regulated by internal factors as physiologic and pathologic conditions, as well as by the external environment *(19, 20)*, or by stochastic factors *(21)*.

Gene expression control at the chromatin level is essential for all eukaryotic organisms, and it is important especially in multi-cellular organisms to orchestrate the biological processes, such as differentiation, imprinting, X-chromosome inactivation and aging. Cellular differentiation is the best example to understand how epigenetic changes work. In fact, although all cells in an organism contain essentially the same DNA, cell types and functions, they differ because of qualitative and quantitative differences in their gene expression. Control of gene expression is therefore at the heart of differentiation and development. The patterns of gene expression that characterize differentiated cells are established during fetal development, when each cell gains a specific set of epigenetic marks, that will be maintained as the cells divide by mitosis. However some of these marks will change all lifelong responding to environmental stimuli while the cellular genome will be still intact *(22)*. Therefore, if we imagine the ball that rolls down the hill as a cell, the epigenetic landscape would represent all possible paths the cell can take leading to differentiation and lineage commitment (**Figure 1.2)**.
The key epigenetic marks are DNA methylation, histone tails modifications and non-coding RNAs silencing *(23)*.

DNA methylation is a process whereby a methyl (CH_3) group is added, most commonly to a cytosine of a DNA sequence. In vertebrates, DNA methylation occurs almost exclusively at CpG islands and correlates with transcriptional repression *(24)*. Lower eukaryotes show either no or very little traces of DNA methylation. Nonetheless, three kinds of modifications (5-methylcytosine, N4-methylcytosine and N6-methyladenine) are found in various bacteria, where they are associated with restriction enzyme-based defense.

Histone modifications. The organization of the eukaryotic genome into chromatin allows DNA to fit inside the nucleus while also regulating the access of proteins to the DNA to facilitate genomic functions such as transcription, replication and repair. The basic unit of chromatin is the nucleosome, comprised of five histone molecules. Interestingly, the N-terminal tails of histones can be subjected to at least 8 types of known post-translational

modifications. The modifications include acetylation, methylation, phosphorylation, sumoylation, ubiquitination, ADP-ribosylation, deimination and prolineisomerization *(25)*. Covalent post-translational modifications can impact the conformation of the nucleosome-nucleosome architecture within chromatin and influence its function such that some modifications are associated with an active chromatin state and others with a repressive state *(26)*.

Noncoding RNA. Recent evidence has emerged showing that noncoding RNAs regulate multiple epigenetic phenomena. Non coding RNAs can be divided in two classes: microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). Roles for miRNAs and lncRNAs have been demonstrated in the regulation of a broad range of biological activities and diseases *(27)*

1.3 EPIGENETICS AND GENE REGULATION

Epigenetic modifications are broadly involved in three key activities:
control of chromosome architecture ensuring stability and proper chromosome segregation during cell division; regulation of the silencing of repetitive elements; and finally, they can initiate and maintain the activity and silencing of genes or their clusters *(26)*.

Gene expression is a complex process involving numerous steps. Transcription, translation and protein modification represent the fundamental steps to transfer genetic information from the store copy of DNA to mRNA, broadly with the following protein synthesis *(28)*.

Epigenetic processes influence gene expression mostly at the transcription level.

In general, DNA methylation is associated with gene repression *(29)* while the histone modification can be related to both silencing and activation of gene expression *(30)*. DNA methylation leads to transcriptional silencing by means of a mechanism involving a group of protein known as DNA methyl-binding proteins (MBPs) that are transcriptional repressor proteins *(31)*. These patterns can also be maintained after DNA replication and mitosis.

In contrast to DNA methylation the effects of histone modifications are more complex and the scientific literature is constantly expanding. Early studies indicated that histone acetylation positively correlates with transcription (as opposed to low levels of acetylation associated with transcriptionally silent chromatin).

Acetylation of lysine residues neutralizes the positively charged histone side chains, reducing the strength of the binding of histone tails to negatively charged DNA, 'opening' the chromatin structure and facilitating transcription, similarly to lysine phosphorylation *(30)*.

Of the various methylated residues that have been identified, several have been highly characterized.

The consequences of methylation as well as ubiquitylation can be either positive or negative with respect to transcriptional activity, depending on the position of the modified residue, whereas lysine sumoylation is always connected to gene repression. The stability of these modifications is variable. Acetylation and ubiquitylation are dynamic and transient, whereas methylation is stable and longer lasting *(32)*.

In summary, the covalent modification status of histone proteins, together with nucleosome composition and arrangement comprises an epigenetic layer of information that facilitates or inhibits gene expression.

Discovered less than a decade ago, miRNAs, the best characterized of all non-coding RNAs, have emerged as important regulators of gene expression. miRNAs repress gene expression degrading target mRNAs and/or inhibiting their translation. Nonetheless RNA-based mechanisms of epigenetic regulation are still less well understood than mechanisms based on DNA methylation and histone tails modifications *(33)*.

1.4 EPIGENETICS AND AGING

Aging is associated with the accumulation of DNA mutations both at nuclear and mitochondrial level *(34)*. Although the accumulation of these mutations has been correlated with aging, no experiment has demonstrated that a reduction in DNA mutations leads to an extension of lifespan. As such, there is currently much interest in the role of epigenetics as a mediator of the aging process *(35)*.

The relationship between epigenetics and aging was proposed many years ago. Pioneering studies showed that genomic global DNA methylation decreases with age in spawning humpbacked salmon and in rat brain and heart *(36, 37)*. More recently, this trend has been confirmed in various mouse tissues and human cells. The definitive validation was provided in 2008 through a longitudinal study of DNA methylation in more than 100 individuals *(38)*.

Moreover the evidence about the relationship between epigenetics and aging come from studies on multiple model organisms, suggesting this as an evolutionarily conserved process *(39)*.

It was observed that the aging clock of *C. elegans* can be halted by environmental influences; this suggested that the causes of aging may be largely epigenetic. The hypothesis is further supported by the finding of transgenerational epigenetic inheritance of extended lifespan in *C. elegans (40)*. Aged cells show several distinctive features on their chromatin and not only a global reduction in DNA methylation. Depletion of DNMT1, the enzyme responsible for maintenance of cytosine methylation, can be related to the observed age-associated increase in stochastic gene expression (transcriptional noise) in some aging tissues *(41)*. The sirtuin family of NAD+-dependent lysine deacetylases has also long been associated with the control of longevity, although the precise mechanisms remain controversial *(42)*.

Furthermore studies on mono- (MZ) and di-zygotic (DZ) twins provided significant evidence that epigenetic variants accumulate during aging independently of the genetic sequence *(43)*. It was observed that lower epigenetic differences occur between MZ than DZ twins. Despite MZ twins are epigenetically indistinguishable during the early stage of life, older individuals exhibited significant differences in their epigenome, elucidating the effect of environmental characteristics on gene function and phenotype *(44)*.

Recent researches on *C. elegans* indicate that the miRNA might also become altered with aging. However, very little is known about miRNA function *(45)*.

Epigenetic changes are, therefore both responsive to, and effectors of the aging process *(46)*. With DNA damage and environmental stresses leading to changes in chromatin, the epigenome adapts to age-related changes as a sensor of cellular

dysfunction, sensing changes that accompany aging. However, the epigenome is also an effector of the aging process, enforcing different patterns of gene expression resulting in cellular phenotypes associated with aging such as senescence and metaplasia *(47)*.

CHAPTER 2: METHYLATION OF THE RIBOSOMAL RNA GENE PROMOTER IS ASSOCIATED WITH HUMAN AGING AND AGING PHENOTYPES

2.1 INTRODUCTION

2.1.1 The Ribosome: Structure and Function

Life, as we know it, is possible thanks to interactions between nucleic acids, proteins and lipids. Protein biosynthesis is an essential process borne by ribosomes, a molecular machine in charge for translating genetic information into proteins *(48)*.
Ribosomes are ribonucleoprotein complexes lie in ribosomal RNA (rRNA) and over 50 ribosomal proteins. Albert Claude first discovered rRNA during his experiments on sarcoma virus infected cells in the late 1930s *(49)*. Later George Palade characterized the ribosome, a new cytoplasmic component with a small, round body of about 100 to 150 Å in diameter *(50)*, and after a few years, protein synthesis was shown to take place on the ribosome *(51)*.
Ribosomes are composed of two unequal subunits called small and large subunit respectively (**Figure 2.1**). The structure has been conserved throughout the kingdoms *(52)*, in fact the ribosomal subunits of prokaryotes and eukaryotes are quite similar. However, the size is different, and the difference is due to the protein and rRNA composition. Prokaryotes have smaller ribosomes with a sedimentation coefficient of 70 Svedberg units (70S) consisting of a small (30S) and a large (50S) subunit, compared to eukaryotic ribosomes of 80S composed of a small (40S) and a large (60S) subunit *(53)*.
Eukaryotic large subunit contains a 5, 25, 28, and a 5.8S rRNA *(53)*, and about 49 proteins, while the small one contains an 18S rRNA and about 33 proteins *(54)*. Ribosomal proteins work in a cooperative way; this means that no one protein alone is able to catalyze an enzymatic reaction. Moreover in the later years a more important role has arisen for rRNAs, in fact, evidence has shown their plausible catalytic activity inside the ribosome, displayed as a super-enzyme complex *(55)*.
Ribosomes are abundant cellular elements, placed in several cellular compartments:

in cytoplasm, where they synthesize cytoplasmatic, nuclear, mitochondrial and perossisomal proteins, implicated in the post- translational targeting;

on the endoplasmic reticulum (ER), originating the rough ER. Here membrane-bound proteins, proteins residing in the endoplasmic reticulum (ER), Golgi and endosomes are synthesized, following the co-translational targeting;

inside mitochondria and chloroplasts, where proteins codified by their own genes are synthesized.

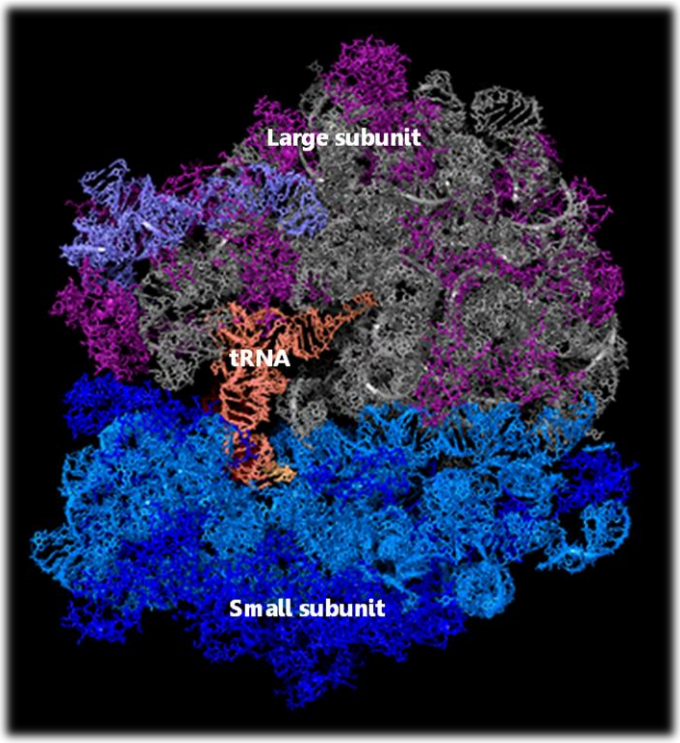

Figure 2.1 Ribosome structure

Ribosomes are complex molecular machines that synthesize proteins in all living cells. This view of a ribosome shows its components in different colors: the small subunit on the left contains an RNA molecule (cyan) and proteins (dark blue); the large subunit on the right contains RNA molecules (grey and slate) and proteins (magenta). The image also shows a transfer RNA (orange) bound to the active site of the ribosome. Source *(56).*

In both prokaryotic and eukaryotic cells from 20000 to 50000 ribosomes are present, depending on protein synthesis activity. In fact cells must govern both the amounts of specific proteins synthesized and the total protein synthesized in response to environmental signals and internal programming *(57).*

2.1.2 Ribosomal DNA (rDNA) arrangement

The rRNA genes are the most conserved DNA sequences in cells, also in distantly related organisms. For this reason, rDNA has been a useful tool in taxonomy and phylogeny studies *(58)*.

Most prokaryotes have three rRNAs, called the 5S, 16S and 23S rRNA organized in operons. Genes for ribosomal RNAs in eukaryotic genomes have been found in separate clusters, consisting of several hundred tandemly repeated copies of the transcription unit and non-transcribed spacer (**Figure 2.2**) *(59)*. The number of copies of this transcription unit may be variable within different species.

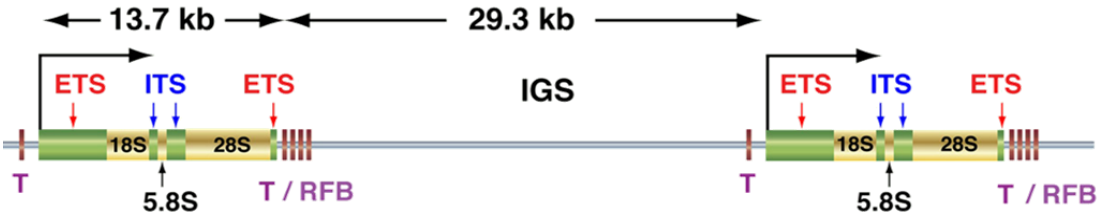

Figure 2.2 Organization of the human rDNA repeats in eukaryotes.

The coding regions for the 18S, 5.8S and 28S rRNA are shown as yellow boxes. The green boxes represent external (ETS) and internal (ITS) transcribed spacer regions. The start site and direction of transcription are indicated by the arrow. Tandemly repeated rRNA coding units are separated by nontranscribed intergenic spacers (NTS/IGS) *(60)*.

rRNA genes are arranged as clusters of tandem repeats. One cluster consists of hundreds of copies of the 5S gene. Human 5S RNA genes are arranged as tandem repeats in a large cluster on chromosome 1. The number of repeats is variable from 35 to 175 copies. The other genes are found in similar structures to the bacterial operons on the short arms of five chromosomes, which consist of repeated sequences, including rDNA and several kinds of satellites *(61)*. In humans, there are five clusters of rDNA located at the p region of 13, 14, 15, 21, and 22 chromosomes. These regions are called nucleolar organizer regions (NORs), where nucleolus originates. The nucleolus is a dense region of the nucleus where massive transcription of ribosomal RNA genes takes place *(62)*.

There is considerable variation in the size of a transcription unit from one species to the next. This fluctuation is due to the length of the external and internal transcribed

sequences (ETS) and (ITS) (**Figure 2.2**). RNA precursor of about 13,000 bp, known as 47 RNA, originates the remaining 3 RNA molecules, 28S, 18S, 5.8S *(63)*.

2.1.3 Structure and function of Ribosomal RNA

Analyzing the primary rRNA structure, it appears as a hodgepodge of evolutionarily conserved interspersed within non-conserved sequences *(64)*. Efforts to predict the secondary and tertiary structure of rRNAs has been made since the late 1970s.

The rRNA secondary structure is mostly characterized by double strand segments, however many hairpin loops have been found. The secondary structure is highly conserved (**Figure 2.3**), for instance, the *E. coli* 23S molecular structure was found to be conserved in the yeast 25S rRNA with extra blocks of sequence and it was also reported in the 28S molecule of multicellular eukaryotes with other extra blocks inserted into the structure. These extra blocks, which occupy the same relative positions in all organisms, have been called expansion segments or variable regions. The increase in size from the *E. coli* 16S and 23S rRNA to the mammalian 18S and 28S rRNA has been attributed to the differences in the number and size of the expansion segments. The small rRNAs of the ribosomal large sub unit LSU (5.8S and 5S) do not contain significant expansion segments as is the case for the large rRNA molecules. However, as mentioned earlier, the 5.8S rRNA is not free but is associated with the 25-28S rRNA *(64)*. Studies on the primary and secondary structures of 5S RNAs also demonstrated that the nucleotide sequence is highly conserved in the course of evolution.

Modeling of eukaryotic ribosomal RNA (rRNA) has been limited to available crystal structures from eubacterial and archaeal sources. Although recent progresses have been made, it is still missing approximately 50% of the rRNA sequence because of expansion segments *(64)*. The tertiary structure of the rRNAs available from X-ray diffraction analysis has shown that the substitution rates, during evolution, are generally low near the centre of the ribosome, where the essential nucleotides for its function are situated, and that they increase towards the surface *(65)*.

The tertiary structure of the eukaryotic ribosomal 5S rRNA, examined using chemical modifications and enzymatic cleavage reactions, revealed a structure

resembling a lollipop. Although the tertiary structure of 5S rRNA has been obtained further studies are still required *(66)*.

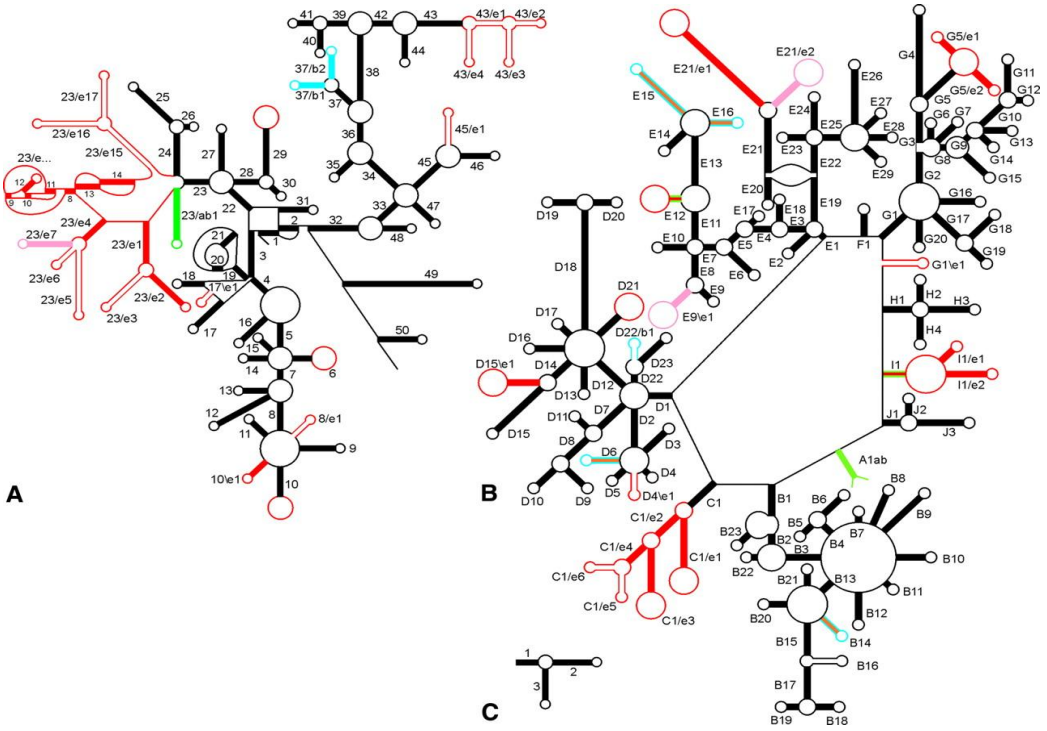

Figure 2.3 Secondary rRNA structures.

Structure and helix numbering of (A) SSU rRNA, (B) LSU rRNA (C) 5S rRNA. The presence of helices in the three domains is indicated as follows: Bacteria only, blue; Eucarya only, red or pink; Bacteria + Archaea, green; Eucarya + Archaea, orange; all three domains, black. Source *(65)*.

Efforts to identify the responsible components for the different ribosomal functions have been made since the discovery of the role of the ribosome in the translation process.

In the early studies the researchers focused their attention on the ribosomal proteins as carriers of the enzymatic functions. The biological role of ribosomal RNA was commonly accepted to be only structural *(67)*. However, after the discovery of catalytic RNAs and RNA catalytic function of RNase P the role of rRNA in translation has been investigated *(68)*.

During the last forty years many experiments have been made to better understand the rRNAs activity, identifying many important features. Shine and Dalgarno, in

1974, discovered that 3' region of the 16S rRNA was involved in mRNA binding and this sequence is still known as Shine and Dalgarno sequence *(69)*.

Later it has been demonstrated that mutations in 16S or 23S RNA affected the resistance to several antibiotics *(70)*. Moreover the 16S rRNA molecule is involved at the A site and it takes also contact with the tRNA molecule at the P site, the crystal structures directly showed the participation of 16S rRNA in tRNA binding to the 30S P site *(71, 72)*.

In 1992, Noller and colleagues showed that the 50S subunit of *T. thermiphilus* retained a peptidyl transferase activity after proteins degradation, referring the catalytic functionality of rRNA *(73)*.

A functional role of 5.8S rRNAs, instead, has emerged more recently, suggesting that the 5.8S rRNA plays a direct role in ribosome translocation *(74)*.

The 5S rRNA molecule is the most highly conserved rRNA, with an important role in ribosome function, coordinating the multiple events catalyzed by the ribosome. Evidence demonstrated that if 5S molecule is not incorporated into the LSU, the LSU becomes unstable and quickly degrades *(75)*.

All these proofs show the functional roles of rRNAs, which are constantly increasing. Thus, rRNA is involved in all the translation process: mRNA and tRNA binding, peptidyl transferase activity, association of ribosomal subunits, translocation, and termination as well as in drugs interaction.

2.1.4 Ribosomal RNA transcription

The nucleolus is a nuclear compartment formed around the ribosomal DNA repeats, called nucleolar organizing regions (NORs). Nucleolus can be considered as a factory where these ribosomal RNAs are transcribed, processed and assembled into ribosomal subunits *(76)*.

In higher eukaryotes, a single transcription unit encodes a precursor transcript (45S pre-rRNA) containing the sequence for three rRNA molecules (5.8S, 18S and 28S).

rRNA synthesis, by means of transcriptional activity changes, meets the demand for ribosome production and protein synthesis. rRNA genes activity during development and differentiation of each cell type requires a strict regulation through a wide range

of pathways among which JNK2-MAPK-mTOR, MYC, PKC, p53 and RAS/ERK *(62, 77–80)*.

The transcription of rRNA genes can be defined as a check point for the ribosome biogenesis. The number of active rRNA genes varies between different cell types, and during different stages of cell development and differentiation. It is highly regulated and also linked not only to cellular growth, proliferation and stress response, but also to energetic requirements of cells *(77, 81)*. Nutrients availability, growth factors, and ATP levels alter rDNA transcription, mainly modulating the activity of TIF-IA *(77)*. Transcription initiation factor TIF-1A was, in fact, suggested to be a key player in regulation of rDNA transcription *(82)*.

The rate of cellular growth is directly dependent on the rate of protein synthesis, which, itself, is intricately linked to ribosome synthesis and rRNA transcription. Several reports suggested that yeast rRNA synthesis could be modulated by varying the transcription rate of rDNA genes or by varying the number of active genes, although an a electron microscopy study revealed that the overall initiation rate determines the rate of rDNA transcription *(83, 84)*.

Almost all signaling pathways that influence growth converge at the RNA polymerase I (Pol I) transcription machinery *(62)*. However not only alterations in the amounts or activity of trans-acting factors, but also the gene promoter chromatin accessibility by Pol I regulate rDNA transcription in eukaryotes *(76)*.

The rDNA promoter contains two domains, the core promoter element (CPE) and the upstream promoter element (UPE). The other sequences involved in promotion of rDNA transcription include the promoter proximal terminator elements and the enhancers. The mammalian rRNA gene core promoter is located from -45 to +18 relative to the start site (+1), that is essential for accurate transcription initiation. The Upstream Promoter Element (UPE), from -156 to -107, shows a regulatory role. The crucial role of both of them has been demonstrated for a well-functioning of the transcriptional machinery *(85, 86)*.

In mammalian cells, efficient transcription of rDNA requires the interaction of the core promoter element with three major proteins:

pol I complex, a large complex enzyme with an approximate molecular weight of 500-600000 Daltons,

upstream binding factor (UBF), that binds as a dimer to the CPE, UPE, spacer promoters and the enhancer repeats in the intergenic spacer,

selectivity factor termed SL1 in humans or TIF-IB in mouse cells. Selectivity factor in turn is a protein complex consisting of TATA- binding protein (TBP), three transcription activating factors (TAFs), transcription initiation factors TIF-IA and TIF-IC and several others *(81, 87).*

2.1.5 DNA methylation

DNA methylation together with histone tail modification is the most important DNA epigenetic change. In eukaryotes it involves the covalent addition of a methyl group to the carbon 5 position of the cytosine ring. This modification is generally found on cytosines followed by a guanine, in a sequence called CpG dinucleotides (CpGs) *(88)*. CpG dinucleotides are not evenly distributed throughout the mammalian genome and are also greatly underrepresented *(89).* For instance, human genome presents only 5-10% of the CpG dinucleotides compared to what would be statistically predicted *(88).* One possible explanation for this distribution is that over a long evolutionary period, the number of CpG dinucleotides has declined as a result of the conversion of CpG to TpG because of the trend of methylated cytosines to deaminate to timine *(89).*

Despite their underrepresentation into the genome, CpG dinucleotides can be collected in small DNA sections. GC-rich sequences are frequent in satellite repeat, rDNA, centromeric repeat sequences and CpG islands (CGIs) *(90).*

DNA methylation prevalently involves repetitive sequences, broadly derived from transposable elements, as well as intergenic and intronic CpG-poor regions. Unmethylated CpG dinucleotides are instead gathered in the CpG islands (CGIs); sequences of about 0.5-1 kb in length, with a CG content, higher than 55%, often associated to the gene promoter regions and to the first exons of many genes among which most housekeeping genes and half of all tissue-specific genes *(91).*

In humans a very low percentage of all cytosine bases are methylated. However considering only CpGs dinucleotides, this number grows to approximately 70-80% *(92)*. Moreover embryonic stem cells seem to have also a substantial amount of non-

CpG methylation in promoter regions of different genes. This kind of methylation has been also reported in plants and in the D-loop of human mitochondrial DNA, although its biological significance is unclear, it seems to be a part of a specific pluripotent cell mechanism. After differentiation of the ESC line, cytosine methylation in non-CpGs disappeared, while it is present lifelong in the mitochondrial control region *(93, 94)*.

Basically two enzyme families are involved in DNA methylation establishment: DNA methyltranferases (DNMTs) and DNA demethylase. DNA methylation is mediated by a family of DNA methyltransferases (DNMTs) that includes DNMT1, DNMT3A, and DNMT3B. These enzymes transfer a methyl group from S-adenosylmethionine (SAM) to deoxycytosine, producing 5-methylcytosine and S-adenosylhomocysteine (**Figure 2.4**).

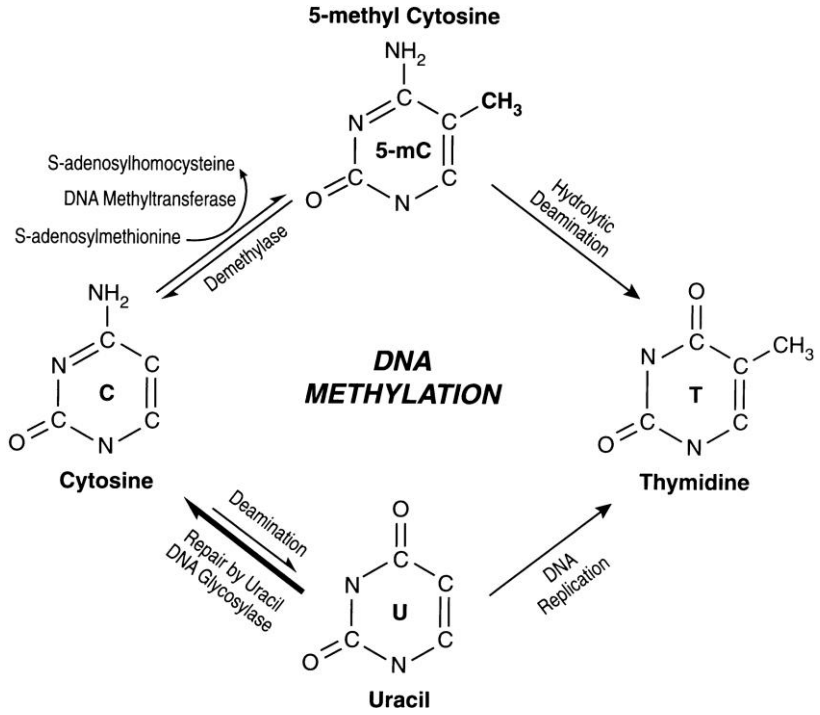

Figure 2.4 Schematic representation of the biochemical pathways for cytosine methylation, demethylation and mutagenesis of cytosine and 5mC.

Cytosine can be methylated to form 5-methylcytosine, which deamination rises to thymine. Deamination of cytosine rises to uracil that however it is recognized by the uracil-DNA glycosylase. Source *(88)*.

DNMT1 is responsible for maintaining CpG methylation during DNA replication. It acts on the hemimethylated sites of DNA produced during the replication process, maintaining the methylation pattern after DNA syntesis. DNMT3A and DNMT3B, homologous enzymes are in charge for *de novo* DNA methylation during development. While DNMT3B is prevalent in the early stages of embryonic development, DNMT3A acts later and it is also involved in the gametes methylation patterns establishment. DNA methylation patterns are also determined by DNA demethylases that operate in several ways *(95)*. More recently another epigenetic marker, 5-hmC, is emerging and it seems to have important roles in epigenetic reprogramming and regulation of tissue-specific gene expression *(96)*.

In a methylated DNA sequence, the methyl group is located in the major groove and does not interfere with the base pairing. DNA methylation, in the promoter regions, can repress gene transcription interfering with transcriptional activators or favoring the formation of repressive chromatin by methyl DNA-binding proteins. Almost all housekeeping genes and the majority of genes with tissue-specific expression contain one or more CpG islands in their promoter region. The methylation of the CpGs in these promoter regions correlates inversely with promoter activity and gene expression *(97)*.

In higher eukaryotes, DNA methylation is crucial for a wide range of cellular activities such as genome stability and protection, imprinting, X-chromosome inactivation, tissue specific gene regulation, carcinogenesis and aging *(98)*.

In mammals, DNA methylation is of vital importance for genome stability, as confirmed by homozygous mice with a disruptive mutations in the *Dnmt1* gene, they die at the embryonic stage *(99)*.

Changes in DNA methylation are involved in the pathogenesis of many human diseases. Differences in the global methylation pattern between cancers and their healthy tissue have been reported. DNA methylation has also been correlated with several non-malignant diseases, and physiological conditions such as the aging process *(100)*.

2.1.6 DNA methylation and aging

The aging process, as described in the first chapter, is characterized by a gradual deterioration of the functional capabilities, with an increased susceptibility to environmental challenges and illnesses, leading to an unavoidable consequence, the failure of survival and finally, the death.

Acquired changes in epigenetic marks have been suggested to be a part of the aging process as well as age-dependent onset of many human diseases *(101)*.

Several experiments have been made over the years, demonstrating the strong association between epigenetic changes and aging. One of the hallmarks of epigenetic drift is a progressive change in DNA methylation. In general two specific modifications of DNA methylation occur during aging: a solid and progressive increase in DNA methylation levels through lifespan in many specific loci and a hypomethylation, across all genome, of repetitive elements. In humans, studies on mono- (MZ) and di-zygotic (DZ) twins provided significant evidence that epigenetic variants accumulate during aging independently of the genetic sequence *(43)*. It was also observed that lower epigenetic differences occur between MZ than DZ twins. In fact DNA methylation changes in aging exhibit familiar clustering, suggesting that the DNA methylation stability is partially genetically determined *(38)*.

Despite MZ twins are epigenetically indistinguishable during the early stage of life, older individuals exhibited significant differences in their epigenome. Remarkably, those twin pairs who reported having spent less of their life together demonstrated the maximum differences in these global epigenetic marks. This suggests that different amount of shared environment might explain the observed differences in the pattern of epigenetic marks, elucidating the effect of environmental characteristics on gene function and phenotype *(44)*. One DNA methylation feature is the age-associated increase of methylation of the regulatory regions of specific genes, partially due to over expression of *DNMT3B (102–104)*.

An example is the increased methylation of the estrogen receptor (ER) gene promoter in colon. It was the first showing an association between aging and promoter DNA methylation. Since then, a growing number of specific loci have been described to become hypermethylated with aging. Hypermethylation was found in genes encoding for ribosomal DNA clusters and for DNA binding and transcription

regulatory proteins. In turn both rRNA and regulatory proteins can affect protein synthesis and biogenesis in general, as well as a huge range of pathways involved in a wide variety of cellular functions *(105, 106)*.

Genes involved in tumor suppression, development and growth, cell-cell adhesion, metabolism, DNA repair, control of signal transmission exhibit altered DNA methylation patterns in aging, in some cases displaying also tissue and cellular specificity *(107)*. Remarkably, methylation changes of certain genes such as the *EDARADD, TOM1L1, NPTX2, ELOVL2* , *FHL2*, and *PENK* can be age predictive *(108)*. Moreover global genome methylation gradually decreases with age in the majority of tissues. Loss of 5-methylcytosine content occurs mainly into non CpG-islands within repeated sequences such as interspersed ripetitive sequences (IRSs), Alu and human endogenous retrovirus K (HERV-K). This might be associated with a reduced expression of DNMT1, the enzyme responsible for maintenance of cytosine methylation *(103, 104)*. As demonstrated by Dnmt1+/- mice, they show insufficient DNA methylation, and it has been associated to immunosenescence *(41)*.

It should be considered that significant inter-individual differences in DNA methylation have been discovered in longitudinal studies with both increase and decrease of the global genome methylation over the period of more than 10 years.

It is evident how the effect of age on epigenetic marks can be difficult to interpret, as several possibly confounding genetic and environmental variables remain unaccounted for. Furthermore, as described above, changes in epigenetic marks are not all uni-directional. All these studies demonstrated a loss of the epigenetic control in aging, suggesting a correlation with pathological and physiological processes, among which the frailty phenotype. In particular, Bellizzi et al. reported that global DNA methylation levels were correlated to the frailty status in middle/advanced-aged subjects (**Figure 2.5**) *(38, 109)*.

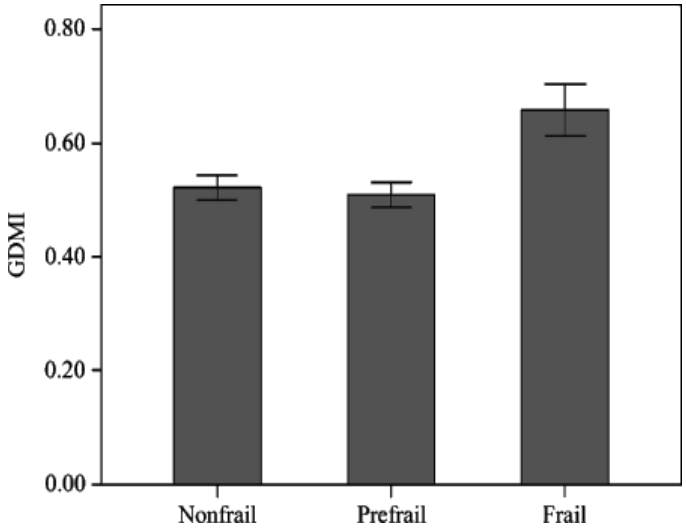

Figure 2.5 Distribution of methylation pattern in a sample of old subjects arranged according to frailty.

The human life expectancy has known a remarkable increase during the last century. Advances in biomedical technology with improved healthcare and disease treatment has to be considered in explaining these changes. However other factors have to be evaluated because genetic and environmental factors work together to determine many phenotypes. As described so far, several evidence gave weight to the epigenetic influence in the aging process as a bridge between aging itself and the environment. One of the most important environmental factors involved in the epigenetic control of aging is nutrition; dietary constituents can affect many phenotypes acting on the DNA methylation status, affecting also aging. These are enzyme co-factors such as folate and vitamins B12 and B6, as well as methyl group donors such as methionine, choline, betaine and serine that increase methylation, and selenium, green tea polyphenols and bioflavonoids that reduce methylation. Maternal behavior or diet can affect epigenetic patterns in her offspring. A study carried out on Agouti pregnant mice showed that a diet rich in methyl donors influences coat color, body weight, and health of their progeny, as well as a diet supplemented with antioxidants like folate, choline and vitamins B6 and B12 have been demonstrated to be effective in aging prevention. Calorie restriction (CR) is the most effective environmental manipulation that can extend maximum lifespan in different species. Recent data suggest that DNA methylation modifications, involving specific genes, play an important role in CR-dependent aging and longevity *(110)*.

2.1.7 Ribosomal DNA methylation

In mouse, about half of the rRNA genes are always transcriptionally silent. Although all the rDNA transcription units of a cluster are almost identical, an important part is generally silenced by means of epigenetic marks and thus, not all rRNA genes are available for transcription. Two classes of rRNA genes exist: active rRNA genes are characterized by an 'open' chromatin structure defined by DNA hypomethylation, acetylation of histone H4 and dimethylation of histone H3 and associated with emerging pre-rRNA, on the other hand silent rRNA genes are inaccessible, they present CpG hypermethylation, histone H4 hypoacetylation, methylation of H3K9 and H4K20 and they are not bound to transcription factors or Pol I (**Figure 2.6**) *(62)*.

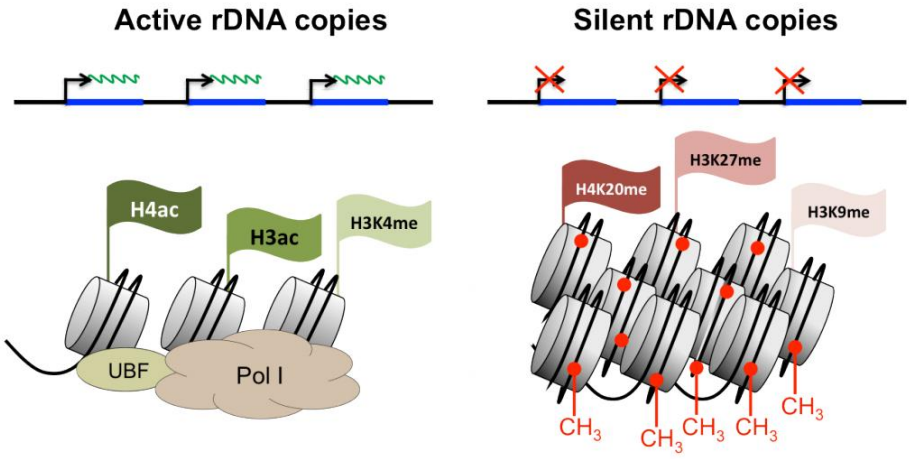

Figure 2.6 The two chromatin states of rDNA repeats.
The two classes of active and inactive NORs are epigenetically distinct. Potentially active rRNA genes exhibit an open chromatin structure, they are associated with Pol I, UBF and nascent pre-rRNA (green lines) and are characterized by DNA hypomethylation, acetylation of histone H4 (Ac) and methylation of H3K4me2. Epigenetically silenced rRNA genes are demarcated by CpG hypermethylation (CH3), histone H4 hypoacetylation, and methylation of H3K9 and H4K20 (Me). Modified from *(111)*.

DNA methylation is commonly associated with gene silencing. Vertebrate rDNA regulatory elements and transcribed sequences show an unusual pattern. They are both rich in CpG dinucleotides and densely methylated. In the early studies, the methylation status of CpGs within the sequence CCGG of mouse or rat rDNA were

determined using the isoschizomers *Hpa*II and *Msp*I. These studies showed an interesting link between the activity state and the methylation levels of the rRNA genes. The methylated sequences matched the silent repeats present in the promoter and enhancer of inactive genes (**Figure 2.6**) *(112)*.

The correlation between promoter methylation and transcriptional silencing was further investigated; finding that 5-aza-2-deoxycytidine (aza-dC) was able to incentivate rDNA transcription, suggesting that transcriptional silencing of rDNA was due to DNA methylation. However methylation did not prevent transcription directly, but only when DNA was assembled into chromatin, indicating a connection between DNA methylation and chromatin modifications to silence the rDNA units *(112)*.

It has been demonstrated that rRNA synthesis is determined by the RNA polymerase I activity rather than the number of active genes. However the coexistence of active and silent rDNA units in each cell implied that only through some phases of early development all gene copies might be activated; as during the oogenesis, for example, where large quantities of ribosomes and proteins are required. This thesis is supported by the lower level of global CpG methylation in germ line cells compared with somatic cells, indicating a cell- or tissue-specific epigenetic difference. The same mechanisms may be related to the augment ribosome production in cancer cells, where rDNA methylation is decreased and the number of active rRNA genes is increased *(111)*.

In mice, CpG dinucleotide at position –133 within the UCE is sufficient to compromise the UBF transcription factor and the Pol I to nucleosomal rDNA binding, preventing initiation complex formation. Impairment of UBF binding to methylated rDNA promoter was specific to rRNA genes assembled in chromatin. This implied that cytosine at position -133 is exposed on the surface of the positioned nucleosome and that the methyl group represents a steric barrier for the binding of UBF transcription factor. In human cells, there are at least 25 CpGs residing within the bounds of the Pol I promoter, although only the CpG methylation at positions -60 and -68 seems to act similarly to the mouse -133 CpG dinucleotide *(113)*.

Mouse and human promoters of active rDNA are associated with Pol I transcription complex and with active histone marks such as acetylated histone H4 (H4Ac) and

histone H3 dimethylated (H43K4me2). On the contrary, silent rRNA genes are associated with histones presenting repressive marks such as H3K9me2 and H4K20me3, and with the heterochromatin protein 1 (HP1) *(111)*. Thus, active and silent rRNA genes are demarcated both by their pattern of DNA methylation and by specific modifications of their associated histones, linking the 'histone code' to the 'cytosine methylation code'. In addition, nucleosome positioning was recently proposed as further characteristic feature of active and silent rDNA chromatin. The positioning of a nucleosome over the promoter region of silent genes was found to be mediated by the nucleolar remodeling complex NoRC. In fact, in both human and mouse, the rDNA transcriptionally silent state is established by this complex. It is a member of ATP-dependent chromatin-remodeling machines, consisting of the ATPase SNF2h and the nucleolar protein TIP5. NoRC interacts with DNA methyltransferases (DNMTs), histone deacetylases (HDACs) and histone methyltransferases (HMTs). Histone deacetylation, DNA methylation and transcriptional silencing are closely interconnected and in higher eukaryotes, NoRC coordinates epigenetic events lead to transcriptional silencing.

The nucleolar remodeling complex is recruited to rDNA by TIP5. The C-terminal part of TIP5 carries a finger/bromodomain that interacts specifically with histones, recruiting SNF2 and chromatin-modifying enzymes to the rDNA promoter. They induce rDNA silencing by histone hypoacetylation, DNA methylation and ATP-dependent nucleosome remodeling. rDNA promoter silencing is initiated by recruitment of NoRC to rDNA, then TIP5 interacts with a complex containing HDAC1, with histone methyltransferase(s), and with DNMT(s) to modify histones and to methylate rDNA. Thus, NoRC operates as a scaffold organizing several complexes that act in nucleosomes remodeling, histones modification and DNA methylation generating a compact chromatin structure *(114)*. Furthermore, tumor studies support the existence of a correlation between promoter methylation status and the transcriptional silencing of rDNA. In cancer cells, rDNA transcription is raised, contributing to increased ribosomes and proteins production necessaries for tumor proliferation. According to this evidence, a lower content of rDNA methylation was reported for several tumors. Moreover, comparing ovarian cancer patients with long and short progression survival, researchers found an higher level

of rDNA methylation in the former group compared with the latter; indicating that rDNA silencing levels may influence tumor proliferation and its outcome *(115)*. Hypomethylation of rRNA genes correlates also with decreased genomic stability. Silencing of rDNA involves the assembly of a repressive chromatin structure. It has been demonstrated that knock-out of Dnmt1 in human cells led to rDNA demethylation, enhancing the binding of Pol I transcription machinery to rDNA as well as wide nucleolus disorganization. Searching for alterations of DNA methylation led to the discovery of an rDNA region hypomethylated through age in both spermatozoa and liver of male rats. rDNA methylation seems to be susceptible to the age process *(106)*.

2.1.8 Aging and ribosomal RNA

The importance of rDNA in the aging process was established in yeast, where the rDNA copy number strongly correlates with rDNA instability and cellular aging. According to the "rDNA theory of aging", dysfunction of DNA repair system is a cause of increased rDNA instability that in turn will prescribe the stability of the whole genome *(116)*. In yeast, mutations in genes implicated in rDNA transcription and elongation have been associated to longevity. In both yeast and humans, indeed, mutations within DNA repair genes result in a reduced lifespan. In humans premature aging phenotypes, related to pathologies such as the Werner syndrome, are mainly associated to RecQ homolog helicases mutations, involved in rDNA repair *(117)*. Unlike yeast, where a clear link exists between rDNA stability and aging, few studies in mammals are reported on this field, because of the difficulties in studying this genome complex section.

In yeast the rDNA cluster represents about 10% of the whole genome. For this reason it seems to be clear that rDNA copy number and its stability can influence the cellular health. In mammal, instead, the rDNA comprises only 0.3% of the genome. Nevertheless, the mammalian nucleolus has been reported to influence various cellular functions sequestering or releasing important factors for cellular processes, regulating senescence *(116)*. Changes in nucleolar morphology are detected in aging cells. While pre-senescent cells show a higher number of smaller nucleoli, senescent

cells have a single prominent nucleolus *(118)*. It has been observed that inhibition of rRNA synthesis and thus ribosome biogenesis pushes cells into the arrest phase, implicating the nucleolus in regulation of cell survival and proliferation. Indeed, some proteins such as the tumor suppressor protein ARF and nucleophosmin are regulated basing on their position into the nucleolus. Notoriously these proteins are involved in the cellular senescence process, mediating p53 stability. The tumor suppressor factors p53 as well as the retinoblastoma protein (RB) regulate the cell cycle checkpoints, inducing if necessary cell cycle arrest, senescence or apoptosis *(119)*. Therefore, the nucleolus plays an active role in the development of the senescent phenotype.

rDNA transcription have been shown to be prerequisites for G1-S progression, although, the exact molecular mechanisms remain largely unknown. The inhibition of Pol I transcription induces senescence in solid tumor cell lines and it is able to induces premature senescence in immortalized primary human fibroblasts (BJ-TERT), with also an up regulation of p53 and p21 protein levels *(120)*.

Previous studies have shown increased methylation of the 5′ end of the rDNA repeat unit during aging in mice. The rDNA role in premature and normal human aging was investigated by Machwe and colleagues. They measured rDNA gene copy number, the level of rDNA methylation, and rRNA expression during the *in vitro* senescence of primary fibroblasts from normal donors (young and old) and from Werner syndrome (WS) patients. WS fibroblasts grew slowly and reached senescence after fewer doublings if compared to the control. However the rDNA copy number did not change significantly throughout the lifespan of both normal and WS fibroblasts.

Using isoshizomeric restriction enzyme analysis, it has been shown that senescence of fibroblasts is associated to a significant increase of CpG methylation at rDNA genes. Methylation of cytosine residues in 28S rDNA increased significantly during aging in both wild type and WS fibroblasts. The maximum difference in methylation of rDNA between the early and senescent passages was observed in WS fibroblasts. CpG methylation in rDNA, hence, seems to be accelerated in senescent WS fibroblasts.

Increased CpG methylation is usually related to the lower expression of genes transcribed by Pol II, by means of a chromatin condensation process. Thus, changes

in the methylation state of rDNA could theoretically influence rDNA transcription by Pol I and, in turn, ribosomal function and protein synthesis, although it has to be further investigated.

Protein synthesis rates have been shown to decrease (40–70% in mammals) with aging, although the reasons are still obscure, they have been generally ascribed to decreased efficiency of ribosomes. Increased methylation of rDNA genes with age could result in altered ribosomal function and decreased protein synthesis, since increased methylation could cause an increased rate and accumulation of mutations in rDNA genes with a consequent transcription of mutated rDNA genes and a nonfunctional rRNAs production. 5-methylcytosine residues in fact are considered hot spots of mutation. They are deaminated at a higher rate than unmethylated cytosine. Deamination of 5-methylcytosine generates a potentially mutation, and about one-third of the point mutations responsible for human genetic diseases are exactly C-T changes at CpG sites *(121)*.

In conclusion increased CpG methylation of the rDNA is associated with cellular senescence; however it has to be better investigated if CpG methylation represents a consequence or cause of cellular senescence. Doubtless the aging process is related to the loss of epigenetic balance, and a growing role is covered by epigenetic modifications in rDNA units.

2.1.9 Aim of the study

The aim of this work was to investigate the cytosine methylation within the promoter region of the human ribosomal RNA genes, analyzing the methylation status of CpG dinucleotides.
Our attention has been focalized on this genome region because of:

- the crucial role of ribosomal RNA into cell functions. Transcription of nuclear ribosomal RNA (rRNA) genes, in fact, it is a key control point, highly regulated in ribosome biogenesis which in turn is linked to cellular adaptation, stress response, cellular growth and proliferation as well as to energetic requirements of cells;
- the high conservation of the rRNA genes throughout the kingdoms. These are the most conserved DNA sequences in cells;
- the rDNA genes arrangement. rRNA genes are found in similar structures to the bacterial operons, as clusters of tandem repeats on the short arms of five chromosomes;
- the peculiars pattern of the rDNA regulatory elements. Vertebrate rDNA regulatory elements and transcribed sequences show an unusual pattern; they are both rich in CpG dinucleotides and densely methylated.

Methylation levels of the promoter region of the human ribosomal RNA genes have been evaluated in blood DNA samples collected from human individuals of different age, showing different aging phenotypes through a MassARRAY EpiTYPER Sequenom platform, wishing to elucidate a potential association between rDNA methylation, aging and aging phenotypes.

2.2 MATERIALS AND METHODS

Population sample

Subjects were recruited between 1996 and 2008 in Calabria as part of different recruitment campaigns and included 334 unrelated individuals, of which 52 were very elderly cases (>95 years), 151 were in the age range 70–95 years, 85 were in the age range 45–70 years, and, finally, 46 were younger than 45 years.

The sample has been recruited as part of different recruitment campaigns carried out by our research group between 2002 and 2007.

The recruitment of subjects older than 90 years was carried out between 2002 and 2005 through the population registries of Calabrian municipality. For this purpose, in 2002 all 409 Calabrian municipalities were initially contacted asking for the lists of all persons born before 1912 or earlier and with residence in those municipalities. 321 of 409 (78.4%) municipalities sent the relevant list. From a population of 12,630 nonagenarians, 1,291 subjects were contacted between 2002 and 2007. 681 subjects were dead when we called, 81 subjects were excluded for dementia, 400 accepted to participate, while the others refused to enter the study.

Subjects in the age range 65–85 years were recruited between 2004 and 2007 as part of a survey aimed at monitoring the health status of this population segment in Calabria. These subjects were invited to participate by general practitioners who explained to them the aim of the project. http://biologia.unical.it/echa/results.htm.

Finally, the sample including subjects in the age range 20–60 was collected as part of a recruitment campaign focused on students and staff of the University of Calabria.

The recruitment campaigns and subsequent analyses received the approval of the Ethical committee of the University of Calabria. All subjects provided written informed consent for studies on aging carried out by our research group. White blood cells from blood buffy coats were used as a source of DNA.

Subjects older than 60 years (n=265) underwent through a geriatric assessment carried out by a geriatrician and a person (usually a biologist) trained to conduct a structured interview including the administration of a questionnaire validated at European level. The questionnaire collected socio-demographic information,

anthropometric measures and a set of the most common tests to assess cognitive functioning, functional activity, physical performance, and depression. In addition, common clinical hematological tests were performed. Subjects with dementia and/or neurologic disorders were not included. Phenotypic information was collected by using the questionnaires available at http://biologia.unical.it/echa/results.htm.

In a previous work, by using a hierarchical cluster analysis (HCA) which availed of specific geriatric parameters, the sample including subjects older than 60 years was used to identify specific aging phenotypes *(122)*. In particular, the sample was analyzed considering two different age groups. The first (S1) included 217 subjects (94 males and 123 females) 65–89 years old (median age 75 years); the second (S2) included 101 subjects (50 males and 51 females) older than 90 years (median age 99 years). By using this approach in the S1 sample, three clusters were identified: nonfrail (the cluster with subjects showing the best scores for the classification variables), frail (the clusters with subjects showing the worst scores for the classification variables), and prefrail (the cluster with subjects showing intermediate scores for the classification variables). In the S2 sample, two clusters were identified. Similar to the first classification, the two clusters obtained were defined as frail (the cluster with subjects showing the best scores for the classification variables) and very frail (the cluster with subjects showing the worst scores for the same variables). The diagnostic and predictive soundness of these classifications were confirmed by a 3-year longitudinal study. In fact, a detailed survival analysis showed higher survival chance for subjects characterized by lower frailty in these classifications.

DNA samples

Six milliliters of venous blood were drawn from each subject. Plasma/sera were used for routine laboratory analyses, while DNA was extracted from buffy coats following standard procedures.

Working principles of quantitative DNA methylation analysis using the MassARRAY system (SEQUENOM)

Quantitative assessment of DNA methylation was performed using the Sequenom MassCLEAVE™ assay. DNA samples for analysis are initially bisulfite-treated, resulting in the conversion of unmethylated cytosines to uracil, whereas methylated cytosines remain unchanged. This conversion reaction allows for accurate discrimination between methylated and unmethylated cytosines at CpG dinucleotides. Following bisulfite treatment, genomic DNA consists of two non-complementary single-stranded DNA populations. Subsequently, PCR primer pairs for a region of interest are designed to amplify both the forward and reverse strand of double-stranded genomic DNA. A T7 polymerase promoter tag is added to the 5′ end of the reverse primer to facilitate *in vitro* transcription and a 10-mer tag is added to the 5′ end of the forward PCR primer to minimize melting temperature differences between both primers during PCR cycling. Following PCR, unincorporated dNTPs are dephosphorylated by treatment with SAP. Reverse transcription is performed using a chemically modified T7 RNA polymerase which utilizes a mixture of ribonucleotides and deoxyribonucleotides when synthesizing the RNA strand. In parallel with the reverse transcription the cleave reaction is achieved using the pyrimidine specific Ribonuclease A (RNaseA) enzyme which cleaves at pyrimidines (C and T) only on the newly synthesized transcript. By incorporating a non-cleavable dCTP (deoxyribonucleotide) into the transcript, RNaseA is unable to cleave at C and can only cleave at T (T specific cleavage) yielding a population of single-stranded cleavage fragments. A methylated cytosine is represented by a G nucleotide in the cleavage fragment, whereas an unmethylated cytosine is represented by an A nucleotide. The mass difference of 16 Da between G (329 Da) and A (313 Da) is easily detected by MALDI-TOF MS. Depending on the number of methylated CpG sites within a cleavage fragment, the difference in mass will increase in 16 Da units.
In the following procedure this methylation specific difference is not used for sequencing but for generating methylation depending mass differences to be analyzed by mass spectrometry (**Figure 2.7**).

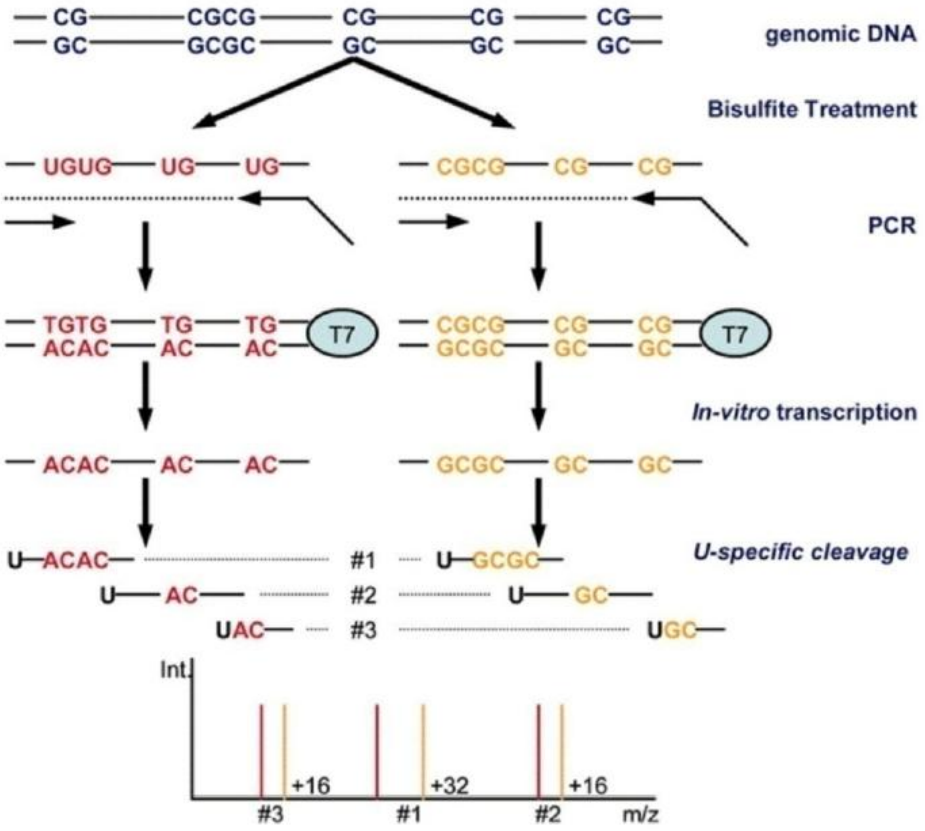

Figure 2.7 Schematic outline of the EpiTYPER process

Genomic DNA is treated with bisulfite and amplified using specific primers with one primer tagged with a T7 promoter sequence. PCR products are subsequently transcribed into RNA, followed by RNase cleavage after every uracil residue. Cleavage products are then analyzed by MALDI-TOF MS. In the unmethylated template (red) cytosine residues are deaminated into uracil and therefore appear as adenosine residues after PCR. Cytosine residues of a methylated template (yellow) remain cytosines. The conversion of guanine to adenine yields 16 Da mass shifts. Cleavage product 1 comprises two CpGs and the mass difference constitutes 32 Da if both CpGs are either methylated or unmethylated. Cleavage products 2 and 3 each contain only one CpG site that is differentially methylated and therefore yield a 16 Da mass shift *(123)*.

Primer design for EpiTYPER assay

The region of 239 bp of the human rRNA promoter harboring a CpG island containing 28 CpGs was amplified using the following primers:

RibFor 5'-GTCCTTGGGTTGACCAGAGG-3'
RibRev 5'-CTCCGGGTTGGAGAGGCTG-3'.

A T7-promoter tag (CAGTAATACGACTCACTA TAGGGAGAAGGCT) was added to the reverse primer and a 10-mer tag sequence (AGGAAGAGAG) was added to the forward primer to balance the PCR primer length.

Bisulfite treatment and PCR conditions

Bisulfite conversion of each DNA sample was performed by using EZ-96 DNA Methylation-Gold kit (Zymo Research), according to the manufacturer's protocol. Briefly, 1 µg of genomic DNA was added to 130 µl of CT Conversion Reagent in a final volume of 150 µl. The mix was incubated at 98°C for 10 minutes and, successively, at 64°C for 2.5 hours. After adding 400 µl of M-Binding Buffer to the wells of the Silicon-A Binding plate, each sample was loaded into the wells and centrifuged at 3,000 g for 5 minutes. After adding of 400 µl of M-Wash Buffer to the wells and a centrifugation at 3,000 g for 5 minutes, 200 µl of M-Desulphonation Buffer were added to each well and incubated at room temperature for 20 minutes. Then, the solution was removed by centrifugation at 3,000 g for 5 minutes and the wells were washed twice with 400 µl of M-Wash Buffer. Deaminated DNA was eluted in 30 µl of M-Elution Buffer.
The PCR reactions were carried out in a total volume of 5 µl using 1 µl of bisulfite-treated DNA, EpiTaq PCR Buffer 1X, 0.4 µM of each primer, 0.3 mM dNTP mixture, 2.5 mM of $MgCl_2$, 0.005 U TaKaRa EpiTaq HS (TaKaRa). The thermal profile used for the reaction included a 4-minute heat activation of the enzyme at 95°C, followed by 45 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, then one cycle at 72°C for 3 minutes. 0.5 µl of each PCR product were electrophoresed on 1.5% agarose gel to confirm successful PCR amplification and amplification specificity.

Dephosphorylation of unincorporated deoxynucleotide triphosphates and in vitro transcription and RNase A cleavage

Unincorporated dNTPs in the amplification products were dephosphorylated by adding 1.7 μl DNase free water and 0.3 μl (0.5 U) Shrimp Alkaline Phosphatase (SAP) (Sequenom, Inc., San Diego, CA, USA). Each reaction was incubated at 37°C for 40 minutes and SAP was then heat inactivated for 5 min at 85°C. Subsequently, samples were incubated for 3 hours at 37°C with 5 μl of T-Cleavage reaction mix (Sequenom), containing 3.21 μl RNAse-free water, 0.89 μl 5x T7 Polymerase Buffer, 0.22 μl T Cleavage Mix, 0.22 μl 100 mM DTT, 0.40 μl T7 RNA & DNA Polymerase and 0.06 μl RNAse A, for concurrent *in vitro* transcription and base-specific cleavage. The samples of cleaved fragments were then diluted with 20 μl water. Conditioning of the cleavage reaction was carried out by adding 6 mg of Clean Resin.

Mass spectrometry

10 nl of the resultant cleavage reactions were spotted onto silicon matrix-preloaded chips (Spectro-CHIP, Sequenom) using a MassARRAY nanodispenser (Sequenom), and analyzed using the MassARRAY Compact System matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF) (Sequenom). The spectra's methylation ratios were calculated using EpiTYPER software v1.0 (Sequenom). The method yields quantitative results for each of the sequence-defined analytic units referred to as unit CpGs, which contain either 1 individual CpG site or an aggregate of downstream CpG sites. Triplicate independent analyses from sodium bisulfite-treated DNA sample were undertaken.

The effectiveness of the entire experimental procedure was also assayed by analyzing as control CpGenome^{TM} Universal Unmethylated DNA (Chemicon) and CpGenome^{TM} Universal Methylated DNA (Chemicon) in a serial mixture of methylated and unmethylated products, with 10% methylation increments.

Statistical analysis

SPSS v.20 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Non-parametric two-sided Mann-Whitney-U test was used to verify if the patterns of methylation were different between sample groups.

2.3 RESULTS

More than 300 copies of rRNA genes lie in the human genome, they are located in the nucleolar organizer regions (NORs) on the short arms of five pairs of acrocentric chromosomes. Each unit contains a coding region of about 13.7 kb, encoding the 18S, 5.8S, and 28S rRNAs, and a non-coding intergenic spacer (IGS) of about 27.3kb, consisting of enhancers, promoter spacers and the core promoter of the next coding sequence (**Figure 2.8**) *(60)*.

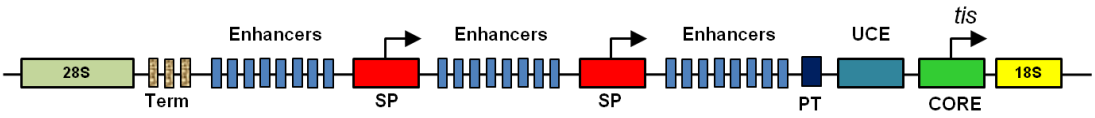

Figure 2.8 Basic structure of the regulator region of the eukaryotic rRNA genes.
IGS contains the promoter and a variable number of sub-repeats such as enhancers, promoter spacers, as well as the termination sequence, that greatly influence IGS length. Modified from *(124)*.

RNA polymerase I (Pol I) transcribes each 13kb unit into a pre-rRNA. The gene expression levels vary depending on the cell requirement. Short-term regulation of rRNA production occurs predominantly modulating the rDNA transcription rates, whereas long-term regulation also involves changes in the number of transcribed rRNA gene copies. Although it is a matter of housekeeping genes, only a small fraction, 20–50%, of all rRNA genes is transcriptionally active. Active rDNA and silent rDNA copies are respectively hypo- and hyper-methylated at CpG sites located within the promoter region. Therefore, it is evident that rDNA gene expression largely correlates to the epigenetic status of this region, even though the exact control mechanism is still unclear.

2.3.1 Methylation levels of the rRNA gene promoter correlate with aging

In this study, a 239 bp region of the rDNA promoter including the core and UCE was analyzed. This region is involved in the regulation of all pol I transcribed copies of rRNA by methylation *(125)*. It is highly enriched in CpG dinucleotides, containing 28 CpGs, 25 upstream and 3 downstream of the starting site (**Figure 2.9**).

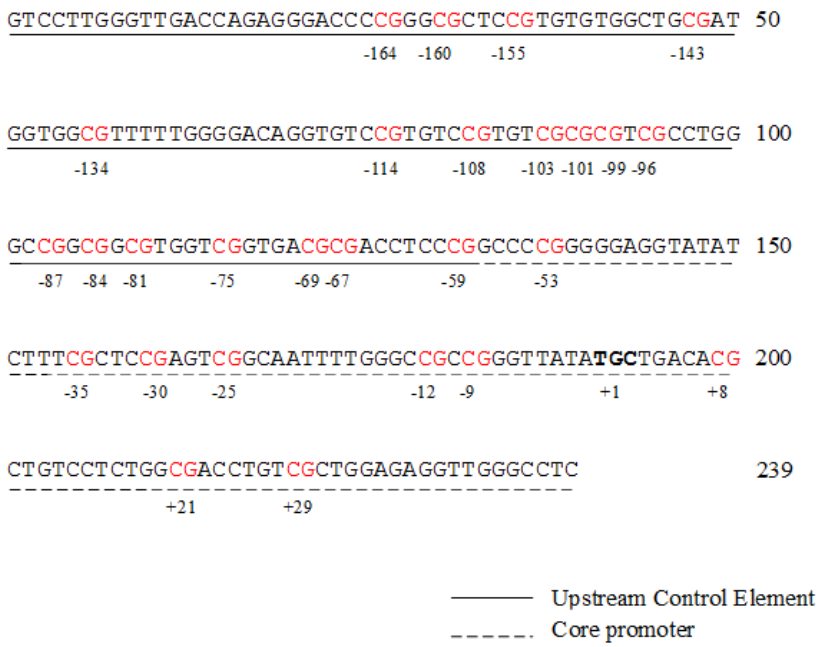

Figure 2.9 Sequence of the rDNA promoter region.
The starting codon is displayed in bold, while the CpG dinucleotides are highlighted in red. Their relative positions were indicated with respect to the starting site. The continuous and the dotted lines indicate the UCE and core promoter, respectively.

DNA methylation analysis was carried out using the EpiTYPER system from Sequenom. This system is a tool for the detection and quantitative analysis of DNA methylation using base-specific cleavage of bisulfite-treated DNA and MALDI-TOF mass spectrometry. Specific PCR primers for bisulfite-converted DNA were designed using the EpiDesigner software. T7-promoter tags are added to the reverse primer to obtain a product that can be transcribed *in vitro*, and a 10-mere tag is added to the forward primer to balance the PCR conditions. One amplicon including the entire 239 bp region was analyzed, where the 28 CpG sites have been grouped by the

software analysis into 11 unit CpGs. The experimental procedure was carried out in DNA samples extracted from peripheral venous blood collected from 334 differently-aged individuals (20- to 105-year-old subjects) showing specific aging phenotypes. A strict quality control was performed to remove potentially unreliable measurements. The unit CpGs that failed to produce data from more than 20% of samples (unreliable unit CpGs) and samples missing more than 20% of the data points (unreliable samples) were discarded.

Hence, a final set, including 4 unit CpGs that comprise 7 CpG sites, was considered. We investigated the association between DNA methylation levels of the above units with age. Obtained results of the two unit CpGs showing significant results are shown in **Figure 2.10**.

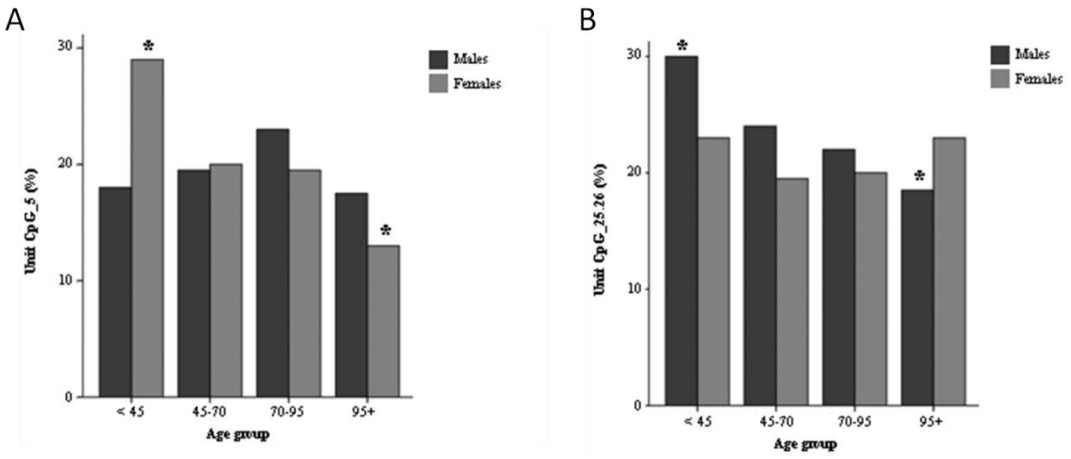

Figure 2.10 Median percentage of DNA methylation at each of the unit CpGs located within the rDNA promoter.

Methylation (%) is shown on the y-axis and the age groups on the x-axis.

Two units, CpG_5 and CpG_25-26, showed a significant hypomethylation with age. More specifically, unit CpG_5 methylation levels decreased from the younger subjects (median percentage 38%) to the older ones (median percentage 13%) in females, unit CpG_25-26 methylation levels also decreased from the younger subjects (median percentage 30%) to the older ones (median percentage 18%) in males. On the contrary, no significant difference was observed in the methylation levels for the CpG_18-19, and CpG_23-24 units (data not shown).

In addition, we investigated the relationship between methylation levels of the unit CpGs with the gender. A significant difference in these levels was observed between males and females in the four unit CpGs only in the youngest group of the sample. In fact, a hypermethylation and hypomethylation trend was observed in females with respect to males for the CpG_5 and CpG_25-26 units, respectively.

These results suggested that DNA methylation levels of the rDNA promoter region correlated both with the age and with the gender of analyzed samples.

2.3.2 Methylation levels of the rRNA gene promoter are associated of with frailty

Subsequently, we wondered whether the methylation percentages of the unit CpGs located within the rDNA promoter region correlated to the frailty status. To answer this question, we availed of the HCA classifications reported in *(122)*, that allowed us to classify this sample in different aging phenotypes (see "Materials and Methods"). In particular, we subdivided these subjects in two age-related groups, <90 and +90 years old. Obtained results are shown in **Figure 2.11**

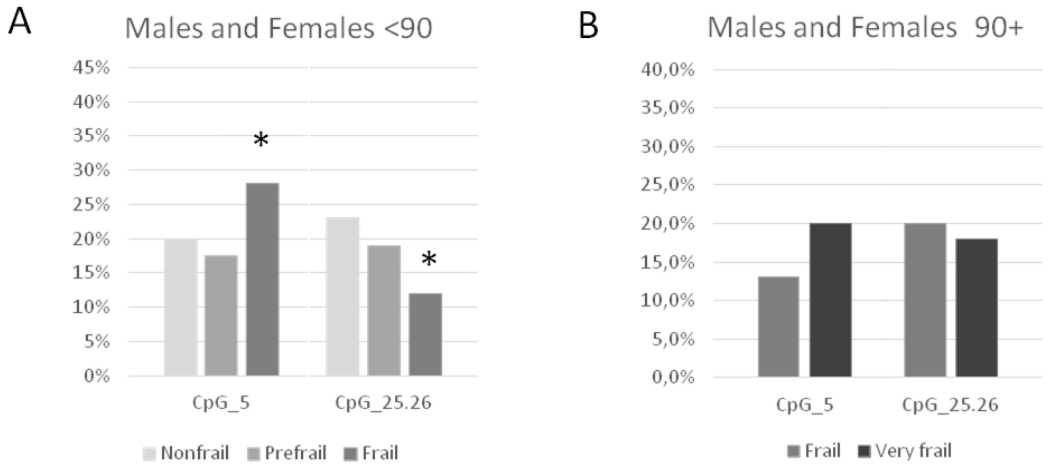

Figure 2.11 Correlation of the methylation levels of the rDNA promoter with frailty in two age-related groups, <90 (A) and 90+ (B) years old.

We observed that the methylation levels were correlated with the degree of frailty in the group of younger subjects. In particular, frail subjects exhibit significantly higher methylation percentages of the CpG_5 unit than those prefrail (27% vs 20%) and nonfrail subjects (27% vs 18%). On the contrary, frail subjects exhibit significantly lower methylation percentages of the CpG_25-26 unit than those prefrail (12% vs 24%) and nonfrail subjects (12% vs 20%). These results indicated that a correlation between the DNA methylation levels of rDNA promoter and the frailty phenotype exists in middle-aged subjects, but not in ultranonagenarians.

2.3.3 Methylation levels of the rRNA gene promoter in association with frailty shows sex-related differences

After observing that the methylation values were correlated with the degree of frailty, we further divided the two age groups according to the gender of the subjects. In **Figure 2.12** and **Figure 2.13** the correlation of methylation levels with frailty in the gender-divided subgroups < 90 and 90+ years is shown, respectively.

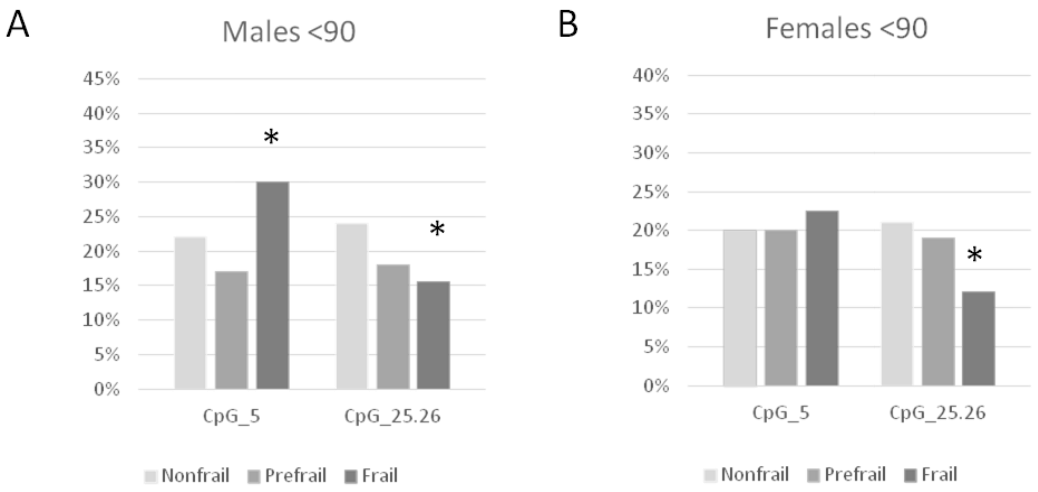

Figure 2.12 Correlation of methylation levels of the rDNA promoter with frailty in the group of <90 years old subdivided in genders

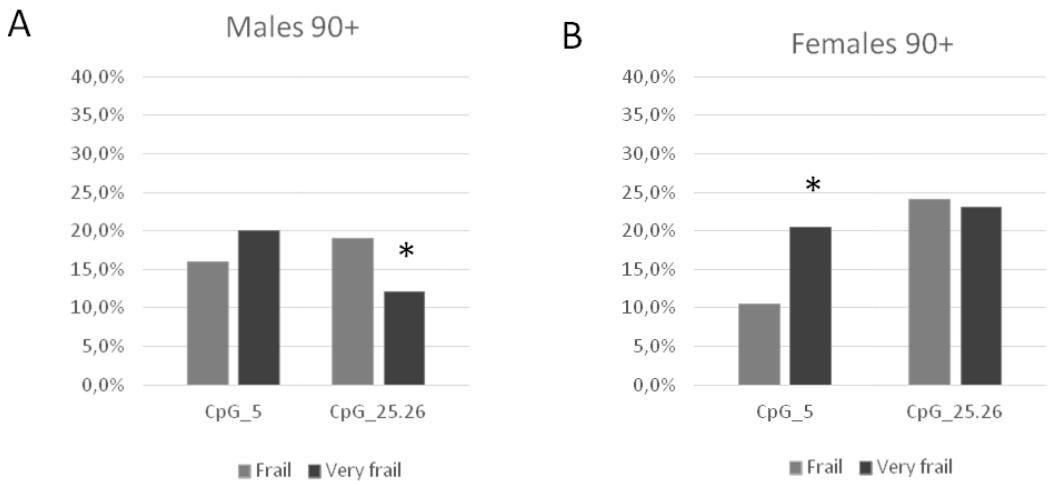

Figure 2.13 Correlation of methylation levels of the rDNA promoter with frailty in the group of 90+ years old subdivided in genders

In the <90 year old group, we observed a significant hypermethylation of the unit CpG_5 in frail subjects (median percentage of methylation 30%) with respect to nonfrail (median percentage of methylation 22%) and prefrail (median percentage of methylation 16%) ones, and a significant hypomethylation of the unit CpG_25-26 (24% vs 18% vs 15%) in males. In females, a hypomethylation was evident at unit CpG_25-26 in frail subjects (median percentage of methylation 17%) with respect to nonfrail (median percentage of methylation 20%) and prefrail (median percentage of methylation 19%) ones.

In the oldest subjects (90+ years old), a significant hypomethylation of the unit CpG_25.26 in very frail subjects (median methylation percentage 12%) with respect to frail ones (median methylation percentage 19%) was observed in males. In females, a hypermethylation was evident for the unit CpG_5 in very frail subjects (median methylation percentage 20%) with respect to frail ones (median methylation percentage 10%).

Therefore, the correlation between the rDNA promoter methylation levels and the frailty phenotype seems to be affected by the gender of the individual.

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2.4 DISCUSSION

Ribosome, a molecular machine in charge of translating genetic information into proteins, is a ribonucleoprotein complex composed of ribosomal RNA (rRNA) and proteins. In the human genome more than 300 copies of rRNA genes are located within the nucleolar organizer regions (NORs) arranged in clusters of tandem repeats of coding regions, parted by non-coding intergenic spacers (IGSs), where the promoter of each gene cluster is located. The gene expression levels vary depending on the cell requirement. However, only a small part of all rRNA genes is transcribed. Active rDNA and silent rDNA copies are respectively hypo and hypermethylated at CpG sites, highlighting that rDNA gene expression correlates to the epigenetic status of the promoter region *(60)*.

Several experiments demonstrated a strong association between epigenetics changes and aging. In general two specific modifications of DNA methylation occur with age: a hypomethylation across all genome and a progressive increase in DNA methylation levels through lifespan in many specific loci. Furthermore the epigenetic control seems to contribute to some age-related phenotypes such as functional and cognitive decline and the development of certain pathologies.

In this study we investigated the methylation levels of cytosine residues, located within the promoter region of the human ribosomal RNA genes, in blood DNA samples collected from human individuals of different age and classified by a precise frailty phenotype based on the individual degree of cognitive, functional, and psychological status *(122)* to elucidate a correlation between rDNA gene promoter methylation, aging and aging phenotypes. To date, although an increase in rDNA methylation in aging mouse tissues has been reported *(126)*, the methylation of rDNA genes during aging has not been examined in detail.

The results here reported are the first demonstrating a correlation between DNA methylation of rDNA promoter region and age in humans. In fact, a unit CpG-specific hypomethylation was observed in ultranonagenarians with respect to younger individuals. Apparently our findings don’t agree with other data obtained in mammals and on human cell lines. In fact, a hypermethylation with ageing was observed in different mouse tissues, in sperm and liver cells of male rats as well as in

human fibroblasts from young and elderly healthy individuals and Werner's syndrome patients *(106, 121, 126)*. It is likely that these data obtained from *in vitro* assays might be different from those we observed in population sample analyses.

Even more interesting is the correlation between rDNA promoter methylation and frailty. Frail and very frail individuals (with high vulnerability) exhibit a unit CpG pecific hyper- and hypo-methylation than those in a better condition (prefrail and nonfrail). Therefore, changes in DNA methylation seem to be specifically associated to the physical functional decline.

The role of ribosomal DNA (rDNA) in human aging has received considerable attention in recent years because of the central role played by its products in protein synthesis. Since methylation is associated to gene silencing, changes in the methylation state of rDNA could potentially influence rDNA transcription and, in turn, ribosomal function and protein synthesis *(121, 126, 127)*. It is unclear from discordant studies whether the level of methylation in the rDNA promoter region affects the transcription of rRNA genes or not *(125, 128, 129)*. More recently, methylation of the CpG dinucleotide at position –133 within the upstream control element, corresponding to unit CpG_5 in our study, has been found to impair binding of the upstream binding factor UBF to nucleosomal rDNA and to abrogate rDNA transcription both in transfection experiments and *in vitro* assays *(112)*. This site has shown to be subject to modifications in methylation status in a differential in relation to chronological age (hypomethylation) or to the functional decline (hypermethylation). On the contrary, no evidence about the functional role of the unit CpG_25.26 is currently available. It is not easy to reconcile these findings with the functional role of the above epigenetic changes *in vivo*. Overall protein synthesis rates have been shown to decrease (40–70% in mammals) with aging *in vivo* and *in vitro (130)*, although, the levels of rRNA do not appear to decrease with aging *(121)*. In addition, lowered protein synthesis during aging has been generally attributed to lowered efficiency of ribosomes and lowered activity of translational elongation factors *(130)*. Therefore, the effects of the methylation of the rRNA genes remain unclear. In any case, our results are in agreement with a series of previous evidence, and suggest that specific age-related phenotypes are modulated not only by genetic

factors but also by the effects of environmental factors through the epigenetic variations on the individual genome.

Therefore, the methylation of specific CpG sites of the ribosomal RNA gene promoter may be a biomarker of aging, connection which is also supported by the finding of accelerated methylation in the rDNA genes of Werner Syndrome fibroblasts *(121),* because it was associated both to the chronological ageing and to physical function, as well as some cancer types, where methylation levels of the ribosomal RNA act as prognostic marker *(131).* This regulation might be related to changes in the production of ribosomes and proteins representing either a strategy to counter act frailty or an effect of it.

CHAPTER 3: SIR2 DEACETILATION ACTIVITY AT SUB-TELOMERIC REGION AFFECTS CELLULAR STRESS RESISTANCE BUT NOT CLS EXTENSION

3.1 INTRODUCTION

3.1.1 Saccharomyces cerevisiae, a model organism in aging research

Saccharomyces cerevisiae, commonly known as budding yeast, was one of the first organisms considered to be a "model" organism for eukaryotic investigations. Yeast has been a successful tool for important discoveries, because it is easy to grow; yeast genome is well mapped and easy to be manipulated by molecular and genetic techniques. Interestingly, approximately 42% of the yeast genome has human similarity. Moreover, its short life cycle facilitates the study of longevity.

In yeast, two methods to measure the aging process exist, called respectively replicative and chronological lifespan. Replicative lifespan (RLS) represents the numbers of daughter cells originate by means of asymmetrical divisions of a single mother cell in the presence of nutrients (**Figure 3.1**), and it is traditionally measured using a micromanipulation procedure on agar plates where the buds are removed from mother cells after each division *(132)*. With replicative aging, cells bud off a limited amount of daughter cells, and then they lose their dividing ability and enter senescence.

On the other hand, chronological lifespan (CLS) represents the survival period of non-dividing cells in the post-diauxic and stationary phases (**Figure 3.1**). Viability can be defined as the ability to restart growing and form visible colonies upon reefing *(133)*. In fact, CLS is measured by monitoring the colonies formation after post-diauxic state, measuring either the proportion of cells capable of reentering the cell cycle and forming a colony when plated on fresh media plates. The two approaches are both powerful tools in longevity studies; whereas it was observed, they can be concordant or discordant in different study cases. Moreover it has been argued that chronological aging is the most relevant model because of non-dividing yeast cells constitute a good model of aging for differentiated non-dividing mammalian cells *(134)*.

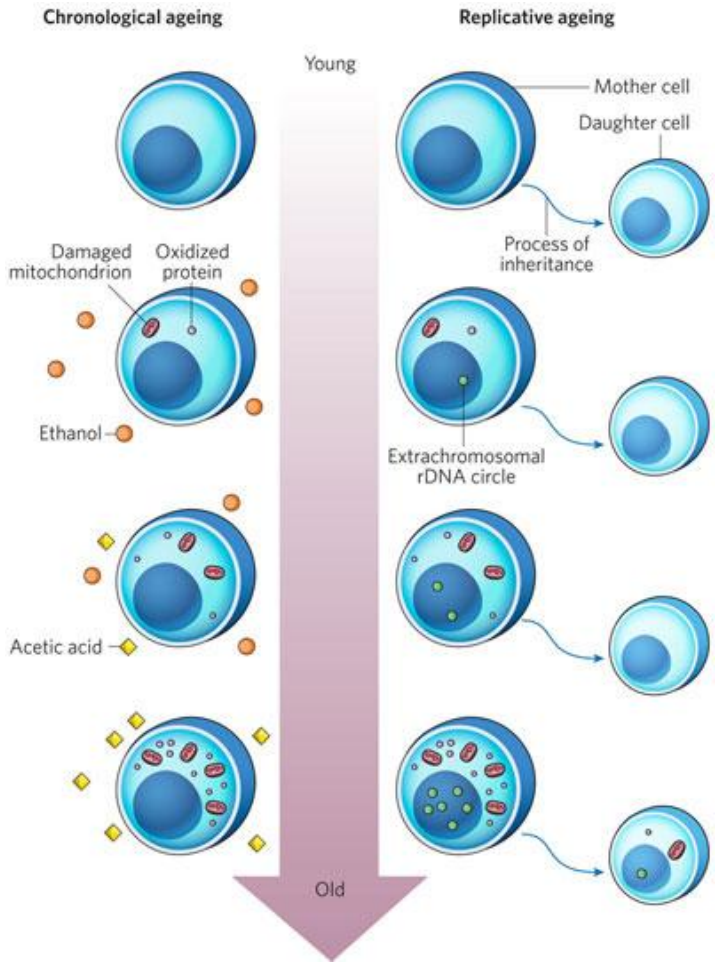

Figure 3.1 The aging models in yeast.

The left panel shows a chronologically aging cell, damages accumulate in these non-dividing cells contribute to chronological senescence. The right one illustrates a replicative aging cell; damage is asymmetrically inherited from the mother cell *(135)*.

When yeast cultures are grown in a rich, glucose-based medium, they exhibit different phases of growth (**Figure 3.2**), among which three are the most important in the aging process. The first phase called exponential phase, occurs in the presence of an abundance of nutrients. As cultures exhaust glucose they enter the second phase of growth, the post-diauxic phase. During the diauxic shift, cells undergo a temporary arrest and begin the physiological shift from fermentation to respiration. This is a very complex point in the growth cycle. The post-diauxic phase begins about 24 hours after the inoculation of culture. Now, cell growth is reduced and the catabolism of ethanol produced from fermentation is the most important source of energy. The third phase, the stationary phase, is a low metabolism state, it starts at

the end of the post-diauxic phase in which many stress responsive genes are up-regulated and there is no net change in cell number, cells die and divide at the same rate *(133, 136)*. This quiescent state has been suggested to resemble the G_0 state in higher eukaryotes *(137)*.

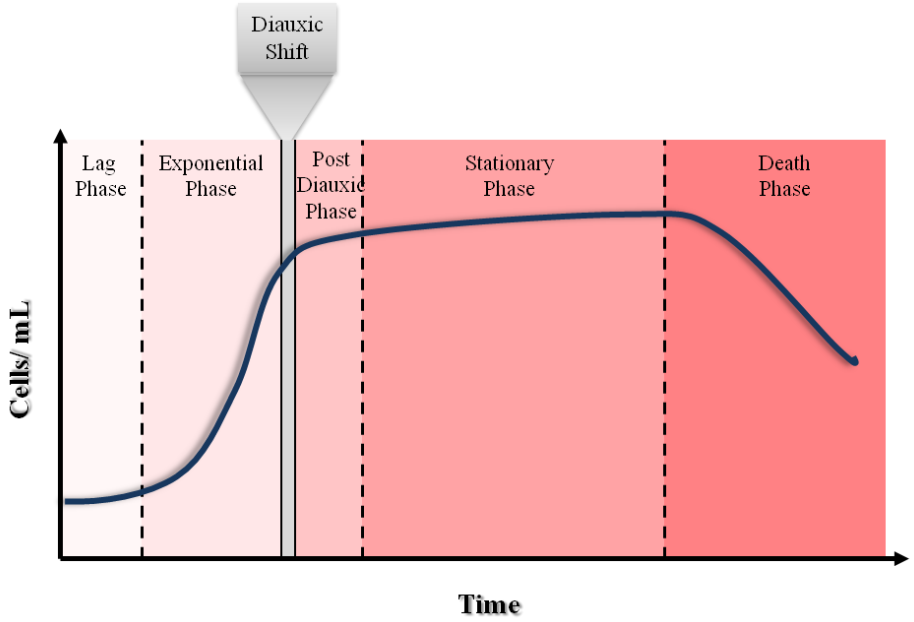

Figure 3.2 Yeast culture growth curve.
Cultures grow exponentially until glucose is exhausted and enter the diauxic shift at approximately 22-24 hours after inoculation. Once exogenous carbon sources are exhausted, 5-7 days after inoculation, cultures enter stationary phase.

3.1.2 Sir2, transcriptional silencing mediator

Exactly fifty years ago Allfrey and colleagues found a correlation between increased histone acetylation and increased transcription levels *(138)*. Since then, several mechanisms by which histone acetylation and deacetilation regulate gene activity have been elucidated.

Acetylation on histone tails occurs most of the time at lysine residues, neutralizing the positive charge of lysine and altering the relationship between nucleosomes and DNA. The destabilized electrostatic bonding makes DNA more accessible to the transcription machinery. In budding yeast, *S. cerevisiae*, Sir (Silent information regulator) proteins are key mediators of chromatin silencing. Among them, Sir2 is a

histone deacetylase essential in the establishment and maintenance of silenced chromatin structure *(139).*

Protein deacetylases are divided into three main classes I, II and III. Sir2 belongs to the last one, which needs nicotine adenine dinucleotide (NAD) as a cofactor, driving a complex reaction that couples lysine deacetylation to NAD hydrolysis. This specific requirement may link Sir2's transcriptional regulation activity to cellular redox state and metabolism.

Sir2 is an evolutionarily conserved protein, from bacteria to human. In mammals seven Sir2 homologous have been identified, which are considered to serve the yeast similar functions *(140).* Sir2 is essential for the correct establishment of silent chromatin structure at three genetic loci in budding yeast, including the cryptic mating type loci (HM loci), rDNA repeats and telomeres, by removing H4 lysine 16 acetylation (H4K16ac) and bringing other silencing proteins (**Figure 3.3**).

HM loci. The *S. cerevisiae* yeast genome includes extra copies of mating type genes in the cryptic mating type loci, *HML* and *HMR*. These loci have to be silenced under normal conditions for yeast mating ability. Sir proteins form a stable complex recruited by silencer binding proteins. The Sir2 protein deacetylates the histones next to the silencers, which in turn promotes the binding of an additional Sir complex to repress the next histones. This sequence of Sir proteins recruitment and histone modifications repeats until the whole *HM* locus is hypoacetylated and then repressed *(56).*

rDNA repeats. In budding yeast, the genes encoding for rRNA (*rDNA*) are organized in tandem arrays of approximately 100 to 200 repeats. Each rDNA repeat contains two rRNA genes separated by non-transcribed spacers. Sir2 operates at rDNA as part of a complex called RENT, limiting the access to RNA polymerase and to the recombination machinery. At this locus an important function of Sir2 is the suppression of intra- and inter-chromosomal recombination. Recombination within the rDNA repeats forms extrachromosomal rDNA circles (ERCs), cause of replicative aging in yeast *(135)*.

Telomeres. Silencing at telomeres is established by means of Sir 2-4 proteins that form a complex and propagate to arrange a silenced chromatin structure. Heterochromatin formation at yeast telomeres is initiated by the TG1–3 sequence-specific binding protein Rap1 (Repressor/activator protein 1). Rap1, in turn, recruits the silent information regulators Sir 2-4 proteins (29–31).

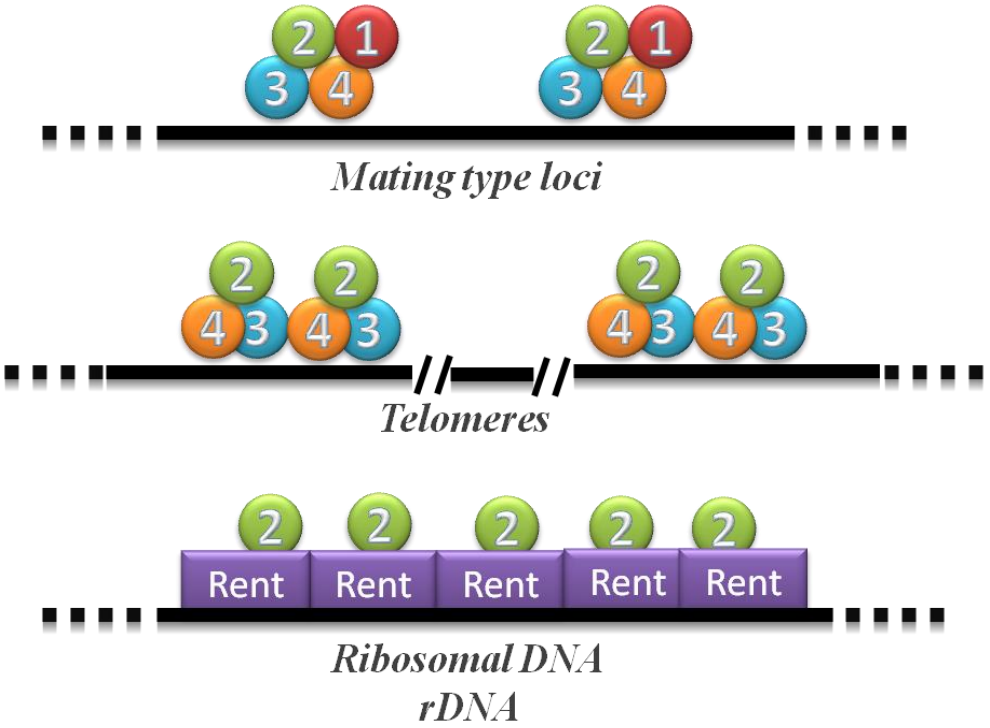

Figure 3.3 Sir2 model of silencing at the mating type loci, telomeres, and rDNA loci. Sir2 associates with Sir1/3/4 to repress the transcription of mating type *loci*, while Sir2/3/4 mediate telomere maintenance. At the rDNA *loci*, Sir2 is recruited to the nucleolus to form RENT complexes, to repress the recombination of rDNA repeats and to maintain genome integrity *(142)*.

3.1.3 Regulation at telomeric and sub-telomeric regions

The telomeres are nucleoprotein structures localized at the ends of eukaryotic chromosomes rich in repetitive sequences that preserve the integrity of chromosome ends from shortening, essentials for chromosomal complete replication and genome stability *(143)*. Telomeres length is a good molecular clock for replicative age, as they shorten with cell divisions in somatic tissues of many organisms. Many immortalized cells maintain telomere length by turning on telomerase. These observations have supported the hypothesis that telomeres may be involved with ageing in some way *(144)*.

In the majority of eukaryotes, telomeres are organized in tandem repeats of short sequences. One of the two strands results to be a single strand G-rich. In *S. cerevisiae* there is a number of repeated sequences found at ends of the chromosome. Some of these are ORFs, others are non-coding sequences. In general there is a mosaic of different repeats at any given end that varies from end to end and from strain to strain at the same end. This complex mixture and the obviously dynamic nature of the region have made difficult to assess the common and possible functional components of the sub-telomeric region.

Most telomere ends have a minimal core X element that range from 0.3 to 3.75 kb, containing the $TG_{1\text{-}3}$ repeats of the actual telomere, and important sequences for DNA replication. Between the telomere and the core X element can be up to 4 tandem copies of the highly conserved Y´ element. Between the X and Y´ are usually found short sub-telomeric repeats that exist in variable copy number and contain degenerate versions of the vertebrate telomere repeat, TTAGGG *(145)*. Various other repeated sequences are found in this junction region. According to several studies the sub telomeric region in yeast consists in 25-30 kb from the chromosome end *(145–147)*. Within these repeats many genes have been found. Moreover the expression of these genes and others adjacent to the heterochromatic telomeres can be repressed because of a phenomenon called telomere position effect (TPE). It has been demonstrated that DNA sequence specificity and reporter genes (such as *URA3*) can be transcriptionally silenced when inserted in a sub-telomeric region *(148)*.

Repression of telomere-proximal genes requires both H4 hypacetylation and H3 hypmethylation.

It is a fact that Sir2 deacetylates H4K16 and with the sir complex silences the telomeres to form a silenced chromatin structure. However the antagonist activity of Sir2 and Sas2, a histone acetyltransferase, generate a gradient of H4K16ac marking the boundary of silencing chromatin near telomeres. Suka and colleagues proposed that global acetylation on H4–Lys16 through Sas2p is a key event that provides a barrier to the spreading of Sir proteins, histone hypoacetylation and silencing into adjacent sub-telomeric chromatin *(149)*.

3.1.4 Sir2, a longevity regulator in yeast

The histone deacetylase Sir2, besides being involved in gene silencing, as described above, plays a central role in several other processes including DNA recombination, apoptosis and aging *(150–153)*.

The significance of Sir2, with regard to aging, was at first indirectly found, through a screen for stress-resistant mutants that also extended RLS *(154)*. Thenceforth the role of Sir2 in the aging process has been deeply investigated, and today it is known that deletion of Sir2 reduces replicative lifespan while its overexpression is able to increase it *(152)*. The proposed mechanism of Sir2-mediated replicative aging is primarily centered on rDNA recombination inhibition. Sir2 silences this area and blocks the extrachromosomal rDNA circles (ERCs) formation. ERCs are self-replicating DNA circles, consisting of individual or multiple rDNA genes that have been excised from the tandem array through homologous recombination. ERCs are replicated in every S-phase, resulting in a growing accumulation in older cells *(155)*. Sir2 deletion causes rDNA hyper-recombination with a consequential ERCs formation increase. Furthermore, increasing SIR2 gene dosage represses rDNA recombination and ERCs formation, extending RLS *(152)*.

A functional role of Sir2 in ageing, by means of different mechanisms, has been demonstrated also in other model organisms including *Caenorhabditis elegans* and *Drosophila melanogaster (156)*. More recent findings have further highlighted the involvement of mammalian Sir2 family proteins (SIRT1-7) in age-associated disorders, such as metabolic diseases, cancer, cardiovascular disease,

neurodegeneration and inflammation, thus suggesting a future role for sirtuins as targets against aging pathologies as well as a probable role in human aging *(157)*.

Sir2 can be considered as a lifespan regulator in yeast. In fact it is involved not only in RLS but also in CLS, however while Sir2 has an anti-aging role in RLS, evidence shows a pro-aging role in CLS *(135, 153)*.

Sir2Δ moderately increases CLS when cells are grown in YPD or SC medium, therefore SIR2 blocks the extraordinary chronological lifespan extension induced by calorie restriction. At the same way, an extreme CLS extension is observed when Δsir2 is added to sch9Δ or Ras/cAMP/PKA mutations that reduce nutrient signaling *(153)*. Considering that both of these pathways extend CLS through the activation of stress response genes, it is probable that Sir2 prevents, at least in part, a complete CLS extension repressing these same genes. This would be consistent with the observation that sir2Δ also confers greater resistance to oxidative and heat stresses how demonstrated in the Longo lab in the last years (**Figure 3.4**).

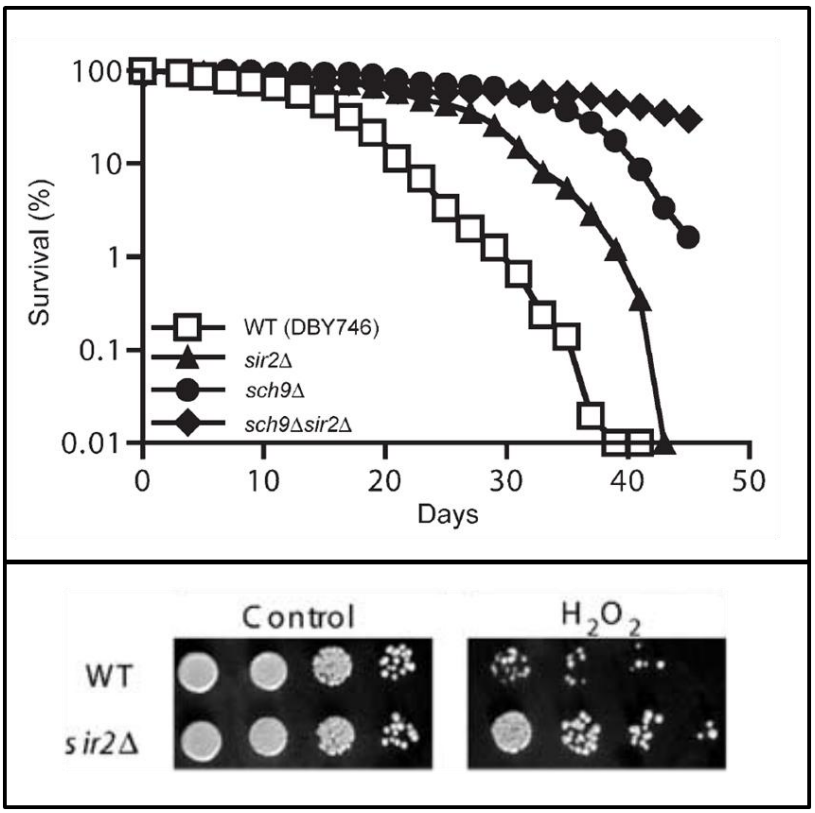

Figure 3.4 Chronological lifespan under calorie restriction and oxidative stress resistance at day 3 of Sir2Δ mutant

Source *(153)*.

Despite all this evidence, the exact mechanisms underlie this phenomenon remain unclear. It has been shown that accumulation of ethanol and acetic acid in the media of yeast cultures can reduce CLS *(158).* Sir2 plays a role in decreasing the rate of consumption of the two substrates regulating the alcohol dehydrogenase (*Adh2),* as well as the inactivation of phosphoenolpyruvate carboxykinase (*Pck1*) *(159).* It seems that the loss of Sir2 drives cellular metabolism from glycolysis toward gluconeogenesis, glycogen and trehalose production during chronological aging, enhancing cellular protection. In fact both pathways are involved in increasing stress resistance against thermo and oxidative stresses of the nondividing cells *(160).* There are strong evidence that oxidatively damaged proteins and other macromolecules accumulate during aging, and may play a causal role in senescence, and the CLS increase observed during superoxide dismutase (Sod) overexpression support this theory *(161).*

Previous studies have identified several changes, environmental and genetic, able to modify chronological or replicative lifespan. Some of that are now known to modulate lifespan also in multicellular eukaryotes *(162, 163).* One of the best examples of environmental intervention acting on longevity is dietary restriction, which has been shown to slow down aging in many different species including yeast, nematodes, fruit flies, rodents, and most recently in rhesus monkeys *(164, 165).*

Dietary restriction can be reached in yeast by reducing the glucose concentration of the growth medium from 2 to 0.5% or lower and it is sufficient to increase both RLS and CLS *(166).*

Sir2 has been proposed as a mediator of CR induced lifespan extension in yeast, *C. elegans* and flies *(167–169).* However, the role of Sir2 in CR is still under debate. In fact CR extends replicative lifespan in a Sir2 dependent and independent manner, while in chronological aging, CR prolongs lifespan especially in the absence of Sir2, or better, the lock of sir2 is effective on longevity under CR *(153, 170, 171).*

The loss of Sir2 expression in yeast correlates with an increase of acetylation at H4K16 at specific sub-telomeric regions, mediated by the histone acetyltransferase Sas2. The antagonistic activity of Sir2 against Sas2 clearly modulates lifespan in yeast regulating the transcriptional activity at certain loci *(172).* In general, it has been established that loss of HDAC activity results in hyperacetylation, which

enhances transcription of antiproliferative genes. On the other hand, loss of HAT activity leads to hypoacetylation, thus contributing to the silencing of cell cycle progression genes *(173)*.

3.1.5 Aim of the study

As described before, in *S. cerevisiae,* Sir2 is strongly associated with lifespan regulation and stress resistance. While the relationship between replicative lifespan and Sir2 is quite known, many unclear features are still present as regard the evidence that Sir2 blocks the extreme chronological lifespan extension. Many theories have been proposed during the years. However no one has been able to elucidate this phenomenon. In the later years a growing body of evidence indicates that chromatin and epigenetic regulation is centrally involved in aging of cells and organisms. More specifically, histone H4K16 is a key substrate of Sas2 and Sir2 activity at sub-telomeric region and through the regulation of histone H4K16 acetylation Sir2 and Sas2 modulate lifespan in *S. cerevisiae.*

Basing on these results, in this study, we analyzed the activity of Sas2 first and then of Rpd3 on the CLS and stress resistance. We also investigated the Sir2's relationship with sub-telomeric genes *(Pkc1 Gtt2, Pre5, Hpa2, Adh7, Git1, Cdc39, Msh3, Kin82, Tup1, Trx3, Ahc2, Srb8, Lys1, Apa2, Gpb1, and Gpb2)* encoding for proteins involved in several cell functions which correlate with aging*;* wishing to find a possible mechanism through which Sir2 regulates chronological lifespan extension and stress resistance in yeast.

3.2 MATERIALS AND METHODS

Yeast strains

Saccharomyces cerevisiae strains used in this study were derived from DBY746 (*MATα, leu2-3, 112, his3Δ1, trp1-289, ura3-52, GAL+*). One-step gene replacement using plasmid pJH103.1 (provided by D. Moadez, Harvard University) was used to disrupt *SIR2* gene. Strains expressing Sir2 protein was obtained by transforming *sir2Δ* mutants with a centomeric plasmid carrying wild-type *SIR2 (p-SIR2- LEU2*) (provided by D. Moadez, Harvard University). Knockout strains were generated by one-step gene replacement as described previously *(174)*.

Growth conditions

Yeast cells were grown in synthetic dextrose complete medium (SDC) containing 2% or 0.5% glucose supplemented with a 4-fold excess of the three essential amino acids tryptophan, leucine, histidine and uracil to avoid possible lifespan modification due to auxotrophic deficiencies of the strains.

Chronological lifespan assay

Yeast chronological lifespan was measured as previously described *(134)*. Overnight SDC culture was diluted (OD 0.1) in to fresh SDC medium to a final volume of 10 ml (with 5:1 flask to culture volume) and were maintained at 30°C with shaking (200 rpm).Twenty four hours later was considered as day1. Every 48 hours, properly diluted culture was placed on to YPD plates and then incubated at 30°C for 2-3 days. Chronological lifespan was monitored by measuring colony-forming units (CFUs). Day3 CFUs was considered as the initial survival (100%) and used to determine the age-dependent mortality. For extreme CR experiment, cells were grown in SDC medium till day1 or day3, centrifuged and washed twice with sterile nano pure water, then added with sterile water. The same procedure was performed every other or four day.

Stress resistance assay

For oxidative stress resistance assays, cells were diluted 10 fold in K-phosphate buffer, pH6.0, and treated with 70 - 300mM H2O2 for 30 min. Serial dilutions (5 fold or 10 fold) of untreated control and H2O2-treated cells were spotted onto YPD plates and incubated at 30°C for 2–3 days.

Quantification of mRNA by real-time PCR

Total mRNA was extracted from cells in expired cultures with a standard phenol/chloroform method. In brief, 2-3 ml culture was collected, centrifuged and washed twice with sterile water. The cell pellets were added with 400 ul TES (10 mM Tris, pH7.5, 10 mM EDTA, 0.5% SDS) buffer and 400ul acidic phenol on ice and vortexed for 15s. Then the mix was incubated at 65-70°C for 20min, vortex for 10s every 5min and afterwards incubated on ice for 5', centrifuged for 5' at 4°C. Transfer the top layer to a new tube and extract once with 400ul CHCl3. The extracted top layer was added with 1 volume 3 M NaOAc (pH5.2) and 3 volume of ethanol and stored at -80°C for 30min. Then centrifuge the mix for 15min at max speed at 4°C, briefly dry and resuspend the extracted RNA in DEPC water. RNA was reversetranscribed using RetroScript II reverse transcription (Invitrogen) and random primers. Real-time PCR was performed using the DNA Engine Opticon 2 (BioRad) with SYBR-green I dye (Bio-Rad laboratory), using specific primers (**Figure 3.5**). Gene expression levels were normalized to housekeeping gene ACT1, encoding for actin and expressed as the ratio to wild type.

PKC1	AGCCGGACAAACAAATGAAC CCTTGCACATTCTTTGCTGA
GTT2	TTAATCGCTGAATGCACAGC TGGATTACGCCTTTTTCCAG
PRE5	GTCGAGGCTATCAGCCAGTC AGCAACAGCTTCTCCATCGT
HPA2	ATAAGGAGGGTTGGCAAAGG GGAATCTGCCGAAATTGAAA
AHC2	TGTATTGGCGGAGAATAGGG TATCTGGGGAATGTCGTCGT
TRX3	GTGGCCCCTGTAAGATGATG TCCTTGCCAAGAACAAAGGT
TUP1	AAGGACGCGTACGAAGAAGA GCAACTGGAACAGATGCAGA
SRB8	GAAAGGAAACGTGGTTGCAT CCTTCTTTTCAAGCCGTGAG
KIN82	AACCGTTCAATACGGCAAAG ATCTGGTTGGTATCGCGTTC
MSH3	TTGCAGAGGATGCAGTAACG TCTGACATCCGGGAAAGAAC
CDC39	TTGGTTTTGGACACGATGAA CCTTGGCAAGTCGCTATCTC
GIT1	TGCGTACGACCAATTGAAAA CACCTGGTCCAGCATTACCT
ADH7	ATGGCTGTGGTCCAGGTAAG CATAAACCTCGGCTCCCATA
APA1	CTCGAAGGCCTTTTCTGATG TGTTTGGGAAACCACATTCC
LYS1	TCAAGCAAACGCATTCTGAC ATCTTCCTAGCGCACCAATG
GPB2	CATATTTGGGGGATTGATGC GTTTGCTAAACCGGCATGAT
GPB1	CAGCGATATGTGGTGGTTTG TGTCGTAGACCCCCGATAAG

Figure 3.5 List of primers utilized for qPCR analysis

Statistical analysis

GraphPad Prism v5.0c was used for the graphic representation and statistical analysis of the data. Longevity curves bar graphs were analyzed by two tailed t test ($P < 0.05$). Statistical significance of qPCR graphs was determined by Turkey Multiple Comparison Test ($P < 0.05$).

3.3 RESULTS

3.3.1 The lack of sas2, the antagonist of sir2, shows divergent effects on CLS and oxidative damage

Yeast Sir2 establishes and maintains chromatin silencing by removing H4 lysine 16 acetylation. Previously, it has been shown that the antagonizing activities of Sir2 and Sas2 regulate the replicative lifespan (RLS) through histone H4 lysine 16 at sub-telomeric regions *(172)*. Sas2 along Sir2, in fact, generate a gradient of H4K16ac marking the boundary of silencing chromatin near telomeres *(149)*. The functional distinction between euchromatic and heterochromatic domains within eukaryotic genomes is essential to maintain gene expression programs that drive development and differentiation in higher organisms. Each expression domain must maintain its identity, and thus junctions exist to separate active from inactive regions and maintain opposing transcriptional states.

Since in Sir2 deficient cells many stress responsive genes are up-regulated, we hypothesized that Sir2 regulates a number of transcription factors, which in turn control the expression levels of the above genes. Therefore, to test if both the oxidative stress resistance shown from Sir2 deficient cells and the extreme chronological lifespan (CLS) extension, similarly to RLS, could be regulated by Sas2 we silenced the *Sas2* gene. Thereafter resistance against hydrogen peroxide and the CLS have been measured.

In our experiment yeast cells were grown in SDC containing 2% glucose, for oxidative stress resistance assays, WT and mutants *Sir2Δ Sas2Δ* cells were diluted 10 fold in K-phosphate buffer, treated with 100mM H_2O_2 for 30 min and then 5 fold serial dilutions of untreated control and H_2O_2-treated cells were spotted onto YPD plates and incubated at 30°C for 2 days until the spots were visible (**Figure 3.6**).

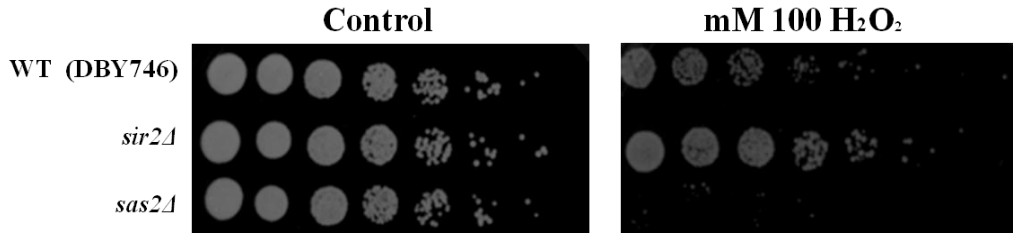

Figure 3.6 Oxidative stress resistance of S*ir2Δ* and S*as2Δ* at day 3

Wild-type (DBY746), *sir2Δ* mutant, and *sas2Δ* mutant cells were treated with 100 mM H_2O_2 for 30 min, serially diluted, and spotted onto YPD plates.

The lack of Sas2 strongly reduced the cellular stress resistance if compared to the control. Therefore, the enhanced cellular protection against oxidative stress following the lack of Sir2 *(153)* can be at least partially related to the sub-telomeric silencing control. In fact the absence of acetylase and deacetylase enzymes seems to be sufficient to affect the oxidative resistance of the cells.

For life-span experiments, cells were grown in SDC or in water (CR), in the second case yeast cells were grown in SDC for 3 days, washed with sterile distilled water, and resuspended in water. They were washed with water every 48h to avoid the accumulation of nutrients released from dead cells. This extreme caloric restriction condition mimics the extreme starvation condition encountered by yeast in wild and causes entry of yeast cells into a low metabolic phase. Chronological lifespan was monitored in expired SDC medium or in water respectively by measuring colony-forming units (CFUs) every 48h on YEPD plates. The number of CFUs at day 3 was considered to be the initial survival (100%) and was used to determine the age-dependent mortality until the end of the experiment at day 40 (**Figure 3.7**).

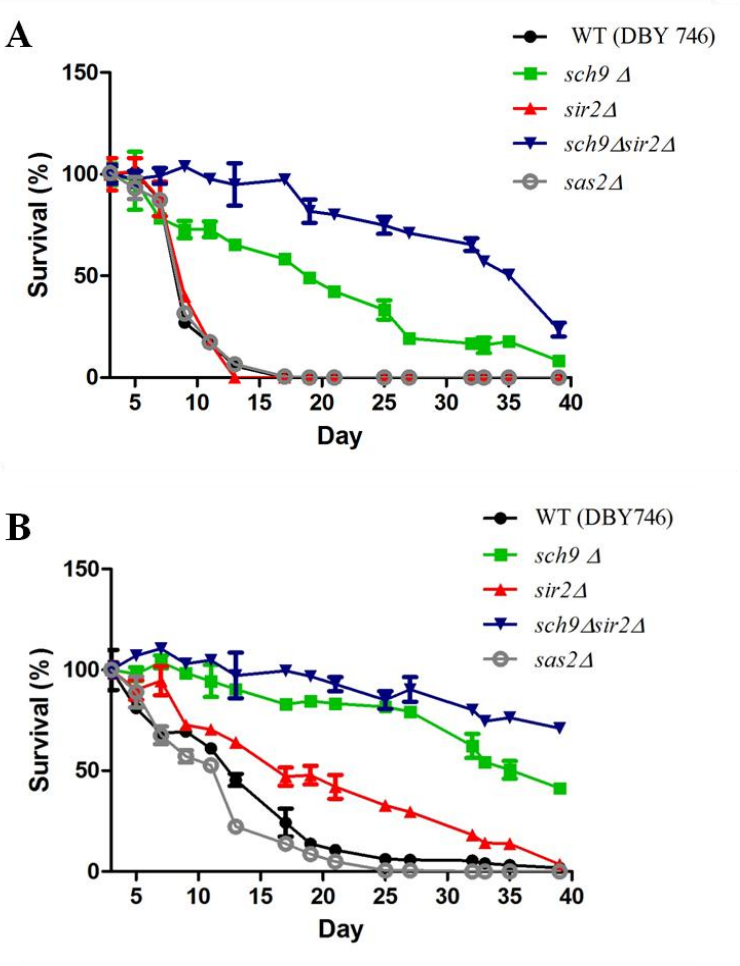

Figure 3.7 8 Chronological Lifespan measurements under standard and CR conditions for *Sas2Δ* compared with S*ch9Δ* e S*ch9ΔSir2Δ* previous described *(153)*.

Chronological lifespan (CLS) of wild-type (WT, DBY746) and *Sir2Δ, Sch9Δ,Ssch9ΔSir2Δ, Sas2Δ* mutants in standard condition (SDC, 2% glucose medium) (**A**) and in extreme calorie restriction (**B**).

Although Sas2 counterweight the Sir2 activity at sub-telomeric region, *Sas2Δ* does not show any differences in chronological longevity both in standard nutritional conditions (2% glucose) and in extreme calorie restriction (CR). CR is still able to increase CLS in *Sas2Δ* and, even if no significant change has been found in comparison to the control, a tiny reduction in CLS between *Sas2Δ* and the wild-type was observed (**Figure 3.7**).

Therefore, what emerges from these graphs is that Sas2 is related to oxidative stress resistance, but it doesn't seem to be involved in the extreme CLS extension observed in absence of Sir2.

3.3.2 Tolerance to oxidative stress is reduced in rpd3Δ mutant in a Sir2 dependent manner

Boundaries between euchromatic and heterochromatic regions have been associated with chromatin-opening activities and in *S. cerevisiae*. The histone deacetylase Rpd3 is required for boundary formation and in a previous work it has been found Rpd3 performs global histone deacetylation at sub-telomeric regions. Rpd3 deletion correlates, indeed, with increased levels of Sir2 at this region.

To further investigate the role of the sub-telomeric region in the higher stress resistance and the extreme CLS extension caused by *Sir2* deletion, after the finding that *sas2Δ* led to a strong reduction of cellular stress resistance, we tested the involvement of Rpd3 by gene silencing and assessing the CLS and resistance to 100 mM of hydrogen peroxide (**Figure 3.9** and **Figure 3.10**).

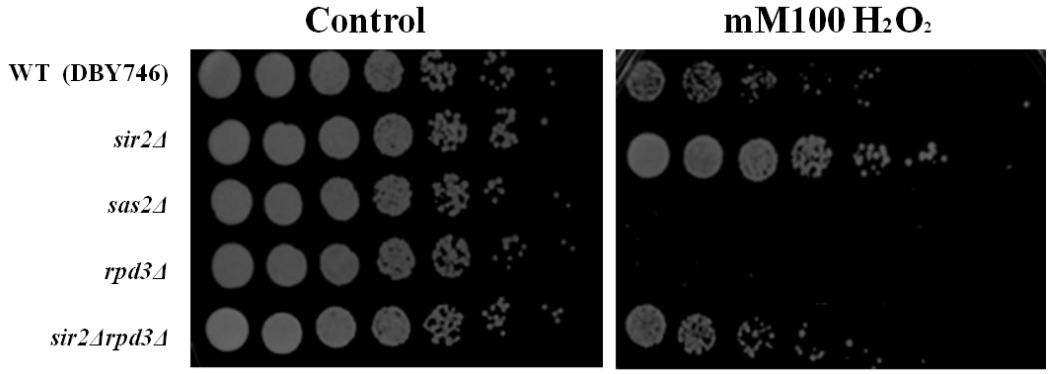

Figure 3.9 Oxidative stress resistance at day 3 of *sir2Δ*, *sas2Δ*, *rpd3Δ* and the double mutant *sir2Δrpd3Δ*.

Wild-type (DBY746), *sir2Δ*, *sas2Δ, rpd3Δ*, and the double mutant *sir2Δrpd3Δ* cells were treated with 100 mM H_2O_2 for30 min, serially diluted, and spotted onto YPD plates. On the left panel it is shown the control, untreated with H_2O_2, while on the right one it is shown the result of the treatment.

The deletion of *Rpd3* is lethal for the cells treated with 100mM H_2O_2, in supporting the initial hypothesis that acetylation and deacetylation of sub-telomeric region is sufficient to affect the oxidative stress resistance of the cells.

Hence, if this supposition is true, the removal of telomeric silencing by Sir2 deletion should abrogate Rpd3Δ lethality. Remarkably, we found that the deletion of Sir2

completely suppresses the lethality of *Rpd3Δ* under H_2O_2 treatment (**Figure 3.9**), showing that the sensitivity of *Rpd3Δ* depends on the Sir2 protein. However, also in this case, similarly to *Sas2* deletion, *Rpd3* deletion does not imply any change in the chronological longevity of yeast when cells are growth both in SDC and in extreme CR (**Figure 3.10**).

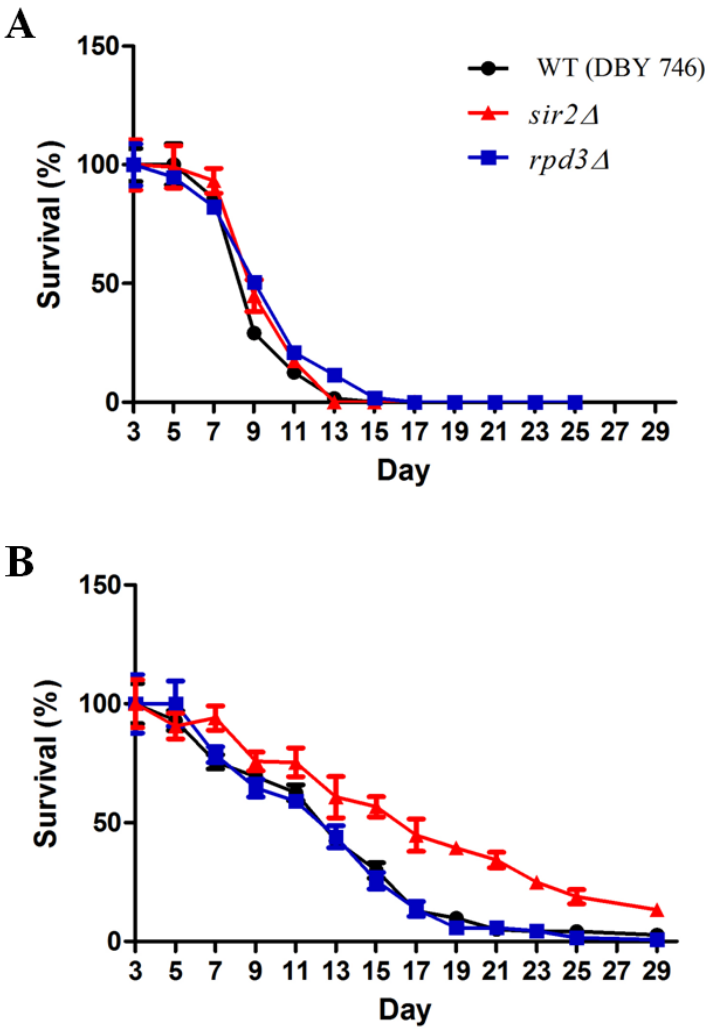

Figure 3.10 Chronological Lifespan assays under normal and CR conditions for *Rpd3Δ*. Chronological lifespan (CLS) of Wild-type (WT, DBY746), *Sir2Δ,Rpd3Δ* in standard condition (SDC, 2% glucose medium) (**A**) and in extreme calorie restriction (**B**).

Therefore, the high oxidative stress resistance exhibited by *Sir2* mutant could be related to the epigenetics status of the sub-telomeric region, although we cannot assert the same for the extreme CLS extension we observed in the Sir2 mutant.

3.3.3 qPCR analysis reveals no difference in sub-telomeric gene expression, but an increasing trend of this expression through aging

The expression of sub-telomeric genes has been analyzed through qPCR reactions. More specifically, we assayed genes involved in metabolism, transcription, growth, and stress resistance that are processes involved in aging. The selection of genes was achieved evaluating: a) their position on the chromosome. In fact several studies demonstrated that the sub telomeric region in yeast consists in 25-30 kb from the chromosome end *(145–147)*; b) their function and c) previous data of a Genome-Wide Screen in *S. cerevisiae* of genes involved in lifespan regulation *(175)*. Total mRNA was extracted from cell cultures with a standard phenol/chloroform method. RNA was reverse transcribed using random primers. Real-time PCR was performed using the DNA Engine Opticon 2 (BioRad) with SYBR-green I dye. Gene expression levels were normalized to housekeeping gene *ACT1*, encoding for actin, and expressed as the ratio to wild type (**Figure 3.11**).

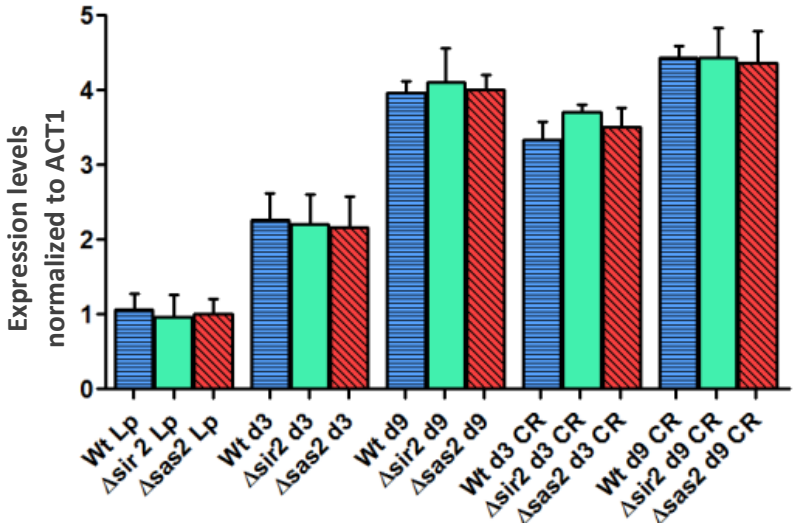

Figure 3.11 Exemplifying graph of the expression levels of candidate genes analyzed by qPCR.

y-Axis represents the gene expression level normalized to the housekeeping gene Actin1 (ACT1). Statistical significance was determined by Turkey Multiple Comparison Test ($P < 0.05$).

Although no significant difference in the expression levels of the sub-telomeric genes was observed in *Sir2Δ* and *Sas2Δ* mutants in comparison to the WT both in SDC medium and in water, a trend during aging was noted. In fact, the expression levels of each gene increased significantly during aging in both wild type and mutants.

3.3.4 CR is still effective in CLS extension of each mutant, although no one of these mutants seems to be involved in Sir2 pathway

In the previous paragraphs it was reported that Rpd3 and Sas2, that exhibit a histone deacetylase and acetyltransferase activity, respectively, and are required to prevent the spread of heterochromatic Sir2 protein into euchromatin at yeast telomeres *(173)*, are also involved in the oxidative stress resistance. In fact both Rpd3 and Sas2 deficient cells display an high sensitivity to hydrogen peroxide, resulting lethal for both of them at 100mM. However *Sir2* deletion abrogates the *Rpd3Δ* lethality, as a signal relieving telomeric silencing suppresses also the lethality induced by H_2O_2. Moreover we noted a trend of increased sub-telomeric gene expression levels through aging.

To determine whether one or more sub-telomeric genes are involved in the regulation of this mechanism, a number of the above genes were silenced and both the stress resistance and the CLS were evaluated (**Figure 3.13**, **Figure 3.14**). Analyzed genes are listed below in **Figure 3.12.**

Apa1	AP4A phosphorylase
Lys1	Saccharopine dehydrogenase
Gpb1	Multistep regulator of cAMP-PKA signaling
Gpb2	Multistep regulator of cAMP-PKA signaling
Adh7	NADPH alcohol dehydrogenase
Gtt2	Glutathione S-transferase

Figure 3.12 Synthetic list of analyzed genes with a short description

Knock-out strains were generated by one-step gene replacement, than WT and mutants cells were treated with 100mM H_2O_2 for 30 min and then 5 fold serial dilutions of untreated control and H_2O_2-treated cells were spotted onto YPD plates and incubated at 30°C for 2 days until the spots were visible.

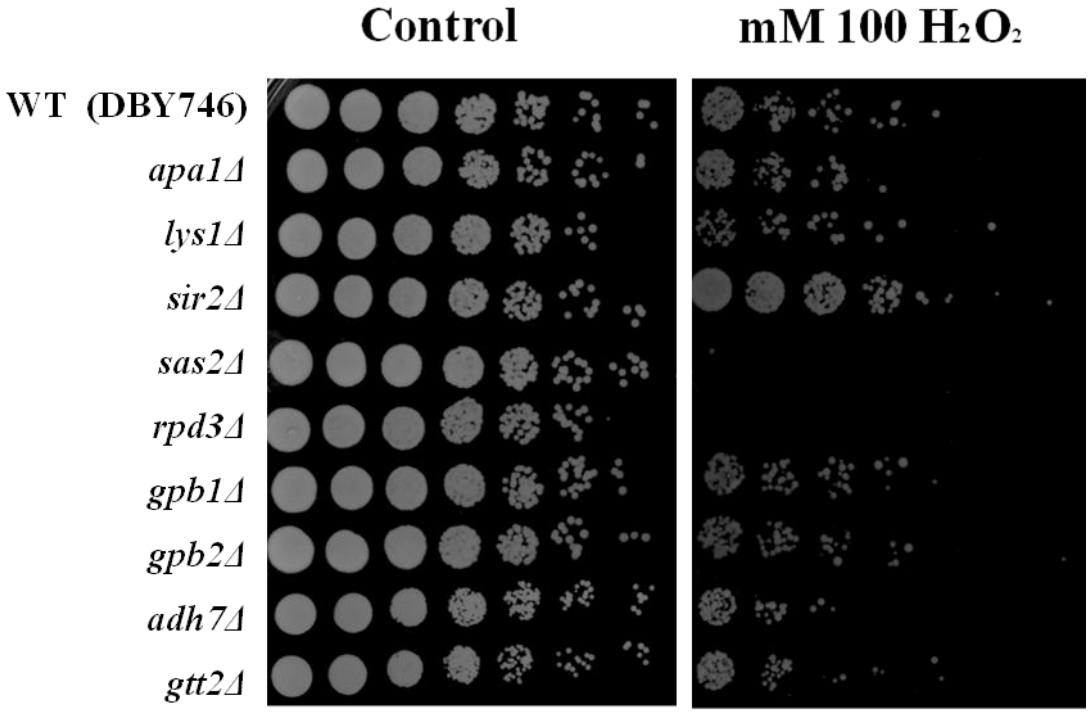

Figure 3.13 Oxidative stress resistance of S*ir2Δ* and S*as2Δ Rpd3Δ* and six sub-telomeric genes deleted mutants at day 3

Sir2, Rpd3 and Sas2 deficient cells still display a pattern similar to that previously reported, while the sub-telomeric gene mutants exhibit the same phenotype of WT cells (**Figure 3.13**). They are not less resistant to oxidative stress as expected, a sign that the activity of the analyzed genes is not crucial in the cellular protection against hydrogen peroxide.

Chronological lifespan was monitored in both expired SDC medium and in water rby measuring colony-forming units (CFUs) every 48h on YEPD plates. The number of CFUs at day 3 was considered to be the initial survival (100%) and was used to determine the age-dependent mortality until the end of the experiment at day 19 and 39 for the cells growth in SDC and water respectively (**Figure 3.14**). The difference

is due to the fact that in SDC medium cells die very fast, so it was useless to proceed until day 39.

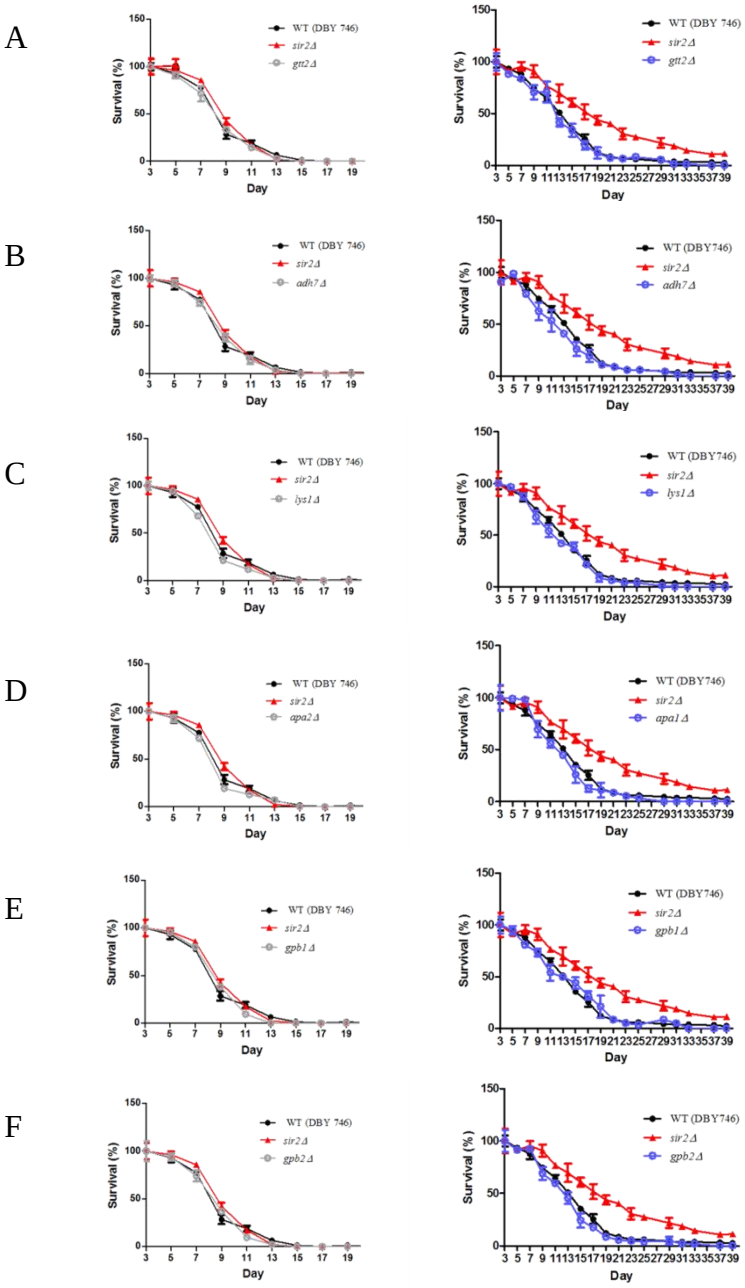

Figure 3.14 Chronological Lifespan assays under standard and CR conditions of six sub-telomeric genes deleted mutants.

Chronological lifespan (CLS) of Wild-type (DBY746), *sir2Δ,* and six sub-telomeric genes deleted mutants: (A) gtt2, (B) adh7, (C) lys1, (D) apa1, (E) gpb1, (F) gpb2; under standard condition, SDC, 2% glucose medium on the left, and extreme calorie restriction on the right respectively.

Even if the CR is more effective in increasing the CLS of each mutant, however no significant change was observed with respect to the control, confirming that even if the sub-telomeric region is involved in regulation of RLS, in cell survival *(172, 173)* and in oxidative stress resistance, to date there is no evidence that links the epigenetic state of this region to the extreme CLS extension occurring in the Sir2 deficient cells.

3.4 DISCUSSION

In yeast, Sir2 is strongly associated with cellular stress resistance and lifespan regulation in both replicative and chronological lifespan. A functional role of *Sir2* in aging has been demonstrated not only in yeast, but also in other model organisms including *C. elegans* and *D. melanogaster (156),* as well as in humans considering the role of the mammalian Sir2 family proteins SIRT1-7 *(157).*

The Longo lab has demonstrated that Sir2 blocks the extreme longevity caused by mutations in the nutrient sensing Tor/Sch9 and Ras/cAMP/PKA signaling pathways and caloric restriction. What is more Sir2 promotes cellular protection against thermo and oxidative stresses *(153).*

In the later years, growing evidence indicates that Sir2 and Sas2 regulate lifespan in *S. cerevisiae* by means the histone H4K16 acetylation at sub-telomeric region. Hence, in this work, we wanted to elucidate whether the sub-telomeric region acts as a link between the lack of Sir2, CLS and the protection against stressors. Our study identifies a novel way through which Sir2 realizes the protection against oxidative damage without affecting the chronological aging, sign that alternative pathways regulate these events.

We observed that the Sas2 deficient cells are more susceptible to the oxidative stress and, taking into account the previous data, it is possible to conclude that the enhanced cellular protection against oxidative stress by lack of Sir2 can be at least in part related to the control of the sub-telomeric region silencing, since Sas2 antagonizes the Sir2 activity exactly at the level of this region. The absence of acetylase and deacetylase enzymes seems to be sufficient to affect cell protection. This finding can be also connected to the recent results reported by Ehrentraut and colleagues, demonstrating that the deletion of Sas2 and Rpd3 is lethal for the cell *(176, 177).*

Following this attractive result, we better investigated the role of the sub-telomeric region by deleting the *Rpd3* gene. Rpd3 deletion is lethal for cells treated with 100mM H_2O_2, supporting the initial hypothesis that the acetylation and deacetylation of sub-telomeric region is sufficient to affect the oxidative stress resistance of the cells. In fact, the removal of the telomeric silencing by deletion of Sir2 abrogates

Rpd3Δ lethality thus indicating that the Rpd3 higher cells sensitivity depends on the Sir2 protein. Despite that, *Sas2* and *Rpd3* deletions do not imply any change in the chronological longevity of yeast when cells are growth both in SDC and in extreme CR. It could be due to the functional redundancy, as demonstrated in a previous work, where *C. albicans* Sas2, the orthologue of *S. cerevisiae* Sas2, has been studied *(178)*. Evaluating these data, our results show that the high oxidative stress resistance, exhibited by Sir2 deficient cells could be related to the epigenetics status of the sub-telomeric region, although we cannot assert the same for the extreme CLS extension.

At this point, it is clear that the epigenetic regulation of the sub-telomeric region plays a crucial role in this phenomenon, and we hypothesized that the increased cell protection could be ascribed to a high expression levels of the sub-telomeric genes, due to the chromatin opening that occurs in the absence of Sir2 *(179)*. No gene among those we analyzed revealed a significant modification of the expression level. This result can provide us information about the real role of these genes in stress cellular protection but also can be attributed to the low number of the examined genes. Although no significant difference in the expression levels of sub-telomeric genes was evident in *Sir2Δ* and *Sas2Δ* mutants with respect to the WT, anyway it was observed an increased trend during aging. In fact, the expression levels of the genes increased significantly during aging in both wild type and mutants. This result could be related to the evidence of a general strong reduction of telomeres silencing through aging *(172, 180)*. However this aspect requires further investigations which have to include an increase of the number of genes. Simultaneously, some of those sub-telomeric genes were silenced, and as expected, the mutants exhibit a similar phenotype of WT cells. They are not less resistant to oxidative stress and no significant change was observed in chronological longevity with respect to the control, thus suggesting that they are not involved in these processes.

Data reported here support the results observed previously *(153, 173)* and provide a partial explanation of the underlying mechanism to the high cell protection in the absence of Sir2.

The importance of this study lies in its contribution not only in elucidating the role of the sub-telomeric region in the Sir2 deletion-dependent increased protection to

oxidative damage, but also into highlighting that oxidative protection and the extreme CLS extension observed into the Sir2 deficient cells are regulated by different pathways, at least in part.

CONCLUSIVE REMARKS

In my dissertation I showed the results of the investigation of two of the most important epigenetic aging-associated features.

The first is the DNA methylation of the ribosomal RNA gene promoter in humans.
The results reported in my thesis are the first demonstration of a correlation between DNA methylation of rDNA promoter region and age in humans. In fact, a unit-CpG specific hypomethylation was observed in ultranonagenarians with respect to younger individuals.
Moreover, we observed that the methylation levels of the rDNA promoter region were correlated also with the gender and with the degree of frailty of sample analyzed, these indicating that it can be modulated not only by genetic factors but also by the effects of environmental influence through the epigenetic variations on the individual genome.
These data represent the first evidence that a site specific methylation on of the ribosomal RNA gene promoter could be a biomarker of the quality of aging, as well as of the aging itself.

The second epigenetic feature discussed in this thesis is the histone tails acetylation at the sub-telomeric region in the budding yeast *S. cerevisiae.*
We found that Rpd3 and Sas2, necessary to prevent spreading of heterochromatic Sir2 protein into the euchromatin at yeast telomeres *(173)*, are also involved in the oxidative stress resistance in a Sir2 dependent manner, without affecting CLS.
Analyzing a group of sub-telomeric genes we didn't find any significant difference in the expression levels of them in *sir2Δ* and *sas2Δ* mutants in comparison to the WT. The mutants of these genes, indeed, exhibited the same phenotype of the control, and then it is evident that they are not involved in these processes.
Our data support the results observed previously and provide a partial explanation of the underlying mechanism to the higher cell protection in the lack of Sir2.
Additionally, our results suggest that the oxidative protection and the extreme CLS extension observed into the Sir2 deficient cells based on different pathways, at least partially.

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APPENDIX A

The Longo lab mainly deals with aging, although from several years, here the beneficial effects of calorie restriction (CR) in association with numerous diseases aging related, such as Alzheimer's, diabetes, and cancer are under investigation.

Cancer is the second cause of death worldwide, following only the heart diseases, although over a third of cancer deaths are due to potentially modifiable risk factors. Cancer cells derive from normal cells that lose their regulation and the ability to divide at a controlled rate.

Despite the simple definition, it is a very heterogeneous pathology; there are several distinct types and subtypes of cancer, which causes may be numerous. However the functions that a cell has to gain to pass from a normal into a malignant cell are almost always the same and it is on these aspects that researchers, involved in the fight against cancer, mainly focus their attention.

It is a long established fact, when the food intake of organisms such as yeast and rodents is reduced; they live longer than organisms fed with a normal diet.

Moreover, it has previously shown that fasting is able to reduce tumor progression by sensitizing cancer cells to chemotherapy and at the same time inducing the protection of normal cells against cytotoxic agents, such as chemotherapy drugs. Nevertheless, until now the molecular mechanisms behind these phenomena remain obscure.

In the fight against cancer, chemotherapy drugs are the most important weapon on hand of medical oncology today, even if they are not effective in the treatment of all cancer types and in all patients. In addition, extremely important is the balance sheet benefits/side effects that all chemotherapeutic agents show on the healthy cells. In the last century the effects of prolonged CR has been highlighted in numerous works. However several problems are associated with CR into a clinical application: long-term CR only delays the progression of malignant diseases and CR is associated with a chronic weight reduction and it may increase frailty incidence *(1)*.

We also know from previous studies that few days of fasting or STS, alternated with a normal diet are able to reproduce or even to enhance the results obtained after months of caloric restriction. Lee et al have shown a significant tumor regression on murine models when the animals were subjected to this treatment, amplifying the effect of several chemotherapeutic, originating a combined phenomenon known as DSS. Tumor cells are more sensitive to drugs if their administration is coupled with

fasting. Despite the discovery of the phenomenon and its correlation with low blood glucose and IGF levels, they have not completely explained causes *(2).*

This effect would seem correlated with the metabolic changes described by Hanahan and Weinberg. In fact, the uncontrolled cell proliferation shown by neoplastic cells requires adjustments of energy metabolism. Cancer cells prefer glycolysis instead mitochondrial oxidative phosphorylation as metabolic program even in the presence of oxygen and for this reason they have to import high quantity of glucose to preserve glycolytic intermediates to amino acids, nucleosides, and macromolecules synthesis, essential for constitutive cell division *(3).*

In this work we tested a diet that mimics the fasting conditions without putting animals to complete fast. The importance of this approach lies in the feasibility of the treatment. In fact, in case of a translational study, we would be in a quandary, patients already weakened by the tumor or chemotherapy. Data obtained support the results observed previously and above all provide a partial explanation of the mechanism by which this dietary intervention is effective in the treatment of cancer, with the immune system in a central position.

The importance of this study, hence, lies not only in the elucidation of the regulation mechanisms of differential stress sensitization shown by the tumor cells, but also into the recognition of an important association between dietary intervention and immunotherapy, that represent the cancer treatment vanguard today.

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APPENDIX B

The Control Region of Mitochondrial DNA Shows an Unusual CpG and Non-CpG Methylation Pattern

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The Control Region of Mitochondrial DNA Shows an Unusual CpG and Non-CpG Methylation Pattern

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Abstract

DNA methylation is a common epigenetic modification of the mammalian genome. Conflicting data regarding the possible presence of methylated cytosines within mitochondrial DNA (mtDNA) have been reported. To clarify this point, we analysed the methylation status of mtDNA control region (D-loop) on human and murine DNA samples from blood and cultured cells by bisulphite sequencing and methylated/hydroxymethylated DNA immunoprecipitation assays. We found methylated and hydroxymethylated cytosines in the L-strand of all samples analysed. MtDNA methylation particularly occurs within non-C-phosphate-G (non-CpG) nucleotides, mainly in the promoter region of the heavy strand and in conserved sequence blocks, suggesting its involvement in regulating mtDNA replication and/or transcription. We observed DNA methyltransferases within the mitochondria, but the inactivation of Dnmt1, Dnmt3a, and Dnmt3b in mouse embryonic stem (ES) cells results in a reduction of the CpG methylation, while the non-CpG methylation shows to be not affected. This suggests that D-loop epigenetic modification is only partially established by these enzymes. Our data show that DNA methylation occurs in the mtDNA control region of mammals, not only at symmetrical CpG dinucleotides, typical of nuclear genome, but in a peculiar non-CpG pattern previously reported for plants and fungi. The molecular mechanisms responsible for this pattern remain an open question.

Key words: mitochondrial D-loop region; 5-methylcytosine; 5-hydromethylcytosine; CpG methylation; non-CpG methylation

1. Introduction

DNA methylation is the most studied epigenetic modification that occurs in all prokaryotic and eukaryotic organisms, with rare exception for yeast, roundworm, and fruit fly.[1] In mammals, it is a post-replication modification in which a methyl group is covalently added to the 5-position of cytosines [5-methylcytosine (5mC)] that are part of symmetrical C-phosphate-G (CpG) dinucleotides. In plant genomes, DNA methylation can occur either symmetrically at cytosines in both CG and CHG (H = A, T, or C) contexts, or asymmetrically in a CHH context.[2] On the contrary, non-CpG methylation in mammals is quite a rare event, although it has been recently described in embryonic stem cells,[3,4] adult mouse brain,[5] mouse germ cells,[6,7] and in promoter regions of different genes.[8–10] Both CpG and non-CpG methylation are determined and maintained by a family of conserved DNA methyltransferases (DNMTs).[11–14] It has been widely demonstrated that CpG methylation status influences chromatin structure, thus regulating the accessibility of transcription factors to their DNA target sequences.[15–17] Conversely,

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the biological significance of non-CpG methylation is currently unknown.

The detection of 5-hydroxymethylcytosine (5hmC) residues in different tissues[18,19] and cells[20] (mainly neurons, brain and embryonic stem cells) has recently been reported. 5hmC is generated through oxidation of 5mC by the TET family of methylcytosine dioxygenases.[21–23] However, a role in 5hmC production was recently also ascribed to DNMT enzymes,[24] suggesting that this species may not be exclusively considered an intermediate of the 5mC demethylation process, but also an important epigenetic marker regulating the pluripotency of stem cells, cellular development, aging, and carcinogenesis.[25]

Previous studies have reported the complete absence of 5mC in mitochondrial DNA (mtDNA) from *Paramecium aurelia*,[26] *Xenopus leavis*,[27] *Neurospora crassa*, and other species,[28] mainly based on the identical restriction patterns obtained with the methyl-sensitive isoschizomers, *Hpa*II and *Msp*I. More recently, the lack of 5mC residues was confirmed by Maekawa *et al.* [29] by analysing cancer cell lines and tissues from patients with gastric and colorectal cancer. Conversely, different amounts of 5mC were observed in mtDNA of many other organisms, such as humans,[30] mice,[31] hamsters,[32] and plants,[33] although the distribution of the methylated cytosines has not been determined in any of these species. More recently, a mtDNA–protein interaction study suggested that this genome may be methylated and DNMTs artificially targeting the mitochondria have access to different sites on the mtDNA depending on the levels of protein occupancy.[34] Finally, Shock *et al.*[35] demonstrated an enrichment of mtDNA sequences by immunoprecipitation against 5mC and 5hmC and the translocation of DNA (cytosine-5-)-methyltransferase 1 (DNMT1) into the mitochondria.

The aim of this study was to investigate the presence of methylated residues of cytosines within mtDNA. In particular, the methylation status of the mtDNA control region (D-loop) was analysed both in blood DNA collected from human subjects and in DNA from cultured cells by bisulphite sequencing and, consecutively, by methylated/hydroxymethylated DNA immunoprecipitation assays. We focused on the above region because it is the control region of the mtDNA, it contains the main regulatory elements for its replication and expression and it is the most rapidly evolving region of this genome.[36] The same analysis was also applied to DNA samples extracted from mouse blood and fibroblast cells. In addition, immunoblotting analyses were carried out to identify which of the three DNA (cytosine-5-)-methyltransferases (DNMT1, DNMT3A, and DNMT3B) and tet methylcytosine dioxygenases (TET1, TET2, and TET3) were located within the mitochondria. Finally, the potential role of the DNMTs in determining D-loop methylation status was investigated by applying the bisulphite sequencing procedure to DNA samples from wild-type (wt) and *Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$ mouse embryonic stem (ES) cells.

2. Materials and Methods

2.1. *Population sample*

A total of 30 unrelated adult subjects (14 men and 16 women), 41–102 years old, participated to the present study. The Ethics committee of the University of Calabria approved the recruitment and the use of the information gathered, as well as the use of the biological specimens collected on 9th September 2004. All subjects lived in Calabria (South of Italy) and their origin in the area was ascertained up to the grandparents' generation. Health status was ascertained by medical visit and at that time peripheral blood samples were also obtained. Before the interview, each subject provided informed consent to permit her/his phenotypic and genetic data to be used anonymously for genetic studies.

2.2. *Cell cultures*

Human skin fibroblasts, HeLa, osteosarcoma 143B.TK$^-$, and murine 3T3-L1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 4.5 g/l glucose and 110 μg/ml pyruvate, supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 50 μg/ml gentamycin (Invitrogen). The Rho^0 cell line, obtained by culturing 143B.TK$^-$ in routine growth medium containing 50 ng/ml ethidium bromide with regular replenishment of medium for about 2 months, was maintained in DMEM supplemented with 10% FBS and 0.2 mM uridine (Sigma).

Wt and *Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$ triple knock-out (TKO) mouse ES cells were grown in gelatinized culture dishes without feeder cells as reported by Tsumura *et al.*[37]

Cells were cultured in a water-humidified incubator at 37°C in 5% CO_2/95% air.

2.3. *DNA samples*

Six millilitres of venous blood were drawn from each subject. Plasma/sera were used for routine laboratory analyses, while DNA was extracted from blood buffy coats following standard procedures.

DNA samples from human skin fibroblasts, HeLa, osteosarcoma 143B.TK$^-$, Rho^0, and murine 3T3-L1 cells were obtained by phenol/chloroform purification, while DNA samples from wt and *Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$ (TKO) mouse ES cells[37] were extracted by the Wizard Genomic DNA Purification Kit (Promega).

Mouse genomic DNA, isolated from whole blood of disease-free mice, was purchased from Promega Corporation.

2.4. *Bisulphite treatment*

Bisulphite conversion of each DNA sample was performed by using the EZ DNA Methylation-Direct Kit (Zymo Research), according to the manufacturer's protocol. Briefly, 1 μg of genomic DNA, previously incubated for 20 min at 50°C in proteinase K and purified by centrifugation, was added to 130 μl of CT Conversion Reagent in a final volume of 150 μl. The mix was incubated at 98°C for 10 min and, successively, at 64°C for 3.5 h. After adding 600 μl of M-Binding Buffer into a Zymo-Spin IC Column, each sample was loaded into the column and centrifuged at 16 000 *g* for 30 s. After adding 100 μl of M-Wash Buffer to the columns and a centrifugation at 16 000 *g* for 30 s, 200 μl of M-Desulphonation Buffer were added to the columns and incubated at room temperature (RT) for 20 min. Then, the solution was removed by centrifugation at 16 000 *g* for 30 s and the columns were washed twice with 200 μl of M-Wash Buffer. Deaminated DNA was eluted in 10 μl of M-Elution Buffer.

To ensure that cytosine conversion was complete, alternative bisulphite modifications were performed by using the EZ DNA Methylation-Gold Kit (Zymo Research), and the Qiagen's EpiTect Bisulfite Kit according to the manufacturer's protocol.

For each procedure of bisulphite treatment, unconverted primers randomly covering the entire mtDNA molecule, including those used for the D-loop analysis, were used in polymerase chain reaction (PCR) reactions, as negative controls.

2.5. *Primer design for PCR reactions*

Six and four sub-regions, covering the entire D-loop of humans (nt 16 024-576) and mice (nt 15 423–16 299), respectively, were isolated by PCR carried out on each bisulphite-converted DNA sample. In particular, as DNA strands are no longer complementary after sodium bisulphite treatment, we designed primers specifically amplifying the top (Light) and bottom (Heavy) strands of the bisulphite-converted DNA (Supplementary Tables S1 and S2). Moreover, some precautions were taken for primer design: (i) cytosines in forward primers and guanines in the reverse primers were replaced with thymines and adenines, respectively; (ii) cytosines within CpG sites were avoided; (iii) when possible, DNA regions characterized by low polymorphism content were preferred; (iv) short size of the amplicons was defined (range: 150–350 bp); (v) a 10-bp tag was added to the 5′- ends of some primers in order to increase the annealing temperature of the A–T enriched primer sequences. In addition, specificity for mtDNA target sequences of designed primers was tested on DNA extracted from osteosarcoma 143B.TK$^-$ Rho^0 cells, completely lacking of mtDNA.

2.6. *Bisulphite sequencing*

The PCR mixture (20 μl) contained 2 μl of bisulphite-treated DNA, Reaction Buffer 1×, TaqMaster PC Enhancer 1×, 0.5 μM of each primer, 0.2 mM dNTP mix (Promega), and 0.05 U of PCR enzyme (5′). The thermal profile used for the reaction included a 4-min heat activation of the enzyme at 95°C, followed by 45 cycles of denaturation at 94°C for 20 s, different annealing temperature (Supplementary Table S1) for 30 s, extension at 72°C for 1 min, then one cycle at 72°C for 3 min.

The obtained PCR products, previously purified by DNA Clean & Concentrator-5 Kit (Zymo Research), were cloned into pGEM-T Easy Vector (Promega) according to the manufacturer's protocol. In the set-up and validation of this procedure, 30 positive clones from five representative human DNA samples were analysed. Once determined the efficacy of the entire protocol, five clones for sample were analysed.

Therefore, plasmids were purified using ZR Plasmid Miniprep Classic (Zymo Research) and analysed by automated sequencing in a ABI PRISM 310 with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

The effectiveness of the entire experimental procedure was also assayed by analysing: (i) CpGenome™ Universal Unmethylated DNA (Chemicon); (ii) unmethylated purified PCR products of each sub-region; and (iii) independent DNA preparations also starting from different tissues.

2.7. *5-methylcytosine immunoprecipitation*

Four μg of DNA extracted from both blood and cell lines were incubated with 40 U of *Alu*I restriction endonuclease in a total volume of 20 μl overnight at 37°C and subsequently at 65°C for 20 min to inactivate the endonuclease. The enzymatic DNA digestion allowed us to obtain fragments of appreciable size compared with the random DNA fragmenting by sonication.

5mC immunoprecipitation was carried out using the EpiQuik Methylated DNA Immunoprecipitation (MeDIP) Kit (Epigentek, NY, USA) according to the manufacturer's specifications. First, wells were washed once with Wash Buffer (WB; CP1) and then incubated at RT for 60 min in the presence of 100 μl of Antibody Buffer (AB; CP2) supplemented with 1 μl of 5mC antibody (or 1 μl of Normal Mouse IgG, as a negative control).

After three consecutive washes of the wells with 150 μl CP2, *Alu*I-digested DNA samples, diluted with

ChIP Dilution Buffer (CP4), were added into the assay wells. The solution was incubated at RT for 90 min on an orbital shaker to allow DNA binding onto the assay wells. Therefore, the wells were first washed six times with 150 μl of the 1× WB, allowing 2 min on a rocking platform for each wash, followed by the addition of 150 μl 1× tris-EDTA (TE) Buffer. Afterwards, 40 μl of the DNA Release Buffer (DRB) containing proteinase K were added to each well and samples were incubated at 65°C for 15 min. Then, samples were incubated in 40 μl of Reverse Buffer (CP6) at 65°C for 30 min, 150 μl of Binding Buffer (CP7) were subsequently added to the wells, and the released samples, transferred to the F-Spin column, were centrifuged at 14 000 *g* for 20 s. After a centrifugation in the presence of 200 μl of 70% ethanol and two consecutive centrifugations in 90% ethanol, at 14 000 *g* for 30 s, purified DNA was eluted in 15 μl of Elution Buffer (CP8).

CpGenome Universal Unmethylated DNA (Chemicon) and CpGenome Universal Methylated DNA (Chemicon) were used as negative and positive controls, respectively, in order to test the effectiveness of the kit.

2.8. *5-hydroxymethylcytosine immunoprecipitation*

5hmC immunoprecipitation was carried out using the EpiQuik Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit (Epigentek), according to the manufacturer's recommendations. Briefly, 100 μl of antibody buffer (AB) were added to each well of the microplates, followed by adding 1 μl of 5hmC antibody (or 1 μl of Non-Immune IgG, as a negative control) and incubation at RT for 60 min. After removing AB and washing of the wells with 200 μl 1× WB, 1 μg of *Alu*I-digested DNA samples was diluted to 10 ng/μl with hMeDIP Solution (HS) and added into the assay wells to be incubated at RT for 90 min on an orbital shaker. Therefore, the wells were first washed five times with 200 μl of 1× WB, and then, with 200 μl of DNA release buffer (DRB). Afterwards, 40 μl of the DRB containing proteinase K were added to each well and samples were incubated at 60°C for 15 min, followed by an incubation at 95°C for 3 min.

The sensitivity of the methods was estimated by analysing the reference DNA fragment containing 5hmC provided by the kit.

2.9. *MeDIP/hMeDIP-PCR*

Immunoprecipitated methylated and hydroxymethylated DNAs were then used as a template for real-time PCRs carried out using the SYBR Green qPCR Master Mix (Promega) in a StepOne Plus machine (Applied Biosystems). In these reactions, PCR primers specifically amplifying D-loop fragments previously detected by bisulphite sequencing as unmethylated and methylated were used (Supplementary Table S3).

The final PCR mixture (20 μl) contained 1 μl of immunoprecipitated DNA, 1× GoTaq® qPCR Master Mix, 0.2 μM of each primer, and 1× CXR Reference Dye. The thermal profile used for the reaction included a 2-min heat activation of the enzyme at 95°C, followed by 35 cycles of denaturation at 95°C for 15 s and annealing/extension at 58°C for 60 s, followed by melt analysis ramping at 58–95°C. All measurements were taken in the log phase of amplification.

Specific PCR primers were used for verifying the enrichment efficiency of methylated and hydroxymethylated positive control DNAs. Fold enrichment was calculated as the ratio of amplification efficiency of the MeDIP/hMeDIP sample over that of non-immune IgG and then, normalizing the data to the nanograms of immunoprecipitated DNA used in PCR. Six independent triplicate experiments were carried out.

2.10. *Dot-blot hybridization of MeDIP and hMeDIP samples*

Dot-blot hybridization was carried out using the forward and reverse primers reported in Supplementary Table S3 as single-strand oligo probes specifically recognizing the H- and L-strands, respectively.

The probes were labelled to the 3′-OH ends using Terminal Transferase (Roche) according to the manufacturer's protocol. Aliquots of four representative human and mouse MeDIP and hMeDIP samples were immobilized on six different N+ membrane (Amersham Biosciences) strips and fixed with ultraviolet light for 5 min. Then, they were blocked in Detector Block Solution (KPL, Inc.) and incubated at RT for 30 min. After the removal of the solution, each membrane strip was incubated with the mix containing the relevant probe at RT for 30 min in the presence of the Detector Block Solution containing 0.5 μg/ml of horseradish peroxidase (HRP) Streptavidin (KPL, Inc.). After the incubation, the Detector Block Solution was removed and the membrane strips were washed four times with Biotin Wash Solution 1× (KPL, Inc.). Immunoreactivity was determined by means of the ECL chemiluminescence reaction (KPL, Inc.).

2.11. *Isolation of mitochondrial protein fractions*

Mitochondrial extracts were prepared using the Mitochondrial Fractionation Kit (Active Motif). 1.5×10^7 HeLa and 3T3-L1 cells were scraped on ice after the addition of 10 ml of ice-cold 1× phosphate-buffered saline (PBS) and then centrifuged at 600 *g* for 5 min at 4°C. Cell pellets were re-suspended in 5 ml of ice-cold PBS and centrifuged at 600 g for 5 min at 4°C. Then, cell pellets were re-suspended in 250 μl of 1× cytosolic buffer included in the kit and incubated on ice for 15 min. Successively, cell pellets were homogenized and the resulting supernatant was

centrifuged at 850 *g* for 20 min at 4°C. After centrifugation, the supernatant, containing the cytosol and the mitochondria, was removed and centrifuged second time at 800 *g* for 10 min at 4°C. Then, the supernatant was again removed and centrifuged at 11 000 *g* for 20 min at 4°C to pellet the mitochondria. Mitochondrial pellets were washed with 100 μl of 1× cytosolic buffer and then centrifuged at 11 000 *g* for 10 min at 4°C. Finally, mitochondrial pellets were lysed by adding 35 μl of complete mitochondria buffer, prepared by adding mitochondria buffer, protease inhibitor cocktail, and dithiothreitol, and by incubating on ice for 15 min.

As a control, whole-protein extracts were obtained according to the standard procedure.

2.12. *Western blotting of DNMT methyltransferases and TET methylcytosine dioxygenases*

Eighty micrograms of whole-protein extracts and 30 μg of cytosolic and mitochondrial protein fractions were resolved on a 7% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred into Hibond-P membranes at 30 V for 2 h at 4°C. Membranes were washed with tris-buffered saline-Tween 20 (TBST) buffer 1× (0.3 mM Tris–HCl, pH 7.5, 2.5 mM NaCl, 0.05% Tween 20) for 10 min and then incubated at RT for 1 h with 5% non-fat dried milk in TBST 1×. Blots were also washed three times with TBST 1× for 10 min and incubated overnight, in TBST containing 1% milk, with anti-DNMT1, DNMT3A, and DNMT3B monoclonal mouse antibodies (1:200), anti-TET1 polyclonal goat antibody (1:200), and anti-TET2 and anti-TET3 polyclonal rabbit antibodies (1:200). Then, anti-mouse, anti-goat, and anti-rabbit (1:5000) antibodies conjugated with HRP (GE Healthcare) were used as secondary antibodies. Immunoreactivity was determined by means of the ECL chemiluminescence reaction (GE Healthcare). Tubulin antibody (1:500) was used as internal control to exclude putative contamination of mitochondrial fraction by cytosolic proteins. Cytochrome c oxidase subunit IV isoform 1 (CoxIV) antibody (1:200) was used as a mitochondrial loading control. All primary antibodies were purchased from Santa Cruz Biotechnology, except for DNMT3A and CoxIV which were from Abcam.

2.13. *Statistical analysis*

Statistical analyses were performed using the SPSS 15.0 statistical software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Student's *t*-test were adopted, with a significance level defined as $\alpha = 0.05$.

3. Results and Discussion

3.1. *CpG and non-CpG methylation patterns in human mitochondrial D-loop*

Bisulphite sequencing was used to investigate the presence of methylated cytosine residues in the Heavy-(H) and Light (L)-strands of the human mitochondrial control region DNA (D-loop).

Results revealed the presence in the sole L-strand of unconverted cytosines, thus indicating the existence of methylation in the D-loop (Fig. 1A and B and Supplementary File S1). In particular, all analysed clones showed identical methylation patterns, with the majority of the methylated cytosines located outside of CpG nucleotides. The global percentage of methylation (the percentage of methylated cytosines with respect to overall cytosine content of the analysed sequence) was around 35% (values ranging from 32 to 37%, standard deviations of 5%) compared with that at CpG sites equal to 17% (values ranging from 16 to 20%, standard deviations of 2.1%) in DNA samples extracted from human bloods (ANOVA test, $P < 0.001$). Similar patterns were also observed in tumour (HeLa and osteosarcoma 143B.TK$^-$) and primary (skin fibroblasts) cell cultures, although they exhibit significant differences in methylation levels at CpG (ANOVA test, $P = 0.002$) and non-CpG sites (ANOVA test, $P = 0.013$). In fact, HeLa cells showed higher overall percentage of methylated cytosines (23 and 27%) compared with fibroblasts (10 and 21%) and osteosarcoma 143B.TK$^-$ (10 and 17%) cells, thus suggesting that the observed methylation pattern might be cell-type specific.

All samples, replicate by alternative bisulphite procedures, gave consistent results. As expected, the analysis of both unmethylated control DNA and of the unmethylated purified PCR products of each sub-region revealed the complete absence of unconverted cytosines, confirming that the bisulphite conversion was efficient.

3.2. *CpG and non-CpG methylation patterns in the mitochondrial D-loop of mouse*

To outline a broader framework of the mitochondrial D-loop methylation pattern, we extended the analysis to the mouse homologous region. Then, we applied bisulphite sequencing to two DNA genomic samples: the first isolated from whole blood, and the second extracted from 3T3-L1 fibroblast cell cultures. As for humans, the analysis demonstrated not only the presence of methylated residues within the L-strand of the murine mitochondrial D-loop, but also that these residues are preferentially located in non-CpG nucleotides (Fig. 2A and B and Supplementary File S2). However, for both the CpG (ANOVA test, $P = 0.029$) and the non-CpG (ANOVA test, $P = 0.005$) sites, the overall

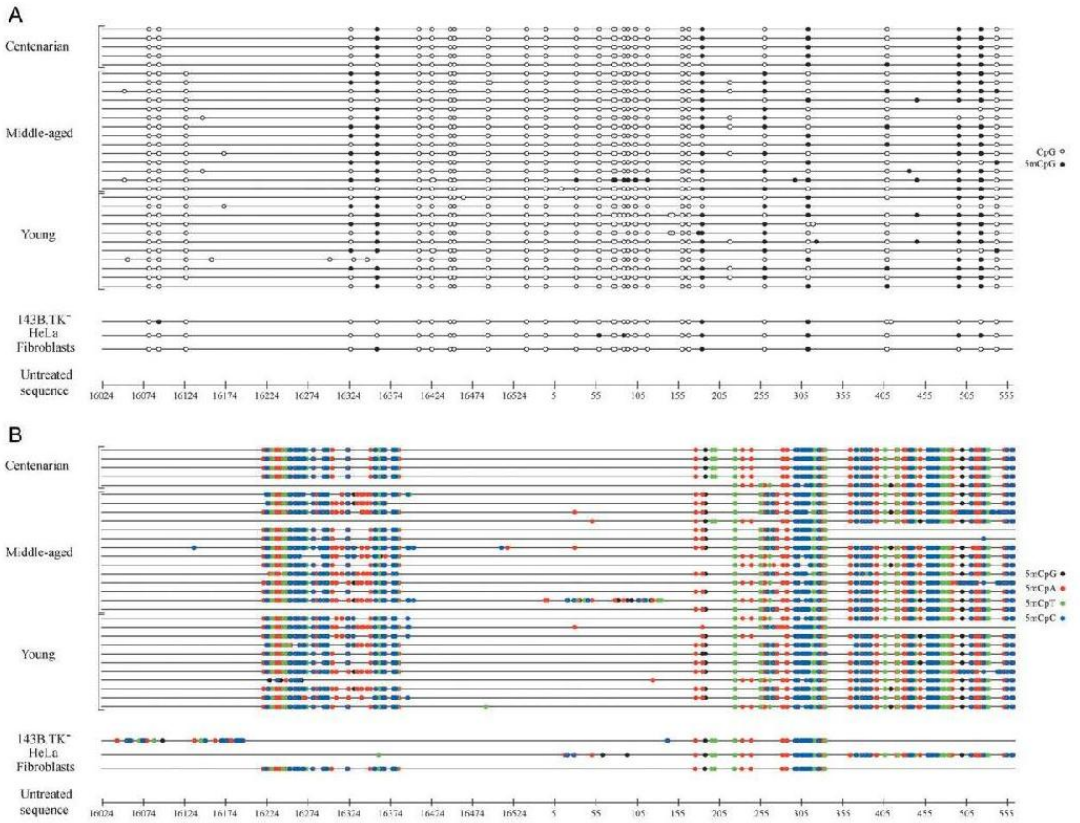

Figure 1. Methylation patterns of the human mitochondrial D-loop in DNA samples from blood and cultured cells. The graphical representation of bisulphite sequencing results was generated by the MethTools software (version 1.2) (http://genome.imb-jena.de/methtools). Bisulphite-generated sequence of each sample (black line) was compared with the untreated sequence of the mitochondrial D-loop, reported here in a base-pair scale. In (A), methylation of cytosine residues located within CpG nucleotides (CpG methylation) is shown. The variability among samples is due to reported polymorphisms (http://www.mitomap.org), which insert or delete cytosines or guanines based on the revised Cambridge reference sequence (rCRS), thus creating/suppressing CpGs. In (B), methylation of cytosine residues located outside of CpG nucleotides (non-CpG methylation) is shown.

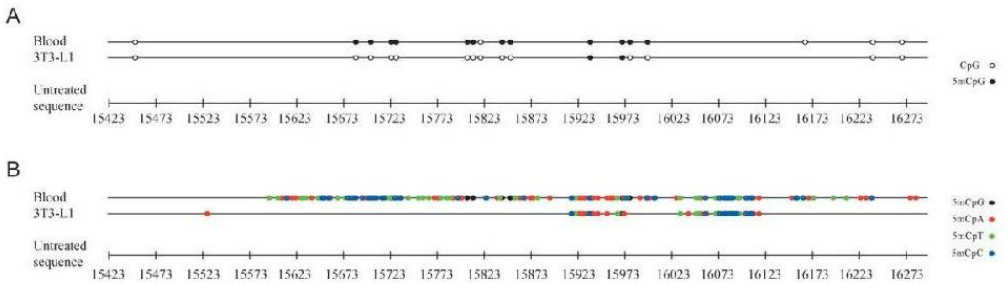

Figure 2. Methylation patterns of the mouse mitochondrial D-loop in DNA samples from blood and cultured cells. In (A), methylation of cytosine residues located within CpG nucleotides (CpG methylation) is shown. In (B), methylation of cytosine residues located outside of CpG nucleotides (non-CpG methylation) is shown.

percentage of methylated cytosines appeared to be higher in the blood DNA samples (70.6 and 58.8%, respectively) than in the fibroblasts (12.5 and 17.7%, respectively).

Overall, the overlapping data obtained in both humans and mice seem to indicate that the methylation of the mitochondrial control region could be a general phenomenon and is established across species, at least in the higher eukaryotes, according to similar patterns.

3.3. *5-methylcytosine and 5-hydromethylcytosine presence in mtDNA*

To confirm the methylation status of the mitochondrial D-loop, we determined the presence of 5mC and 5hmC within mtDNA-specific fragments by immunoprecipitation assays, because sodium bisulphite treatment does not distinguish between these two modified bases. In both humans and mice, a significant enrichment for the two bases was observed in those fragments detected by bisulphite sequencing as methylated. In particular, immunoprecipitated DNA fragments were enriched ~3.5- to 5-folds in humans and 3- to 4-folds in mice for 5mC, relative to the non-immune IgG control and to the immunoprecipitated DNA amount (Fig. 3A). MtDNA immunoprecipitated using anti-5hmC was enriched ~4- to 6-folds in humans and 3- to 8- folds in mice (Fig. 3B). Noteworthy, the human mtDNA fragment 16 037–16 477 was found to be composed primarily of 5hmC residues. Consistently, with both antibodies, no enrichment was observed for those fragments detected as unmethylated by bisulphite sequencing.

Taken as a whole, the results we obtained demonstrated the presence of both 5mC and 5hmC modifications within the D-loop region with a prevalence of 5hmC.

Finally, DNA probes annealing specifically the H- and L-strands were used in dot-blot hybridization experiments on MeDIP and hMeDIP samples in order to validate the presence of asymmetric methylation. We found positivity for those probes specifically recognizing L-strand, thus demonstrating that this strand is enriched, while H strand did not (Supplementary Fig. S4).

3.4. *DNA methyltransferases and tet methylcytosine dioxygenases presence within mitochondrial protein fractions*

We then questioned whether the enzymes involved in the maintenance and *de novo* methylation of the

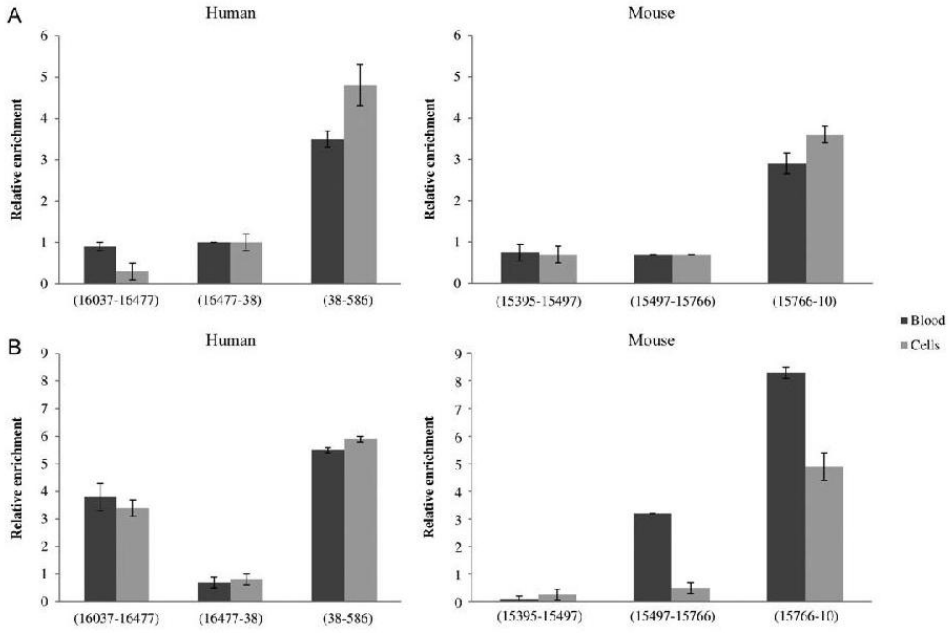

Figure 3. Methylated and hydroxymethylated mtDNA immunoprecipitation. *Alu*I-digested DNA extracted from blood and cultured cells of human and mouse were immunoprecipitated with anti-5mC (A) and anti-5hmC (B). Samples were amplified with primers specific for DNA fragments detected as unmethylated and methylated by bisulphite sequencing. The nucleotide position of these fragments is indicated. Fold enrichment was calculated as the ratio of amplification efficiency of the immunoprecipitated sample over that of non-immune IgG and normalized to the nanograms of immunoprecipitated DNA amount. Data represent the means of six triplicate experiments with standard errors of the mean.

nuclear DNA could localize within the mitochondria. In addition, we investigated the mitochondrial localization of methylcytosine dioxygenases, reported to be involved in the conversion of 5mC to 5hmC. For this purpose, using human HeLa and murine 3T3-L1 cultured cells as model, we performed immunoblotting assays on mitochondrial protein sub-fractions to detect the presence of DNMT1, DNMT3A, DNMT3B as well as of TET1, TET2, and TET3.

As shown in Fig. 4, DNMT1 and very low levels of DNMT3B were observed in the mitochondrial fraction in both humans and mice. As for TET proteins, we found TET1 and TET2 in the mitochondrial fraction of HeLa cells, meanwhile in 3T3-L1 cells only TET1 was detected in the same fraction. On the contrary, we did not find DNMT3A and TET3 in the mitochondrial fraction of the analysed samples (data not shown).

These findings suggest that enzymes involved in nuclear DNA methylation processes can cross the mitochondrial membrane to their final destination inside the mitochondria and, therefore, they might have a potential role in determining the methylation pattern observed in the D-loop.

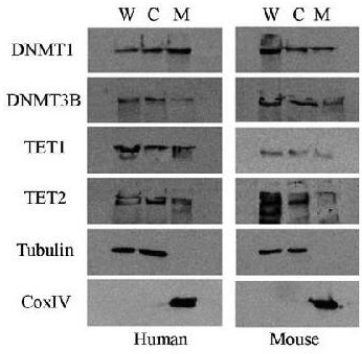

Figure 4. Representative western blot electrophoresis patterns of DNMTs and TETs. Whole cell lysate (W), cytosolic (C), and mitochondrial (M) protein fractions of human HeLa and murine 3T3-L1 cells. CoxIV and tubulin were analysed as mitochondrial- and cytosolic-specific markers, respectively.

3.5. *CpG and non-CpG methylation patterns in the mitochondrial D-loop of* Dnmt1, Dnmt3a, *and* Dnmt3b *(TKO) mouse ES cells*

To demonstrate whether the methyltransferases located within the mitochondria have a role in determining the methylation patterns observed in the D-loop, we examined the methylation status of the L-strand of this region in mouse ES cells deficient for Dnmt1, Dnmt3a, and Dnmt3b. In these cells, the complete lacking of DNA methylation was checked as reported by Tsumura *et al.*[37] (Supplementary Fig. S5).

Bisulphite sequencing analysis revealed the presence of methylated cytosines within the D-loop region in ~50% of methylated clones in both the wt and TKO ES DNA samples (Fig. 5A and B and Supplementary File S3). In these clones, the wt DNA sample shows global methylation percentage both at CpG (50%) and non-CpG (28%) sites higher than TKO one (31 and 23%, respectively; ANOVA test, $P < 0.001$). The persistence of methylated cytosines in the D-Loop region of the TKO DNA is consistent with the idea that DNMTs are not the only enzymes involved in the establishment of the reported methylation pattern. What is more, in both samples, the methylation percentage at CpG and non-CpG sites is significantly lower than that of other murine samples previously analysed (ANOVA test, $P < 0.001$). This result, together with the co-presence of both unmethylated and methylated clones, likely indicates that mitochondrial D-loop methylation depends on the differentiation state of cells.

3.6. *Discussion*

The presence of methylated cytosines within the nuclear genome of mammals has been extensively investigated and validated in the last 20 years by several lines of evidences, particularly in relation to their role in the gene expression silencing that explains the inverse correlation between density of methylation

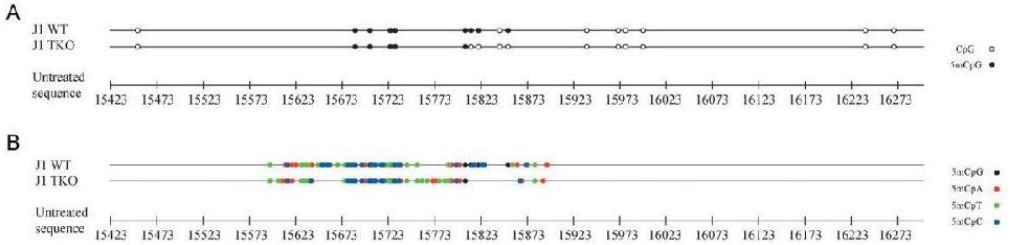

Figure 5. Methylation patterns of the mitochondrial D-loop in DNA samples from wt and *Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$ mouse ES cells. In (A), methylation of cytosine residues located within CpG nucleotides (CpG methylation) is shown. In (B), methylation of cytosine residues located outside of CpG nucleotides (non-CpG methylation) is shown.

within the regulatory sequences of a gene and its transcriptional levels.[16,38–40]

On the contrary, methylation of the mitochondrial genome is largely debated and is far from being elucidated. Indeed, conflicting reports are currently in disagreement over the possible presence of methylated cytosines within mtDNA.[26–35] Nevertheless, a CpG under-representation in mtDNA, first observed by Pollack *et al*. in mouse and successively by Cardon and McClelland in humans, has always suggested a susceptibility to mutation of this dinucleotide also in the mitochondrial genome and, consequently, to methylation.[31,41,42]

Here, we provide evidence for epigenetic modifications in mtDNA as methylated cytosines were detected within the mitochondrial D-loop in DNA samples extracted from blood and cultured cells of both humans and mice. A peculiar aspect of our data is that the majority of the methylated cytosines were located outside of CpG nucleotides. The presence of non-CpG methylated cytosines might suggest that the limited available data about mtDNA methylation can be attributable to the techniques that have been used to date aimed prevalently at identifying methylated CpG dinucleotides located within recognized sequences of methylation-sensitive restriction enzymes.[27,28]

Of note is also the existence of intercellular variability for both CpG and non-CpG methylation, with higher methylation levels in human blood and HeLa cells with respect to fibroblasts and osteosarcoma cells. The same pattern was observed in mouse, supporting the notion of tissue specificity of DNA methylation. On the other hand, no clear age-related differences were observed.

Although recent findings[35] have shown the presence of methylation and hydroxymethylation marks (5mC and 5hmC) in mtDNA by immunological and alternative methods, no data have been documented so far about this presence in DNA samples extracted from blood and cultured cells of humans and mice. The results of our study represent the first evidence of the exact localization and the relative abundance of 5hmC, relative to 5mC in the mtDNA from these tissues and cells. Our findings provide also further support to what has already been reported by Shock *et al*., specifically, that epigenetic modifications of cytosines in the mtDNA are likely much more frequent than previously believed.[35,43,44]

It is important to underline the many control experiments we carried out in order to accurately confirm results we obtained. First, we performed different bisulphite treatments, also repeated many months later, to ensure that cytosine conversion was complete. Secondly, we adopted alternative DNA extraction procedures and extended proteinase K treatment for removing any residual protein detrimental to the above conversion. Using both strategies, the observed methylation patterns were similar. Thirdly, we conducted the bisulphite sequencing procedure by using fully unmethylated DNA samples to exclude potential effects on complete bisulphite conversion by specific structural features of the analysed region. On the other hand, we retain that the presence within the analysed region of unconverted cytosines directly adjacent to other converted cytosines represents a good marker of a successful conversion. Finally, both bisulphite sequencing and MeDIP/hMeDIP-PCR assays were carried out also on DNA extracted from osteosarcoma 143B.TK$^-$ Rho^0 cells, completely lacking of mtDNA, in order to exclude spurious amplification of nuclear mitochondrial pseudogenes (NUMTs), with consistent results.[45]

The presence of DNMT1 and much lower levels of DNMT3B enzymes within the mitochondria of the HeLa and 3T3-L1 cells, used as cellular model systems in this study, would suggest that the methylation patterns we observed in the regulatory D-loop are established by these enzymes. In fact, previous studies have demonstrated the involvement of DNMT methyltransferases in the establishment of methylation in both canonical and non-canonical sites.[12,13] However, the translocation of the DNMTs within the mitochondria is emerging to be tissue-specific, thus we cannot assume that the results observed in HeLa and 3T3-L1 cells are a general phenomenon. However, in spite of these assumptions, *Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$ mouse ES DNA exhibit an analogous methylation pattern, although less marked than that of the wt sample. This finding brought us to believe a non-exclusive involvement of DNMTs in the establishment and maintenance of the methylation patterns we observed within the mitochondrial D-loop. However, more interestingly, the differences in D-loop methylation among the two ES cells with respect to that of other murine cells (blood and fibroblast cells) we analysed as well as the co-presence in ES cells of methylated and unmethylated mtDNA molecules seem to suggest that DNA methylation patterns of adult cells are established at early stage of cell development and can change according to cell and tissue differentiation.

The intra-mitochondrial presence of TET1 and TET2 might imply that the presence of 5hmC within the D-loop could represent an intermediate step in the 5mC demethylation mediated by the above enzymes, thus indirectly regulating the replication or transcription machinery of mtDNA. Therefore, the molecular mechanisms responsible for this methylation, as well as the functional properties of mitochondrial DNMT and TET enzymes within the mitochondria, represent interesting points that deserve further investigation.

Our findings lead us to question on the biological significance of the D-loop methylation. In both human and mouse samples, we identified methylated cytosines in the promoter region of the heavy strand (P_H)

and within conserved sequence blocks (CSBI-III), which are highly conserved sequences located at the 5′-end of the D-loop and considered to be implicated in the processing of the RNA primer during the replication of the H-strand.[46] Therefore, it is possible to hypothesize that D-loop methylation might play an important role in modulating either replication or transcription of mtDNA, two processes widely described as physically and functionally correlated. Indeed, the regions we found to be methylated are involved in forming the DNA–RNA hybrid due to the transcription of the leading (H)-strand origin. It is of note that the formation of this hybrid occurs to initiate the H-strand replication. Likely, the methylation of L-strand, displaying complementary sequence to RNA primer, may be involved in regulating the formation of the hybrid or stabilizing its persistence. We are currently carrying out experiments that could clarify the functional relevance of epigenetic modifications on the above processes, and the mechanisms which make them possible.

Finally, it is might to speculate that a dense methylation of symmetrical (CpG methylation) and non-symmetrical (non-CpG methylation) sites we observed within the mitochondrial D-loop may resemble typical features RNA-directed DNA methylation (RdDM), so far described only in plants and in fungi.[39,47] In fact, RdDM occurs specifically along DNA regions that are complementary to RNA, which directs the formation of a putative RNA–DNA duplex. It does not seem a coincidence to have detected the methylation patterns described above within the D-loop, which is a stable triple-helical structure where a RNA sequence forms a hybrid structure with the L-strand.

Taken together, our data provide unequivocal evidence supporting the presence of both methylated and hydroxymethylated cytosines within the human mtDNA control region (D-loop), with an unexpected prevalence in non-CpG moieties. Since both CpG and non-CpG methylated sites were located within the promoter region of the P_H and in CSBI-III, it is plausible that epigenetic modifications can regulate replication and/or transcription of the mtDNA. From our data, it also emerges that the peculiar methylation patterns we observed were strictly cell-type dependent, and that the methylation of mitochondrial genome seems to be much more similar to the epigenetic patterns taking place in plants and fungi than those characterizing eukaryotic organisms.

Supplementary Data: Supplementary data are available at www.dnaresearch.oxfordjournals.org.

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