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2º CICLO - MESTRADO
MESTRADO EM MEDICINA E ONCOLOGIA MOLECULAR

Impact of mtDNA mutations on chromatin remodeling

Diogo Manuel de Castro Abreu

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ABSTRACT

Mitochondria are considered a crucial regulator of cellular metabolism and signaling, converting so diverse functions such as cell proliferation, cell quality maintenance and apoptosis. Mitochondrial DNA (mtDNA) mutations have been described as characteristic of metabolic and degenerative diseases. However, the mutation associated phenotype only manifests it-self when the mutation levels surpass a certain threshold, leading to mitochondrial dysfunction. This dysfunction is associated with alterations in mitochondrial functions, which could result in changes in the metabolites levels. Interestingly, several metabolites are required for epigenetics –mechanisms of gene expression regulation without entailing alterations in the DNA sequence. Since epigenetic modifications alter gene expression by activating and/or repressing gene transcription, we hypothesize that mtDNA variations (and mutations) could alter the cellular transcriptome, as a result of alterations in the epigenetic pattern.

In order to verify an association between mtDNA variants and cellular transcriptome, we used transmitochondrial cytoplasmic hybrid cells (cybrid cells) as models. These models allow the study of different mtDNA cellular implications with minor inference of nuclear background. In this work, we used cybrid cell lines with exogenous mtDNA carrying different Ins3571C mutation levels (143B CA 80% and 143B CA 60%) from enucleated thyroid cancer cells, carrying the A3243T mutation (143B MELAS) from platelets from a patient with encephalopathy disease, and carrying mtDNA wild-type from haplogroup H and J (143B WT H and 143B WT J).

Initially, we analyzed both the clonogenic potential and cell proliferation of all cell lines. In order to assess changes in the epigenetic mechanisms, we evaluated the global DNA methylation and hydroxymethylation levels, as well as the acetylation (H3K9c, H3K14ac, H3K18ac, H3K27ac and H3K56ac) and methylation (H3K9me3, H3K27me3 and H3K79me) levels of histone H3. For a more specific analysis, we started the analysis of the H3K27ac mark genome-wide distribution in order to map the genome regions that holds this epigenetic modification. Since epigenetics is also crucial for protein expression and, in some cases, for its activation, and because cell proliferation is controlled by cell cycle regulators, we also evaluate the proteins levels from p53 pathway, in particular, p21, p27, p53 and acetylation of p53.

Our results suggest that compensatory mechanism(s) may be activated by cells with mtDNA mutation(s) in order to maintain cell epigenetic pattern and cellular homeostasis. We propose that epigenetic alterations may occur in specific genome regions as opposed to global epigenetic overview.

RESUMO

A Mitocôndria é considerada o principal regulador do metabolismo de diferentes vias de sinalização celular, convertendo funções tão diversas como proliferação e manutenção celular e apoptose. Mutações no ADN mitocondrial (mtADN) são características de doenças metabólicas e degenerativas. No entanto, as alterações fenotípicas só se manifestam quando os níveis de mutação ultrapassam um certo limite, levando à disfunção mitocondrial. Esta disfunção está associada a alterações no funcionamento da mitocôndria, e consequentemente à alteração nos níveis de metabolitos. Curiosamente, estes metabolitos são necessários para a epigenética – mecanismos que regulam a expressão genética independentemente da sequência de ADN. Uma vez que modificações epigenéticas alteram a expressão genética através da ativação ou repressão da transcrição de genes, propomos que variantes de mtADN (e mutações) podem alterar o transcriptoma celular, devido a alterações no padrão epigenético.

De forma a verificarmos a associação entre mtADN e transcriptoma celular, utilizamos linhas celulares híbridas citoplasmáticas trans-mitocondriais (células cíbridas) como modelos de estudo. Com estes modelos é possível verificar as consequências de diferentes mtADN com pouca interferência do ADN nuclear (nADN). Neste estudo utilizamos linhas cíbridas com mtADN exógeno com diferentes níveis de mutação Ins3571C (143B CA 80% e 143B CA 60%) de células enucleadas de cancro da tireoide, com a mutação A3243T (143B MELAS) de plaquetas de pacientes com encefalopatia e com *wild-type* mtADN do haplogrupo H e J (143B WT H e 143B WT J).

Inicialmente, analisamos o potencial clonogénico e a capacidade de proliferação em todas as linhas celulares. De forma a verificamos as alterações nos mecanismos da epigenética, procedemos à quantificação dos níveis globais de metilação e hidroximetilação do ADN, bem como os níveis de acetilação (H3K9, H3K14ac, H3K18ac, H3K27ac and H3K56ac) e de metilação (H3K9me3, H3K27me3 and H3K79me) da histona H3. Para uma verificação mais específica, começamos a analisar a distribuição da marca H3K27ac por todo o genoma de forma a mapear as regiões que apresentam esta modificação epigenética. Como a epigenética é também importante para a expressão proteica e, em alguns casos, para a sua ativação, e como a proliferação celular é controlada por proteínas reguladoras do ciclo celular, analisamos os níveis de proteínas incluídas na via do p53, nomeadamente p21, p27, p53 e a acetilação deste último.

Os nossos resultados sugerem que mecanismo(s) compensatórios podem ser ativados nas células com mutação(ões) no mtADN de forma a manter a homeostasia celular e o padrão

epigenético. Nós propomos que alterações epigenéticas podem ocorrer em regiões específicas do genoma em vez de alterações nos níveis globais da epigenética.

ABBREVIATION LIST

2-HG – 2-hydroxyglutarate
5caC – 5-carboxylcytosine
5fC – 5-formylcytosine
5hmC – 5-hydroxymethylcytosine
5mC – 5-methylcytosine
7BS – seven-β-strand
A3243G – A>G point mutation at nucleotide 3243
ac – acetylation
Acetyl-CoA – acetyl coenzyme A
AMPK – AMP-activated protein kinase
AOX – alternative oxidase
ARBP2 – adipocyte enhancer-binding protein 2
ATP – adenosine triphosphate
ATPase – adenosine triphosphate synthase membrane subunit
BER – base excision repair
bp – base pair
BSA – bovine serum albumin
C-5 – carbon 5 position
CG – cytosine and guanine residue
ChIP-Seq – chromatin immunoprecipitation followed by next generation sequencing
COX/CO - cytochrome C oxidase
CpG – cytosine contiguous to a guanine residue
CTD – carboxy-terminal domain
Cybrid cells - transmitochondrial cytoplasmic hybrid cells
CytB – cytochrome B
DBS - double-strand breaks (DSB)
D-loop – displacement-loop
DMEM – dulbecco's modified eagle's medium
DNA – deoxyribonucleic acid
DNMT – deoxyribonucleic acid methyltransferases
dNTPs – deoxyribonucleotide triphosphate
DPF – double PHD finger domain
DTT – 1,4-dithiothreitol
ECL – western lightning plus-enhanced chemiluminescence substrate
ESC – embryonic stem cell
Etbr - ethidium bromide
EZH – enhancer-of-zest homolog
FAD – flavin adenine dinucleotide
$FADH_2$ – Dihydroflavine-adenine dinucleotide
FBS – fetal bovine serum
FH – fumarate hydratase
FW – forward primer
GNAT – GCN5related N-acetyltransferases
GOT1 – glutamate-oxaloacetate transaminase 1
H1/2A/2B/3/4 – Histone 1; 2A; 2B; 3 or 4
HAT – histone acetyltransferases
HDAC – histone deacetylase
HRP – horseradish peroxidase
H-strand – heavy strand

HSV – herpes simplex virus
HTM – histone methyltransferase
IDH – isocitrate dehydrogenase
IM – inner membrane
Ins3571C – cytosine insertion at the nucleotide 3571
JARID2 – AT-rich interaction domain containing 2
JmjC/JMJD – Jumonji C domain-containing proteins
K – lysine
Kaiso, ZBTB4, and ZBTB38 – zinc-finger proteins
KDAC – lysine deacetylase
KTM – lysine methyltransferases
lncRNA – nong non-coding ribonucleic acid
LSD1/KDM1 – lysine-specific demethylase 1
L-strand – light strand
MBD (MeCP2, MBD1, MBD2, MBD3, and MBD4) – methyl-CpG-binding domain proteins
MDM2 – Mouse double minute 2 homolog
me1/me2/me3 – mono-, di- and trimethylation
MELAS – myopathy, cardiomyopathy, lactic acidosis, and stroke-like episodes
miRNA – micro ribonucleic acid
MMP – mitochondrial membrane potential
mRNA – messenger ribonucleic acid
MTA – methylthioadenosine
mtDNA – mitochondrial deoxyribonucleic acid
NAD^+ – nicotinamide adenine dinucleotide
NADH – Dihydronicotinamide adenine dinucleotide
NAM – nicotinamide
NC – nitroceluloce
ncRNA – non-coding RNAs
ND – nicotinamide adenine dinucleotide dehydrogenase
ND1l – NADH dehydrogenase-like 1
nDNA – nuclear deoxyribonucleic acid
NHEJ - non-homologous end joining
NUMTs – nuclear mitochondrial pseudogenes
O/E – observed/expected
OGT – O-GlcnAc transferase
O_H – heavy-strand origin for mitochondrial DNA replication
O_L – light-strand origin for mitochondrial DNA replication
OM – outer membrane
OXPHOS – oxidative phosphorylation
PBS – phosphate buffered saline
PBS-T – phosphate buffered saline with polyoxyethylene (20) sorbitan monooleate
PCLs – polycomb-like proteins
PCR – polymerase chain reaction
Pen/Strep – penicillin/streptomycin
P_H – heavy-strand promoter
P_L – light-strand promoter
PRC2 – polycomb repressive complex 2
PTM – post-translational modifications
qPCR – real-time polymerase chain reaction
R – arginine
RbAp46/48 – polycomb protein EED and retinoblastoma protein associated protein 46/48
RFU – relative fluorescence units

RNA – ribonucleic acid
RNAi – ribonucleic acid interference
ROS – reactive oxygen species
rRNA – ribosomal ribonucleic acid
RT – room temperature
RV – reverse primer
SAH/AdoHcY – S-adenosyl-L-homocysteine
SAM/AdoMet – S-adenosyl-L-methionine
SDH – succinate dehydrogenase
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sirt – sirtuins
SNPs – single nucleotide polymorphisms
SUZ12 – polycomb repressive complex 2 subunit
TCA cycle – tricarboxylic acid cycle
TD PCR - touchdown polymerase chain reaction
TDG – thymine deoxyribonucleic acid glycosylase
TET - ten-eleven translocation
TRD – transcriptional repression domain
tRNA – transfer ribonucleic acid
$tRNA^{Leu\ (UUR)}$ – transfer ribonucleic acid for leucine UUR
UHRF – ubiquitin-like proteins
WT – wild-type
α-KG – alpha-ketoglutarate
ρ0 – mitochondrial deoxyribonucleic acid depleted cell line

INTRODUCTION

MITOCHONDRIA

Mitochondria are considered key players in cellular metabolism in all nucleated cells. These are double-membrane organelles that possess an outer (OMs) and inner membrane (IMs). It is thought they originated from an endosymbiosis between a α-proteobacteria (precursor of the actual mitochondrion) and an archaeon (nucleus and cytosol) (Andersson, Zomorodipour et al. 1998).

Eukaryotic cells possess multiples copies of circular and double-stranded deoxyribonucleic acid (DNA) – called mitochondrial DNA (mtDNA) – which is characterized by being exclusively maternally inherited; the paternal mtDNA is actively eliminated upon fertilization (Al Rawi, Louvet-Vallee et al. 2011). However, the possibility of biparental transmission of mtDNA was recently observed, which may occur as a result of mutation in nuclear genes involved in the parental mtDNA elimination process (Luo, Valencia et al. 2018).

During evolution, mtDNA size was reduced as a result of transfer of mitochondrial genes to the nucleus (Nunnari and Suomalainen 2012). The integration of mtDNA in the nuclear genome generate nuclear mitochondrial pseudogenes (NUMTs), which is achieved by non-homologous end joining (NHEJ) machinery in double-strand breaks (DSB) and by retrotransposition (Hazkani-Covo and Covo 2008). Mitochondria are so semi-autonomous organelles that require proteins encoded by their mtDNA, but also proteins encoded by the nuclear genome for their normal cellular activities (Taylor and Turnbull 2005). In humans, mitochondrial genome is formed by 16,569 base pair (bp) and encodes 37 genes: two ribosomal ribonucleic acids (rRNAs) - 16s rRNA and 12s rRNA; 22 transfer RNAs (tRNAs); and 13 genes whose proteins are part of the oxidative phosphorylation (OXPHOS) system - NADH dehydrogenase 1 (ND1), ND2, ND3, ND4, ND4L, ND5, ND6, cytochrome B (CytB), cytochrome C oxidase I (COXI/COI), COXII, COXIII, adenosine triphosphate (ATP) synthase membrane subunit 6 (ATPase6) and subunit 8 (ATPase8) (Larsson and Clayton 1995) (Figure 1). Moreover, mtDNA is characterized by a heavy (H) and light (L) strand. H strand is described to be enriched with guanine and encodes 12 of 13 mitochondrial proteins (Dong, Wong et al. 2018). In contrast, L strand is constituted by a higher percentage of cytosine and is responsible for the encoding of ND6 protein (Dong, Wong et al. 2018) (Figure 1). Additionally, the mitochondria genome comprises a noncoding region that regulates the replication and transcription of mtDNA and include the displacement-loop (D-loop) (Falkenberg 2018) (Figure 1). This region is crucial since it holds both origins and promoters strands for mtDNA replication (O_H and O_L) and transcription (P_H and P_L), respectively (Falkenberg 2018) (Figure 1).

mtDNA exhibits a tenfold higher mutation rate comparing with nuclear DNA (nDNA) (Croteau and Bohr 1997). The absence of histones in mitochondria to compact and protect mtDNA allied to the fact that mitochondria is a major responsible for reactive oxygen species (ROS) production make mtDNA more susceptible to damage and more exposed to ROS (Croteau and Bohr 1997). Consequently, these mutations that can occur, generate a state of heteroplasmy in a cell, i.e., a mixture of two or more mtDNA variants (Gorman, Schaefer et al. 2015). Due to the replicative segregation of mtDNA during cell cycle, the percentage of mutation can vary and can increase or decrease until all mtDNA become identical – homoplasmy (Wallace 2018). Although mitochondria energy production commonly decreases with the rise of disruptive mutation levels, the mutation phenotype usually only appears when the mutation levels exceeds a "threshold", which correspond to the minimum bioenergetic threshold for normal functioning (Rossignol, Faustin et al. 2003). It is noteworthy that some mtDNA mutations could lead to different phenotypes, depending on the percentage of mtDNA mutations levels (Wallace 2018). For instance, in case of $tRNA^{Leu(UUR)}$ A3243G mutation, 20% to 30% of heteroplasmy levels result in type 1 or type 2 diabetes or autism; 50% to 80% of heteroplasmy levels result in myopathy, cardiomyopathy, lactic acidosis, and stroke-like episodes (MELAS syndrome); whereas 90% to 100% of heteroplasmy results in perinatal lethal diseases, such is the case of Leigh syndrome (Kopinski, Janssen et al. 2019).

Differences in energy production, in mitochondria function as well as in the influence of nuclear genome can also occur as a result of mtDNA haplogroups, which are groups of single nucleotide polymorphisms (SNPs) accumulated over time (Kenney, Chwa et al. 2014). This type of variations are a consequence of maternally inherited mutations, natural selection and human migration during human history (Mishmar, Ruiz-Pesini et al. 2003, Ruiz-Pesini, Mishmar et al. 2004). Therefore, mitochondrial haplogroups are distributed according to geographic location (Mishmar, Ruiz-Pesini et al. 2003). Accordingly to Siegismund *et al.*, up to 27 variations were identified in Europe by 2016, the most frequent haplogroups being H, V, HV, J, T, U, and K of lineage R and I, W, and X of the lineage N (Siegismund, Schäfer et al. 2016).

Mitochondria are crucial for enzymatic activity regulators of many cellular signaling pathways throughout metabolites. Nearly 350 metabolites were identified, which are generated through metabolic pathways such as OXPHOS, long-chain fatty acid beta-oxidation, amino acid metabolism, urea cycle, one-carbon cycle, and tricarboxylic acid cycle (TCA cycle) (Chen, Freinkman et al. 2016). Since mtDNA mutations could result in alterations in mitochondrial functions, namely in some of the abovementioned, this could result in variations of the levels of certain metabolites. It is then possible that, if the enzymatic activity of epigenetic enzymes is affected, the genetic transcription of nDNA can also be affected (Chen, Freinkman et al. 2016).

In fact, mtDNA mutations can reduce OXPHOS activity. This impairment could result in alterations of cellular respiration, ATP and ROS production (Tuppen, Blakely et al. 2010). Additionally, it has been observed that deficiency in OXPHOS complex I or complex IV result in alterations of the expression of proteins involved in apoptotic, cytoskeletal, OXPHOS, TCA cycle, glycolysis, and oxidative stress responses (Annunen-Rasila, Ohlmeier et al. 2007).

TCA cycle generates essential metabolites for normal epigenetic activity. Wu *et al.* described in cells with impaired OXPHOS (cells with depletion of mtDNA) alterations in metabolites from the TCA cycle, specifically alpha-ketoglutarate (α-KG), fumarate, malate, citrate levels, succinate and 2-hydroxyglutarate (2-HG) (Wu, Sagullo et al. 2016). Moreover, mtDNA-depleted cells demonstrated an increase in one-carbon cycle serine metabolism and transsulfuration, which is vital for generation of co-factors for nDNA and histone methylation related proteins (Wu, Sagullo et al. 2016, Patananan, Sercel et al. 2018). Consequently, these alterations seen in cells depleted of mtDNA seemed to affect gene transcription, by activating or repressing gene expression (Patananan, Sercel et al. 2018).

mtDNA mutations can also influence the transcriptional profile depending on mtDNA mutation levels. For instance, Kopinski *et al.* described that cells with dissimilar $tRNA^{Leu(UUR)}$ A3243G mutation levels feature different gene transcription profile while cells with the same level of heteroplasmy revealed an analogous profile (Kopinski, Janssen et al. 2019). These changes were accompanied by variations of metabolites levels. The authors reported that 20% to 30% of mutation load caused a reduction in the nuclear NAD^+/NADH ratio; 60% to 70% resulted in an increase of nuclear NAD^+ levels, increase in α-KG levels and decay in histone methylation; 90% to 100% of mutations load led to a significant decline in acetyl coenzyme A (acetyl-CoA) levels and decrease in histone acetylation (Kopinski, Janssen et al. 2019).

Overall, variations in gene transcription profiles seems to occur through alterations in the epigenetic marks, considering that several metabolites are key players in the epigenetic activity.

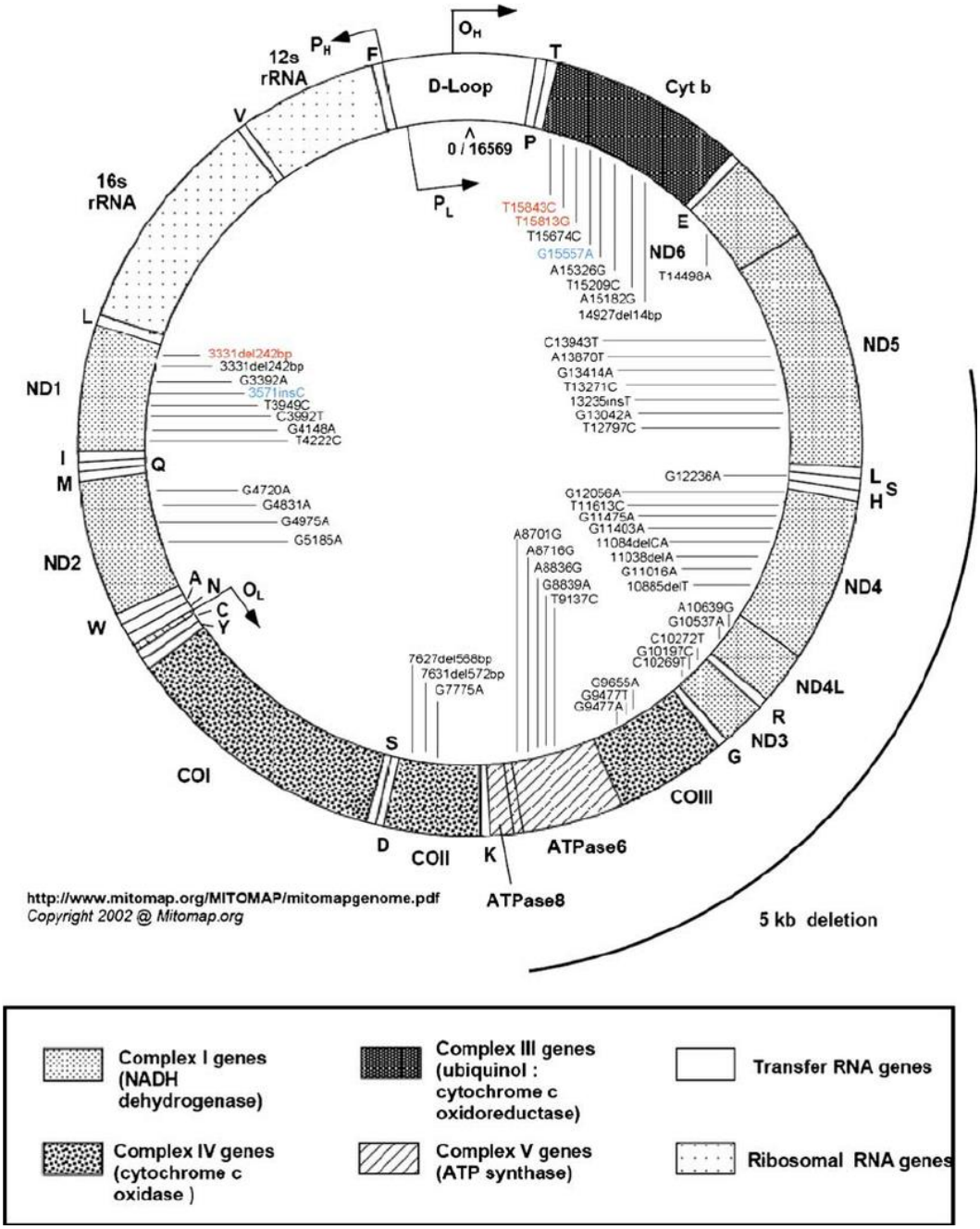

Figure 1 - Figure representative of human mtDNA and examples of somatic mutations in mtDNA. mtDNA is formed by 16,569 bp, a Heavy (H) and (Light) L strand and a noncoding region (which includes the displacement-loop (D-loop)). mtDNA encodes 37 genes: ribosomal RNAs (2 rRNAs - 16s rRNA and 12s rRNA), 22 transfer RNAs (tRNAs) and 13 genes essential for oxidative phosphorylation (OXPHOS) - NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5, ND6, cytochrome B (CytB), cytochrome C oxidase (COXI/COI, COXII, COXIII) and ATP synthase membrane subunits (ATPase6 and ATPase8). (Máximo, Lima et al. 2009)

EPIGENETICS

In 1942, Waddington described epigenetics as "the branch of biology that studies the causal interactions between genes and their products which bring the phenotype into being" (Waddington 2012). In 1957, the same author proposed the term "epigenetic landscape" in which he described that cellular differentiation appears to be an epigenetic event rather

alterations in genetic inheritance (Waddington 1957) (Figure 2). The author reported that, during multicellular organism development, the majority of cells generate a wide range of cell types with several functions and profiles of gene expression, even though parental cells shared the same genotype.

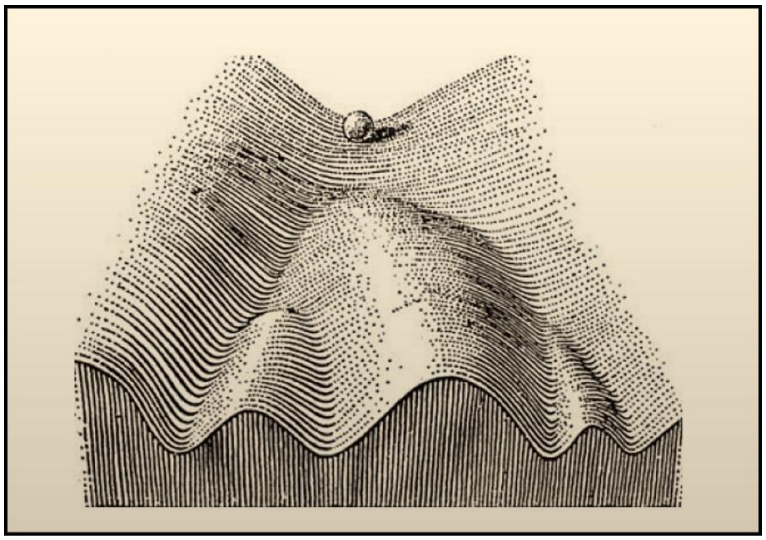

Figure 2 - Figure representation of epigenetic landscape. Cellular differentiation is achieved through epigenetic events, which could lead to different cell fates (Waddington 1957)

In the last decades, epigenetic has been accepted as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Wu and Morris 2001). These changes include DNA methylation and demethylation, post-translation modifications (PTMs) of histones, incorporation of histone variants and gene regulation by non-coding RNAs (ncRNA) (Allis and Jenuwein 2016).

nDNA Methylation – Methylation and Demethylation

nDNA methylation is one of the best characterized epigenetic mechanisms, which is conserved throughout evolution (Feng, Cokus et al. 2010). In prokaryotes, this process takes place in cytosine and adenine bases and makes it possible to differentiate self-DNA from host-DNA in a host restriction system (Jeltsch 2006). In mammalian, methylation is catalyzed by DNA methyltransferases (DNMTs) - DNMT1, DNMT3a, DNMT3b, and DNMT3L - which transfer a methyl group from a cofactor S-adenosyl-L-methionine (SAM or AdoMet) (Robertson 2001) (Figure 3A). The methyl group is added to carbon 5 position (C-5) in a cytosine contiguous to a guanine residue (CG dinucleotides - CpG) forming a 5-methylcytosine (5mC) and S-adenosyl-L-homocysteine (SAH or AdoHcy) (Robertson 2001) (Figure 3A and 4). DNMT2 is also a methyltransferase enzyme, however DNMT2-based DNA methylation is minimal (Lewinska, Adamczyk-Grochala et al. 2018).

nDNA methylation is a mechanism that influences transcription among different cell types by structural changes in chromatin structure (Cheng and Blumenthal 2010, De Carvalho, You et al. 2010). This epigenetic process is a key player in parental imprinting, in chromosome X inactivation, development of the immune system, in cellular reprogramming and protection of endogenous retroviruses and transposons (see (Maresca, Zaffagnini et al. 2015) for a comprehensive review on this subject). Besides the contribution to normal development, changes in nDNA methylation are also associated with diseases, namely in cancer. Aberrant nDNA (de)methylation is commonly found in human tumor samples or during tumor development, as a result of hypomethylation of oncogenes and/or hypermethylation of tumor suppressor (Lian, Xu et al. 2012, Wu and Ling 2014).

In the gene promoter region, nDNA methylation usually leads to repression of gene expression. Two explanatory models have been proposed. One model explains the transcriptional repression through the recruitment of reader proteins that act as a repression tag, inhibiting the binding of transcription factors to that site – "indirect" model (Nan, Cross et al. 1998, Dhasarathy and Wade 2008). The second defends a direct inhibition in the access of transcription factors, repressing this way gene transcription – "direct" model (Watt and Molloy 1988, Iguchi-Ariga and Schaffner 1989).

In the gene body, nDNA methylation demonstrates an opposite effect, revealing a positive correlation with gene transcription (Greenberg and Bourc'his 2019). Gene body methylation is more commonly at exons (Greenberg and Bourc'his 2019). However, the biological consequence of nDNA gene body methylation is not yet completely established. One possibility is that methylation in gene body promotes transcription elongation and/or co-transcriptional splicing, affecting gene expression (Greenberg and Bourc'his 2019). On the other hand, nDNA methylation in gene body inhibits intragenic promoters (promoters that initiate transcription of alternative truncated mRNA isoforms) (Greenberg and Bourc'his 2019).

CpG islands are formed at the minimum of 500bp with a 55% percentage of CG base composition and CpG frequency (observed/expected, O/E) ratio of 0.65 (Takai and Jones 2002). These CpG islands are frequently found unmethylated in 70% of gene promoters, particularly in housekeeping genes (Yang, Lin et al. 2016). However, CG sites are poorly found in the genomes, since 5mC have higher mutagenic propensity in comparison to unmethylated cytosines (Pfeifer, Tang et al. 2000). Interestingly, in humans, 60-80% of the predicted 56 million CpGs are methylated (Laurent, Wong et al. 2010).

The mechanisms behind *de novo* methylation are still unclear. Morris *et al.* described that methylation in specific sites of nDNA is accomplished through RNA interference (RNAi) mechanisms that targets DNMTs to those sequences (Morris, Chan et al. 2004). Despite the fact

that RNAi interferes with nDNA methylation in plants, there are no sufficient evidence that the same mechanism occurs in mammalian cells (Moore, Le et al. 2013). Moreover, Brenner *et al.* reported a different mechanism for *de novo* nDNA methylation. According to their study, transcription factors or components of repressor complexes bind to DNMTs, which target nDNA sequences for methylation (Brenner, Deplus *et al. 2005)*. On the other hand, some authors described that CpG sites, manly at CpG islands, are protected by transcription factors binding, preventing *de novo* nDNA methylation, even when transcription factor binding sites are mutated (Gebhard, Benner et al. 2010, Lienert, Wirbelauer et al. 2011). Furthermore, during differentiation, *de novo* nDNA methylation in specific genes occurs as a result of downregulation of transcription factors, exposing the CpG sites (Lienert, Wirbelauer et al. 2011).

DNMT3a, DNMT3b and their cofactor DNMT3L, a catalytic inactive regulatory factor, catalyze *de novo* patterns of DNA methylation in hemimethylated and unmethylated DNA during development and cell fate determination (Okano, Bell et al. 1999, Hata, Okano et al. 2002) (Figure 3B). DNMT3a and DNMT3b are structurally and functionally similar, but demonstrate distinct expression patterns and are essential in different stages of differentiation. Whereas *DNMT3a* is ubiquitously expressed, *DNMT3b* expression is lower in most of differentiated tissues, except for thyroid, bone marrow and testes (Xie, Wang et al. 1999). Additionally, Okano *et al.* reported that DNMT3a and DNMT3b are necessary in cellular differentiation and early development, respectively, since *DNMT3a* knockout mice originated a smaller mice that survived for approximately four weeks and *DNMT3b* knockout mice resulted in embryonic death (Okano, Bell et al. 1999). In the case of DNMT3L, it is a cofactor with no catalytic domain that promotes DNMT3a and DNMT3b activity (Suetake, Shinozaki et al. 2004). It has been observed that DNMT3L is mainly expressed in early development, whereas in adulthood is only found in germ cells and thymus (Aapola, Kawasaki et al. 2000, Aapola, Lyle et al. 2001). In mice, it was reported that DNMT3L is crucial for establishing both maternal and paternal genomic imprinting, for methylating retrotransposons, and for compaction of the X chromosome in mice (as gather in the (Moore, Le et al. 2013)).

On the contrary, DNMT1 is responsible for the conservation and dissemination of nDNA methylation during nDNA replication and repair, preferentially in hemimethylated DNA (Loughery, Dunne et al. 2011, Kar, Deb et al. 2012) (Figure 3B). *DNMT1* expression is regulated in a cell-cycle-dependent manner. It is highly expressed in proliferating cells, particularly during S-phase, and is present at low levels in non-dividing cells (Robertson, Uzvolgyi et al. 1999, Kimura, Seifert et al. 2003). DNMT1 location also appears to change during cell cycle. During interphase, it is diffusely expressed in the nucleus, while during early and mid S-phase it is

present at the DNA replication foci where newly synthesized hemimethylated DNA is assembled (Easwaran, Schermelleh et al. 2004).

DNA methylation maintenance can be achieved through the intervention of the methylation reader proteins. Some examples of these methylation reader proteins are the methyl-CpG-binding domain proteins (MBD) family protein members (MeCP2, MBD1, MBD2, MBD3, and MBD4; a family of proteins that contain a conserved methyl-CpG-binding domain), the zinc-finger proteins (Kaiso, ZBTB4, and ZBTB38), the UHRF1 and UHRF2 (ubiquitin-like, containing PHD and RING finger domain 1 and 2 – multidomain proteins), among others (Kimura and Shiota 2003, Filion, Zhenilo et al. 2006, Hashimoto, Horton et al. 2009). The MeCP2 contains a transcriptional repression domain (TRD), a domain that is important to MeCP2 to recruit and binding of DNMT1 to hemimethylated DNA (Kimura and Shiota 2003). Similar to MeCP2, MBD1 and MBD2 proteins also include a TRD domain. Via MBD domain, these proteins have higher affinity to bind to nDNA in methylated CpG sites, whereas TRD domain allows binding to repressor complexes, resulting in repression of gene expression (Nan, Ng et al. 1998, Ng, Zhang et al. 1999). Like MBD proteins, the Zinc-finger proteins (containing a zinc-finger domain) prevent gene expression by binding to methylated DNA (Filion, Zhenilo et al. 2006, Lopes, Valls et al. 2008). UHRF1 and UHRF2 also bind to DNMT1, mainly during DNA replication (Achour, Jacq et al. 2008, Hashimoto, Horton et al. 2009). Muto et al described that UHRF1 deletion results in embryonic death, a phenotype similar to Dnmt1 deletion (Muto, Kanari et al. 2002).

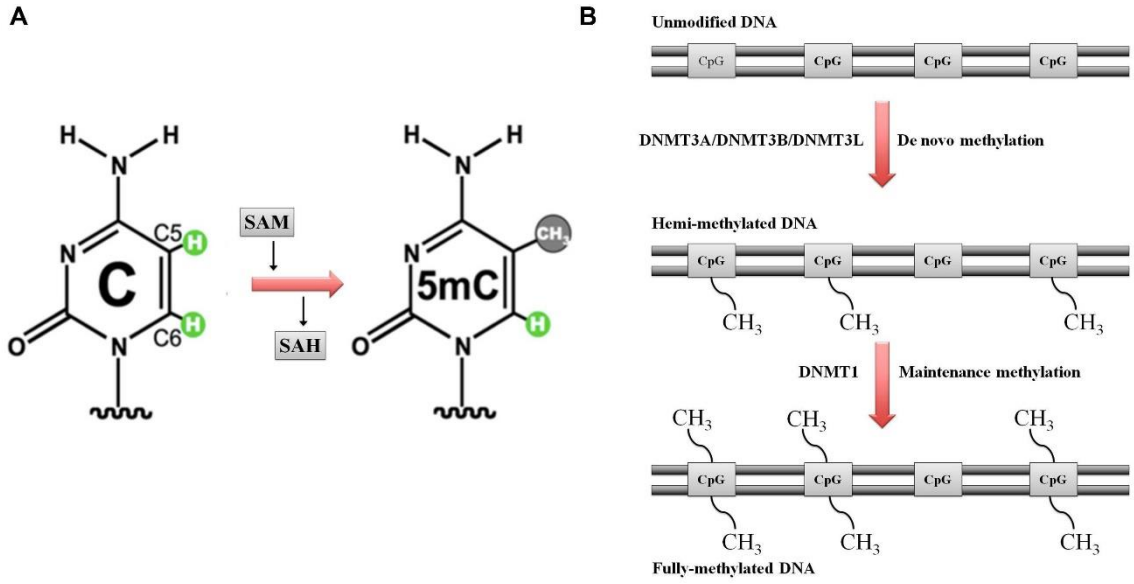

Figure 3 - Schematic representation of de novo and maintenance of nDNA methylation. **A –** DNA methyltransferases (DNMTs) transfer a methyl group from S-adenosyl-L-methionine (SAM or AdoMet) to the carbon 5 position in a cytosine contiguous to a guanine residue forming, as products, 5-methylcytosine (5mC) and S-adenosyl-L-homocysteine (SAH or AdoHcy) **B –** Schematic representation of de novo methylation performed by DNMT3a, DNMT3b and DNMT3L in hemimethylated or unmethylated DNA and methylation maintenance achieved by DNMT1 in hemimethylated DNA. (Maresca, Zaffagnini et al. 2015)

Ultimately, nDNA methylation is dynamic and reversible (Wu and Zhang 2010). The reversion is called demethylation and can occur through passive or active mechanisms. Passive demethylation is characterized by inhibition or dysfunction of DNMT1 activity, interfering with maintenance of nDNA methylation, resulting in loss of 5mC during successive cell divisions (Zhu 2009). Active demethylation requires a series of enzymatic reactions, since there is a strong covalent carbon-to-carbon link between the methyl group and cytosine (Zhang, Pomerantz et al. 2007). Ten-eleven translocation (TET) enzymes (TET1, TET2, and TET3) are methylcytosine dioxygenases responsible for the active demethylation through oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) (Tahiliani, Koh et al. 2009) (Figure 4). For this enzymatic reaction, TET requires, as cofactors, oxygen (O_2), αKG and iron (II) (Fe(II)), resulting in demethylation of 5mC, as well as succinate and carbon dioxide (CO_2) (Filipp 2017) (Figure 4). Furthermore, TET enzymes have the capability to oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Ito, Shen et al. 2011) (Figure 4). Subsequently, both forms are recognized and excised by human thymine DNA glycosylase (TDG), and replaced to a unmodified cytosine by base excision repair (BER) mechanism (Maiti and Drohat 2011) (Figure 4). On the other hand, nDNA demethylation can be achieved through passive dilution of 5fC and 5caC during nDNA replication (Inoue and Zhang 2011).

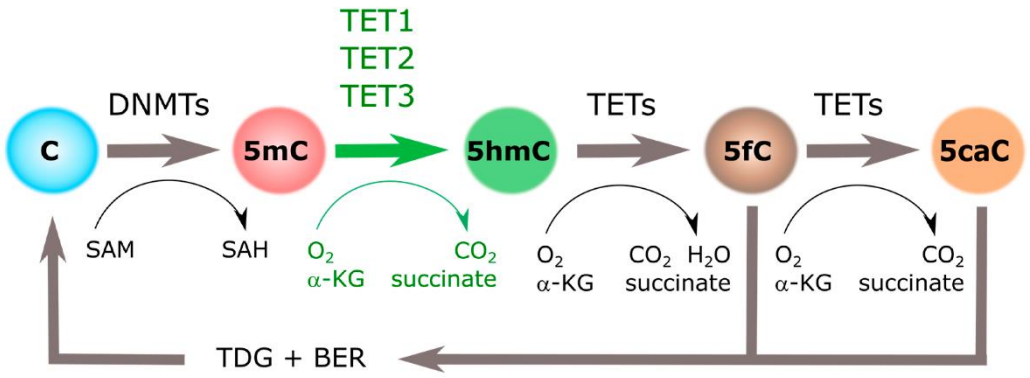

Figure 4 - Schematic representation of cytosine methylation and active demethylation. DNA methyltransferases (DNMTs) transfer a methyl group from cofactor S-adenosyl-L-methionine (SAM/AdoMet), generating 5-methylcytosine (5mC) and S-adenosyl-L-homocysteine (SAH/AdoHcy). Ten-eleven translocation (TET) enzymes are responsible for the active demethylation using, as cofactors, oxygen (O_2), alpha-ketoglutarate (α-KG) and iron (II), generating 5-hydroxymethylcytosine (5hmC) and succinate and carbon dioxide (CO_2) as byproducts. TET enzymes can continue the demethylation by oxidating 5hmC, forming 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Thymine DNA glycosylase (TDG) can recognize and excise 5fC or 5caC and an unmodified cytosine (C) is added by base excision repair (BER) mechanisms (Adapted from (Ecsedi, Rodríguez-Aguilera et al. 2018)).

Shen *et al.* reported that active demethylation may be an important process occurring in cell differentiation and stem cell maintenance (Shen, Song et al. 2014). Additionally, some authors described a genome-wide presence of 5hmC and its derivatives, which indicates that active demethylation might have a critical role in a variety of cellular functions (Inoue and Zhang 2011, Yu, Hon et al. 2012). Downregulation of TET and consequently decrease of 5hmC levels

have been detected in some cancer types - melanoma, hepatocellular carcinoma, and hematopoietic malignancies - manly in tumor progression (James, Mazurier et al. 2008, Ko, Huang et al. 2010, Lian, Xu et al. 2012, Liu, Liu et al. 2013). It indicates that the presence of demethylated nDNA might be used in cancer diagnosis and prognosis purposes.

HISTONE MODIFICATIONS

The nucleosome structure, the basic unit of chromatin, is formed by 147 bp of DNA involved around a histone octamer (two copies of H2A, H2B, H3, and H4) (Clapier and Cairns 2009). This structure is described to interfere with the DNA-binding capacity of several proteins (Clapier and Cairns 2009) (Figure 5A). Nucleosomes positive charge connects to the negative charge of DNA, impeding binding of transcription factors (Kimura 2013). Additionally, nucleosomes are coupled trough binding of DNA linker (50–100 bp long) with the linker histone H1, which stabilizes nucleosome and chromatin structure (Hergeth and Schneider 2015). Histones N-terminal tail can be exposed to PTMs that allow the regulation of chromatin packaging and structure (Kouzarides 2007, Clapier and Cairns 2009) (Figure 5). In fact, histone PTMs, usually called as epigenetic marks, can contribute to activation or repression of transcriptional activity. They predominantly occur in histone 3 and 4 (H3 and H4) in certain amino acids as a result of strong complex formation with DNA (Kimura 2005, Kouzarides 2007). These modifications are also crucial for other cellular processes, such as DNA replication and repair and cell-cycle control (Kouzarides 2007, Allis and Jenuwein 2016). Thereby, a deficiency in the histone modifications machinery could lead to some pathologies, such as cancer.

At least fifteen different PTMs in histones have been identified. Methylation and acetylation are key epigenetic marks in gene expression regulation (Kimura 2013, Huang, Sabari et al. 2014) (Figure 5B).

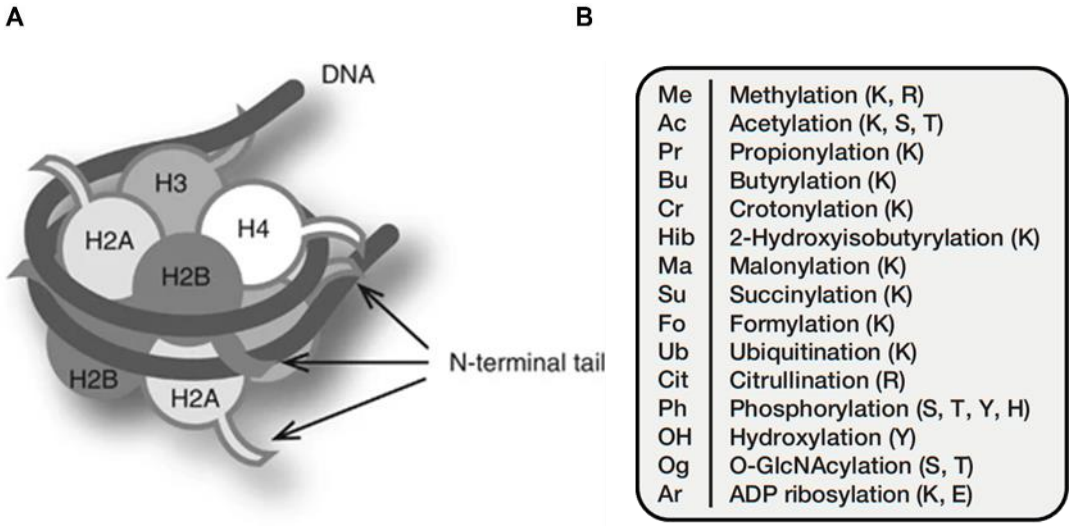

Figure 5 - Figure representation of nucleosome structure and post-translational modifications (PTMs). **A –** Nucleosome is assembled by 147 bp of DNA involved around a histone octamer (two copies of H2A, H2B, H3, and H4). Each histone features a N-terminal tail, which can be exposed to PTMs. **B -** Post-translational modifications (PTMs) in specific residues identified in human, mouse, and rat. (Adapted from (Kimura 2013, Huang, Sabari et al. 2014))

HISTONE METHYLATION

Histone methylation is an epigenetic process that occurs in the lysine (K) and arginine (R) amino acids. It is responsible for enhanced basicity, hydrophobicity of histone tails and change protein affinity that regulate gene expression (Teperino, Schoonjans et al. 2010). Specifically, lysine methylation is an epigenetic mark that can occur in different states in the ε-group of the lysine residue - mono-, di- and trimethylation (me1, me2 and me3, respectively) (Black, Van Rechem et al. 2012). This epigenetic mark could lead to either activation or repression of transcription activity. In particular, methylation in H3K4 and H3K79 are linked to transcription activation, whereas methylation in H3K27 and H3K9 lead to transcriptional silencing (Hyun, Jeon et al. 2017). Histone modification co-operates with other epigenetic marks, including nDNA methylation, which allows a better regulation of gene expression (Hyun, Jeon et al. 2017).

Histone lysine methylation is achieved through histone methyltransferases (HTMs) enzymes, particularly lysine methyltransferases (KTMs) (SET domain lysine methyltransferases and non-SET domain lysine methyltransferases) that are considered the “writers” enzymes (Teperino, Schoonjans et al. 2010). All KTMs use SAM as a methyl group donor (Figure 6), but they differ in structure, mechanism and substrate recognition (Smith and Denu 2009). Additionally, these enzymes exhibit specificity, i.e. they recognize specific lysine residue(s) of a particular histone (Teperino, Schoonjans et al. 2010) (Figure 7).

SET-domain KTMs contain a conserved 130 amino acid catalytic domain and their activity is characterized by interaction with short, linear peptides containing the target lysine (Black, Van Rechem et al. 2012, Wu, Connolly et al. 2017). These enzymes also target non-histone proteins, such as tumor suppressors (p53 and pRb) and estrogen receptor α (Huang, Dorsey et al. 2010, Munro, Khaire et al. 2010). On the other hand, the non-SET domain KTMs are described to have twisted β-sheet structure (seven-β-strand (7BS) methyltransferase family) and are characterized by recognizing their substrates as tertiary protein structures (Falnes, Jakobsson et al. 2016).

The structural modification induced by KTMs in the lysine residue enables binding of "reader" proteins - MBD proteins -, resulting in a biological response. MBD proteins are characterized by a hydrogen bond network, which is responsible for the specific recognition of the different states of methylation (Li, Fischle et al. 2007). As KTMs could methylate nonhistone proteins, MBD proteins can also interact with these modifications, specifically L3MBTL1. This MBD protein is responsible for the p53 and Rb regulated genes repression due to the link of L3MBTL1 with two mono-methylated lysine sites (K382me1 and K860me1, respectively) (Saddic, West et al. 2010, West, Roy et al. 2010). Other example of a reader protein is 53BP1 that can also bind to p53 in two demethylated lysines (K370 and K382) (Huang, Sengupta et al. 2007, Kachirskaia, Shi et al. 2008). In case of binding at the demethylated K370 residue, this protein promotes transcription of p53-target genes, whereas binding at the demethylated K382 residue, 53BP1 activate p53, as a consequence of DNA double-strand break signals (Huang, Sengupta et al. 2007, Kachirskaia, Shi et al. 2008).

Similar to nDNA methylation, histone methylation is also a reversible process. The "eraser" proteins – histone demethylases (HDMs) – are the responsible enzymes (Figure 6). In particular, lysine-specific demethylase 1 (LSD1/KDM1) is a key player in demethylate mono- and di-methylation in H3K4me2 and H3K4me1 (Shi, Lan et al. 2004) (Figure 6 and 7). In their enzymatic activity to oxidate the methylated lysine, they reduce FAD to $FADH_2$ (Smith and Denu 2009) (Figure 6).

Tri-methylated lysine demethylation is only possible though Jumonji C domain-containing proteins (JmjC/JMJD) (Klose and Zhang 2007) (Figure 7). These proteins require α-KG, O_2, and Fe(II) as cofactors, generating CO_2 and succinate as by-products (Shi and Whetstine 2007) (Figure 6). Although JmjC proteins are key players in demethylation of tri-methylated residues, they could, as well, demethylate other methylation states (Klose and Zhang 2007) (Figure 7).

Identical to KTMs, enzymatic activity of KDMs occur at specific lysine residues and at certain methylated states (Black, Van Rechem et al. 2012) (Figure 7).

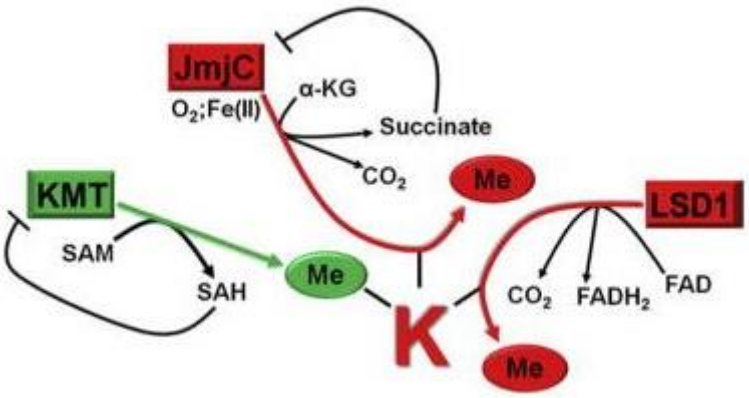

Figure 6 - Schematic representation of KTMs and KDMs activity. KTMs are histone methylation enzymes responsible for histone methylation, which use SAM as cofactor. JmjC are histone demethylation enzymes responsible for demethylate tri-methylated residues that require alfa-ketoglutarate (α-KG), oxygen (O2), and iron (II) (Fe(II)) as cofactors, generating carbon dioxide (CO2) and succinate as by-products. This enzyme can also demethylate mono- and di-methylation. LSD1 are histone demethylation enzymes responsible for demethylate mono- and di-methylation that require FAD and generates COs and $FADH_2$ as by-products. (Black, Van Rechem et al. 2012)

In the particular case of H3K27, methylation is reached through activity of multiprotein enzyme complex – the polycomb repressive complex 2 (PRC2) (Margueron and Reinberg 2011). PRC2 is composed by enhancer-of-zest homolog 1 and 2 (EZH1/2 - SET-domain KTMs), polycomb repressive complex 2 subunit (SUZ12), polycomb protein EED and retinoblastoma protein associated protein 46/48 (RbAp46/48) (Margueron and Reinberg 2011) (Figure 7). In fact, methylation of H3K27 is only possible as a consequence of PRC2 activity, since EZH1/2 isolated has no enzymatic activity (Pasini, Bracken et al. 2004, Nekrasov, Wild et al. 2005). PRC2 is recruited and regulated by accessory proteins, such as adipocyte enhancer-binding protein 2 (AEBP2), jumonji and AT-rich interaction domain containing 2 (JARID2) and polycomb-like proteins (PCLs) (Alekseyenko, Gorchakov et al. 2014). Furthermore, SUZ12 and EED subunits have the capacity to tri-methylate H3K27 (H3K27me3), an epigenetic mark that leads to transcriptional repression, and to disseminate the repressive mark to adjacent nucleosomes (Margueron, Justin et al. 2009, Yuan, Wu et al. 2012). Additionally, Silva *et al.* showed that inactivation of X chromosome is also a consequence of increased levels of H3K27 methylation, which maintain the inactive chromatin structure (Silva, Mak et al. 2003). H3K27me2 and H3K27me3 demethylation is also achieved through UTX/KDM6A, UTY/KDM6C and JMJD3/KDM6B enzymes that belong to JmjC domain-containing family protein (Agger, Cloos et al. 2007, Walport, Hopkinson et al. 2014) (Figure 7). These marks lead to re-activation of gene transcription.

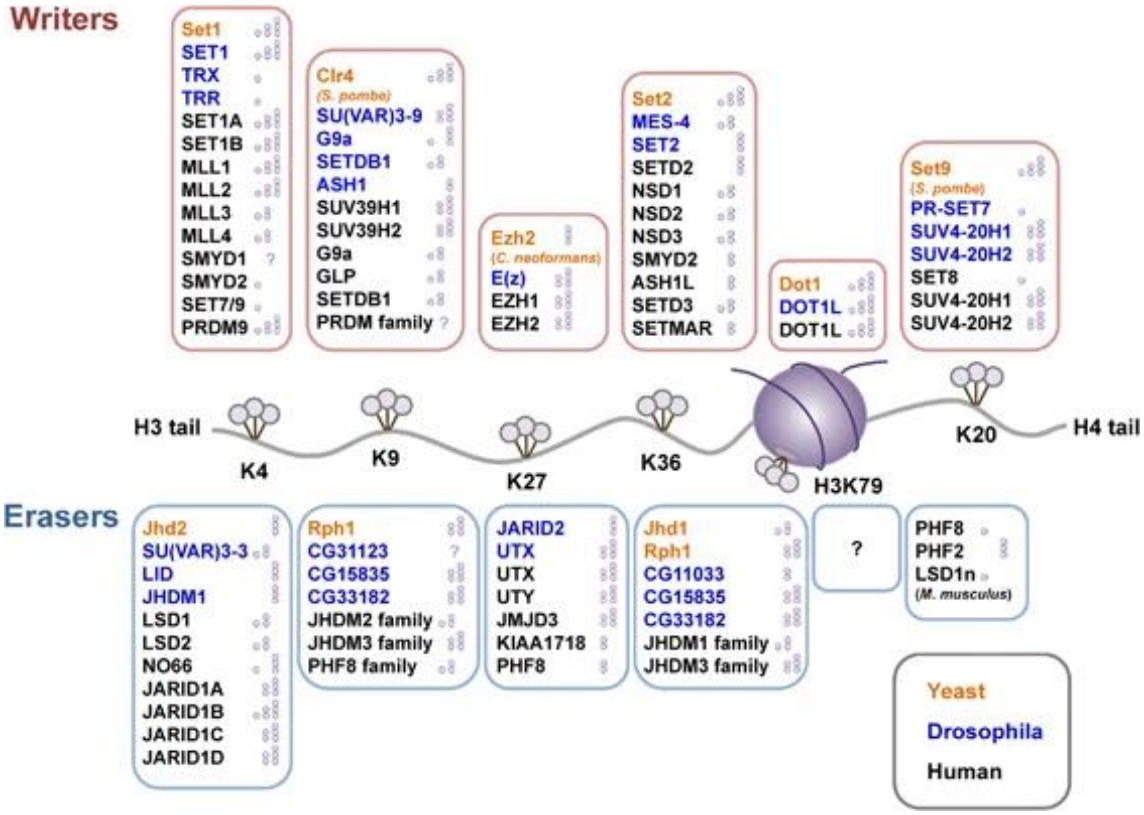

Figure 7 - Schematic representation of key methylation marks in histone 3 and 4 and the methyltransferases (writers) and demethylases (erasers) enzymes at each residue identified in Yeast, Drosophila and Human. Single circles correspond to monomethylation; double circles correspond to dimethylation; triple circles correspond to trimethylation (Hyun, Jeon et al. 2017)

Deficiency in methylation mechanism has been associated with some human diseases. In particular, overexpression of subunit EZH2 of PRC2 complex has been linked to cancer, namely in human breast, prostate and lymphoma (Audia and Campbell 2016). Is it then possible that the inhibition of PRC2 could be a treatment option.

Histone Acetylation

Histone acetylation leads to transcriptional activation. Acetylation neutralizes the positive charges of the target histone residues resulting in a lower DNA-protein affinity (Gelato and Fischle 2008). This epigenetic mark allows the activity of DNA repair mechanisms and has an impact on the activity of DNA replication origins (Murr, Loizou et al. 2006, Norio 2006).

In lysine acetylation, an acetyl group is added to the primary amine in the ε-position of the lysine. It can be achieved by nonenzymatically mechanism and, in most of the cases, through the "writer" enzymes - histone acetyltransferases (HATs) (Ali, Conrad et al. 2018). HATs can be divided in two classes based on subcellular localization. While HATs type A are located at the nucleus and are a key player in regulating gene expression as a result of histone acetylation (Voss and Thomas 2018), HATs type B are localized at the cytoplasm and are responsible for acetylation newly histone preceding nucleosomes assembly (Sobel, Cook et al. 1995). In humans,

the Hats type A are divided in three families: GNAT (GCN5related N-acetyltransferases), CBP/P300 and the MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60). All utilize acetyl-CoA as cofactor, and demonstrate a similar structure - globular α/β fold (Clements, Rojas et al. 1999, Lin, Fletcher et al. 1999, Delvecchio, Gaucher et al. 2013). It has been shown that an equilibrium of histone acetylation is crucial for normal cellular functions, since both higher and lower levels of HATs are associated with poor outcomes in cancer (Hou, Li et al. 2012, Chen, Luo et al. 2013).

HATs also acetylate non-histone proteins, particularly transcription factors. Their acetylation is responsible for altering their activity throughout nuclear translocation or stabilization (Ali, Conrad et al. 2018). For instance, as a result of DNA damage, acetylation in p53 is achieved through p300 and MOZ activity at K120 and K382 residues in its carboxy-terminal domain (CTD) (Rodriguez, Desterro et al. 2000, Rokudai, Laptenko et al. 2013). Consequently, p53 acetylation inhibits MDM2-mediated ubiquitination and degradation, maintaining p53 in the nucleus (Luo, Su et al. 2000, Rodriguez, Desterro et al. 2000).

Similar to other epigenetic marks, lysine acetylation allows the binding of downstream readers proteins that possess specific acetyl−lysine binding domains – the double PHD finger domain (DPF), the YEATS domain and, most frequently, the bromodomain (Dhalluin, Carlson et al. 1999, Zeng, Zhang et al. 2010, Li, Wen et al. 2014). The bromodomain is characterized by, approximately, 110 amino acids and was described to be present in 46 proteins, including nuclear HATs proteins (Ali, Conrad et al. 2018). Most of these proteins are nuclear factors responsible for regulating chromatin structure and, as a consequence, for the regulation of transcription (activation or repression) (Ali, Conrad et al. 2018). In case of acetylation in p53, p300/CPB and KAT4 bromodomains operate as reader proteins that promote acetylation in histones H3 and H4 of p53 response genes, causing cell-cycle arrest or apoptosis (Barlev, Liu et al. 2001, Li, Piluso et al. 2007, Wu, Lin et al. 2014).

Lysine acetylation is also a reversible epigenetic mark. Deacetylation is achieved through lysine deacetylases (KDACs; also identified as histone deacetylases (HDACs)) –class I, II, III and IV KDAC.

KDACs class I (KDAC 1, 2, 3, 8), II (KDAC4−7, 9, 10) and IV (KDAC11) are zinc ion (Zn^{2+}) dependent enzymes, which utilizes H_2O and Zn^{2+} to generate the deacetylated lysine, as well as acetate as byproducts (Audia and Campbell 2016, Ali, Conrad et al. 2018). Normally, these enzymes act through macromolecular complexes responsible for transcription repression (Ali, Conrad et al. 2018). However, these classes present different cellular localizations – class I are predominantly localized at the nucleus, whereas classes II and IV shuttle between the nucleus and the cytoplasm (Ali, Conrad et al. 2018). Additionally, KDACs have the capability to deacetylate various histones sites, since these enzymes present low substrate specificity (Audia

and Campbell 2016). Furthermore, overexpression of KDACs are commonly found in cancer patients (Audia and Campbell 2016). Since KDACs class I, II and IV depend on Zn^{2+}, this dependency is use as target to inhibit the deacetylases activity (Ali, Conrad et al. 2018).

On the other hand, Class III KDACs, also termed as sirtuins (Sirt1-7), are nicotinamide adenine dinucleotide (NAD^+) dependent deacetylases which do not rely on an active site metal to catalyze the deacetylation (Katsyuba and Auwerx 2017). Instead, their enzymatic activity requires acetamide oxygen, H_2O and NAD^+ to generate a deacetylated product and O-acetyl-ADP-ribose and nicotinamide (NAM) as byproducts (Ali, Conrad et al. 2018). Interestingly, sirtuins activity is negatively regulated by their enzymatic byproduct, NAM (Sauve and Schramm 2003, Seto and Yoshida 2014). All sirtuins exhibit some similarity - 22%–50% in the overall amino acid sequence and 27%–88% in the conserved catalytic domain (Seto and Yoshida 2014). However, these enzymes demonstrate dissimilar deacetylase capability – Sirt1, 2 and 3 demonstrate strong deacetylase activity compared to Sirt5, 6 and 7; and Sirt4 do not present deacetylase activity (Ali, Conrad et al. 2018). Furthermore, sirtuins also differ in their subcellular localization. Sirt1 and Sirt2 commute between nucleus and cytoplasm, Sirt3 shuttle between nucleus and mitochondria, Sirt4 and Sirt5 localize in the mitochondria, Sirt6 locate in the nucleus and Sirt7 localize specifically in the nucleolus (Michishita, Park et al. 2005, Seto and Yoshida 2014). Unlike the other KDACs classes, sirtuins do not depend on macromolecular complexes, but are regulated by specific binding partners.

MITOCHONDRIA INFLUENCE

Mitochondria are important players in the control of the cellular epigenetic status through the tight regulation of key intracellular metabolites. As a consequence of mitochondrial activity, there is an oscillation in the concentration of several metabolites that are required for the enzymatic activity of several “epigenetic enzymes” (as substrates or co-factors) (Xu, Wang et al. 2016) (Figure 8). This way, distinct epigenetic modifications occur in nDNA and histones accordingly to the metabolic cellular status and there is a regulation in gene expression (Sakamoto, Hino et al. 2015, Guha, Srinivasan et al. 2016, Tian, Garcia et al. 2016). Thus, mitochondria are considered novel key players in the regulation of gene expression.

SAM is a critical cofactor of DNMTs and HTMs, which is originated in the one-carbon cycle, being one of the intermediates (Figure 8). Singhal *et al.* and Maddocks *et al.* demonstrated that nDNA and histone methylation levels were lower in cells with defective one-carbon cycle, as a result of a reduction of SAM (Singhal, Li et al. 2015, Maddocks, Labuschagne et al. 2016).

In nDNA and histone demethylation, specifically TET and JmjC enzymes, utilizes α-KG as a cofactor, which is an intermediate of TCA cycle (Figure 8). Some studies reported that

deficiency/inactivation of TCA enzymes, namely fumarate hydratase (FH) and succinate dehydrogenase (SDH) led to inhibition of TET enzymes, as a result of accumulation of fumarate and succinate. As a consequence, there was seen by the authors an increase of DNA and histone methylation, activation of hypoxia pathways and, possibly favors tumorigenesis (Hoekstra, de Graaff et al. 2015, Sciacovelli, Goncalves et al. 2016).

Additionally, as previously stated, TET and JmjC activity produces succinate and CO_2. Liu *et al.* reported that different α-KG/succinate ratios affect macrophages activity (Liu, Wang et al. 2017). The authors demonstrated that an elevated α-KG/succinate ratio led to demethylation of H3K27me3 marks, anti-inflammatory macrophages differentiation and tissue repair activity. On the other hand, low α-KG/succinate ratio led to a pro-inflammatory feedback (Liu, Wang et al. 2017). Furthermore, Carey *et al.* described that maintenance of naïve mouse embryonic stem cells is achieved through high values of α-KG/succinate ratio, which result in maintenance of cell pluripotency and enzymatic activity of TET and JmjC (Carey, Finley et al. 2015). However, a reduction of α-KG/succinate ratio affected demethylation processes, resulting in increased trimethylation and decreased monomethylation of H3K9, K3K27, H3K36, and H4K20 and promoting cell differentiation (Carey, Finley et al. 2015). Nevertheless, TeSlaa *et al.* demonstrated different outcomes in primed-state human pluripotent stem cells and mouse epiblast stem cells (TeSlaa, Chaikovsky et al. 2016). The authors demonstrated that high levels of α-KG promoted cell differentiation, as well a reduction in H3K4me3 and H3K27me3; whereas elevated succinate levels increased these epigenetic marks (TeSlaa, Chaikovsky et al. 2016).

Another metabolite associated with interference in demethylation is 2-HG. 2-HG exists as two enantiomers - D-R-2-hydroxyglutarate or L-S-2-hydroxyglutarate –, which are originated as a result of a neomorphic mutations in isocitrate dehydrogenase 1 and 2 (IDH1/2) or by transamination via glutamate-oxaloacetate transaminase 1 (GOT1), respectively (Lu, Ward et al. 2012). This metabolite can compete with α-KG for the active site of TET enzymes and JHDM, inhibiting TET and JmjC activity (Lu, Ward et al. 2012, Lozoya, Martinez-Reyes et al. 2018). Consequently, inhibition of TET and JHDM activity would result in decrease nDNA and histone demethylation (Lu, Ward et al. 2012, Lozoya, Martinez-Reyes et al. 2018).

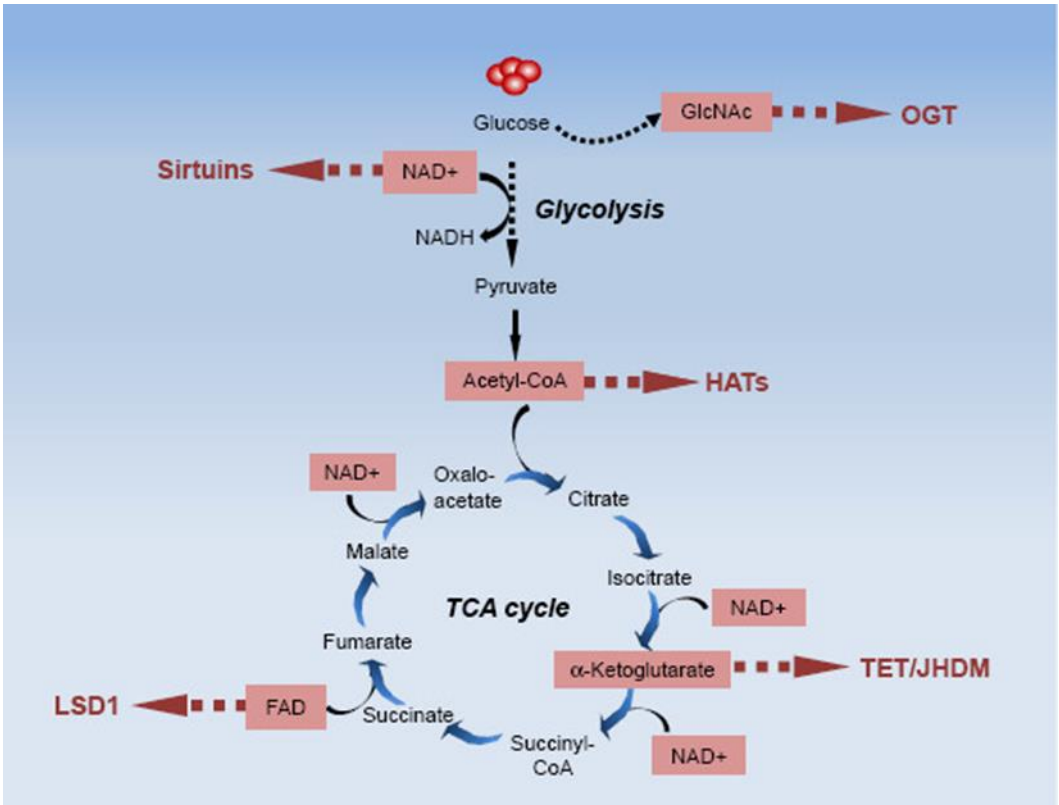

Figure 8 - Schematic representation of glycolysis and tricarboxylic acid (TCA) cycle and the co-factors of histone GlcnAcylation (O-GlcnAc transferase – OGT), histone acetylases (HATs), histone deacetylases (Sirtuins), nDNA and histone demethylases (TET and JHDM, respectively) and histone demethylases of mono- and di-methylation (LSD1) (Xu, Wang et al. 2016)

MTDNA METHYLATION

It is established that nDNA methylation is a crucial epigenetic modulator that have an impact on gene expression. However, this is not equal regarding mtDNA methylation (Matsuda, Yasukawa et al. 2018).

Some studies described that mtDNA methylation is absent or a rare event in cultured cell from different species (including human cells), and animal liver tissue (as gather in the (Maresca, Zaffagnini et al. 2015, Matsuda, Yasukawa et al. 2018)) (Figure 9). Maekawa *et al.* described that mtDNA methylation is a rare event and hypermethylation occurs in a very low frequency, suggesting that mtDNA methylation cannot be used as a cancer biomarker (Maekawa, Taniguchi et al. 2004).

On the other hand, some reports detected mtDNA methylation in a variety of cells as well as in animal tissues (as gather in the (Maresca, Zaffagnini et al. 2015, Matsuda, Yasukawa et al. 2018)) (Figure 9). Interestingly, two studies of Vanyushin and Kirmos described that in beef heart, fish liver (sheat-fish), birds liver (duck and chicken) and mammals liver and heart (rat and ox) had more mtDNA methylated than nDNA (Vanyushin and Kirnos 1974, Vanyushin and Kirnos 1977).

Additionally, some studies reported the presence of DNMTs in the mitochondria of human and mouse cell lines and tissues, evidencing that methylation can occur in the mtDNA (Chestnut, Chang et al. 2011, Shock, Thakkar et al. 2011, Bellizzi, D'Aquila et al. 2013, Wong, Gertz et al. 2013) (Table 1 and Table 2).

In a more recent report, Matsuda *et al.* (Matsuda, Yasukawa et al. 2018) analyzed the possibility of mtDNA methylation in mouse liver, brain and embryonic stem cells (ESCs) through three different methods – Next-generation bisulfite sequencing, methylated cytosine-sensitive endonuclease McrBC treatment and mass spectrometric nucleoside analysis. The authors detected 5mC using the three techniques, however at very low levels (Matsuda, Yasukawa et al. 2018) (Figure 9). Additionally, the authors showed that 5mC do not occur at specific sites in mtDNA, leading the authors to conclude that mtDNA methylation may not be an important player in mitochondrial gene expression and in metabolism (Matsuda, Yasukawa et al. 2018).

Although it seems that methylation of mtDNA methylation occur, its importance is still under debate and there is a need of more studies in this field. The outcomes of this mechanism in mitochondrial processes as well as in the influence in nuclear genome remains unclear. Nevertheless, the existence of this epigenetic marks in the mitochondria could possibly influence mitochondrial gene transcription profile, if this affects, in some way, the mitochondrial energy and metabolite production.

Table 1 - DNA methyltransferase (DNMTs) in Human mitochondria in different cell types/tissues (Maresca, Zaffagnini et al. 2015)

DNA methyltransferase	Mitochondrial localization	Organism	Cell type/Tissue	Reference
DNMT1	Yes	Human	HCT116	(Shock, Thakkar et al. 2011)
DNMT1	Yes	Human	HEK293	(Chestnut, Chang et al. 2011)
DNMT1	Yes	Human	HeLa	(Bellizzi, D'Aquila et al. 2013)
DNMT3A	Yes	Human	HEK293	(Chestnut, Chang et al. 2011)
DNMT3A	Yes	Human	Frontal Cortex	(Wong, Gertz et al. 2013)
DNMT3A	No	Human	HEK293	(Wong, Gertz et al. 2013)
DNMT3B	Yes	Human	HeLa	(Bellizzi, D'Aquila et al. 2013)

Table 2 - DNA methyltransferase (DNMTs) in Mouse mitochondria in different cell types/tissues (Maresca, Zaffagnini et al. 2015)

DNA methyltransferase	Mitochondrial localization	Organism	Cell type/Tissue	Reference
DNMT1	Yes	Mouse	MEF	(Shock, Thakkar et al. 2011)
DNMT1	Yes	Mouse	NSC34 Astrocyte Microglia	(Chestnut, Chang et al. 2011)
DNMT1	Yes	Mouse	3T3-L1	(Bellizzi, D'Aquila et al. 2013)
DNMT1	No	Mouse	Adult skeletal muscle	(Wong, Gertz et al. 2013)
DNMT3A	No	Mouse	MEF	(Shock, Thakkar et al. 2011)
DNMT3A	Yes	Mouse	NSC34 Astrocyte Microglia	(Chestnut, Chang et al. 2011)
DNMT3A	Yes	Mouse	Adult skeletal muscle, brain, spinal cord, heart, testes, spleen	(Wong, Gertz et al. 2013)
DNMT3B	No	Mouse	MEF	(Shock, Thakkar et al. 2011)
DNMT3B	Yes	Mouse	3T3-L1	(Bellizzi, D'Aquila et al. 2013)
DNMT3B	No	Mouse	Adult skeletal muscle	(Wong, Gertz et al. 2013)

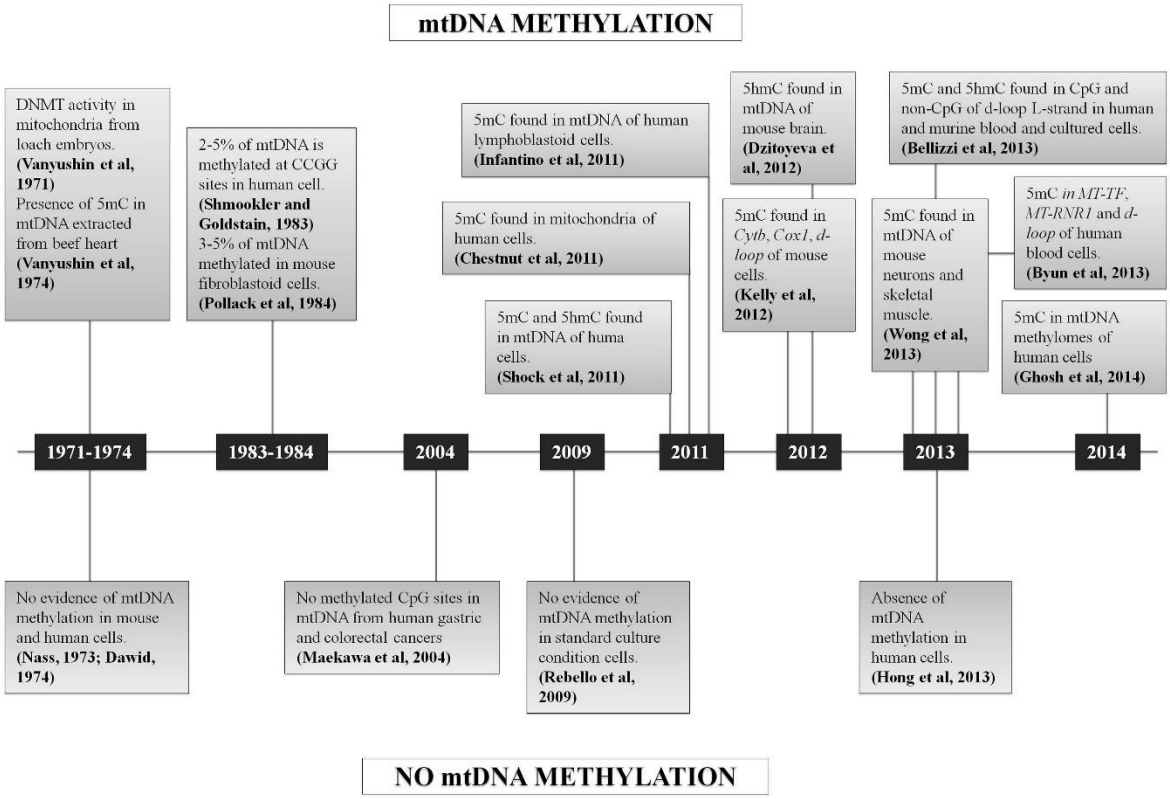

Figure 9 - Chronology sequence of mtDNA methylation reports. (Maresca, Zaffagnini et al. 2015)

CYBRID CELLS

One elegant model to study the cellular and molecular effects of mtDNA are the transmitochondrial cytoplasmic hybrid cells (cybrid cells) (Figure 10). Cybrid cells can be generated to harbor distinct mtDNA derived from patients or cell lines carrying specific mutations (Figure 10). In Table 3, there are some studies performed that used cybrid cell as a model.

King and Attardi (King and Attardi 1989) succeeded to repopulate cells with exogenous human cells mtDNA. To do that, the authors depleted parental cells from their mtDNA (p^0) which became the acceptors cells of exogenous sources to generate cybrid cells. They subjected human osteosarcoma cell line 143B.TK- to low concentrations of ethidium bromide (Etbr) for a long-term, which resulted in mtDNA elimination. Low concentration of Etbr affects mtDNA replication and result in loss of half of mtDNA at each cell division (King and Attardi 1996). As a result, these cells depended only on glycolysis and became uridine and pyruvate dependent, due to mitochondrial respiratory defects (Grégoire, Morais et al. 1984, King and Attardi 1989). Both alterations are used as selectable markers for the mtDNA depletion (and also for mtDNA repopulation). These cells showed no COX activity, confirming mtDNA elimination, since COX is a mitochondrial-encoded protein. Finally, they successfully generated cybrid cells through two different approaches: fusion of cytoplasm with p^0 cells and microinjection of a single

mitochondrion from different cell line (King and Attardi 1989). Comparing with the mtDNA donors, these cybrid cells demonstrated similar respiration capability. In fact, the transformed cells became independent of uridine and pyruvate (King and Attardi 1989).

Saffran *et al.* also described an alternative method to deplete mtDNA (Saffran, Pare et al. 2007). They report that herpes simplex virus (HSV) induces the elimination of the entire mitochondrial genome and mitochondrial mRNA trough the viral UL12 gene. This gene encodes full-length UL12 and a truncated UL12.5 proteins that have endonuclease and 5'-3' exonuclease activity (Reuven, Antoku et al. 2004). They revealed that 143B osteosarcoma cells transfected with a plasmid encoding the UL12.5 lose the entire mtDNA without causing lethal cell damage attributed to deficiencies in mitochondria respiratory chain and without causing damage to the mitochondria (Saffran, Pare et al. 2007). Saffran *et al.* were able to present a faster method to deplete mtDNA, since they observed that 70% of the colonies formed from cells with transfected with UL12.5 expression vector were devoid of mtDNA in 2 days (Saffran, Pare et al. 2007). Moreover, this method causes less side effects compared to long-term exposure of Etbr. Etbr is an intercalating agent which may cause off-target effects in nDNA after long-term exposure and can lead to suspension of cell proliferation (van Gisbergen, Voets et al. 2015, Martínez-Reyes, Diebold et al. 2016).

One of the major advantages of the cybrid cells as models is the fact that these cells share the same nuclear background, since they are generated from the same parental cell. Consequently, the mitochondrial genomes are obtained from a distinct source but inserted in the same parental cell line carrying the same (nuclear) genetic background, making it possible to analyze the different effects of mtDNA and distinguish which genome (nDNA or mtDNA) is causing the changes (King and Attardi 1989, Swerdlow 2007, van Gisbergen, Voets et al. 2015) (Figure 10). Additionally, cybrid cell lines enable the analysis of the effect of different mtDNA mutations in a cancer context with the same nDNA background, as *in vitro* (cell lines) or *in vivo* (xenograft mouse model) approaches (van Gisbergen, Voets et al. 2015).

Nevertheless, nDNA modifications present in the parental cell line could alter (or overlap) the effects created by the fused mtDNA, which can be a disadvantage of these models (Iglesias, Llobet et al. 2012). In this way, to overcome this limitation or to be confident on the observed effects, the generation of different cybrid cell lines carrying the same mtDNA source in different parental cell lines can be done.

Cytoplasmic fusion also features some disadvantages. The reintroduction of mtDNA in ρ0 cells (cells depleted of mtDNA) can also transfer unwanted micro RNAs (miRNAs), long non-coding RNA (lncRNAs), signaling proteins, or other organelles and biomolecules, making it difficult to interpret the results (Patananan, Wu et al. 2016).

So far, it is not possible generate models with exogenous mtDNA through standard transfection methods of mitochondrial genes (Zhou, Kachhap et al. 2007). A standard cloning/transfection methods would implicate alterations in amino acids or transduced proteins, since translation of the mitochondrial genome divers from nuclear genome (Zhou, Kachhap et al. 2007, Máximo, Lima et al. 2009).

Table 3 - Examples of studies that used cybrid cell lines as a model, using different parental cells and different mtDNA mutations.

Model	Mutation/ Haplogroup	Cell line	Observation	References
Prostate cancer	ATPase6 mutation (T8993G)	PC3	Increase of tumorigenicity in nude mice	(Petros, Baumann et al. 2005)
Carcinoma	ATPase6 mutation (T8993G and T9176C)	HeLa	Advantage in the early stage of tumor growth in nude mice	(Shidara, Yamagata et al. 2005)
Lewis lung carcinoma	ND6 mutation (G13997A and 13885insC)	P29 and A11	mtDNA interchanging between the two cell lines resulted in an increase of metastatic potential in P29 cell line	(Ishikawa, Takenaga et al. 2008)
Encephalopathy disease	$tRNA^{Leu(UUR)}$ mutation (A3243T)	143B	Increased motility and migration capacities Higher metastatic potential in nude mice	(Nunes, Peixoto et al. 2015)
Breast cancer	D5 Haplogroup	143B and MDA-MB-231	More susceptible to tumorigenesis	(Ma, Fu et al. 2018)
Type 1 or type 2 diabetes Autism MELAS syndrome Perinatal lethal diseases	$tRNA^{Leu(UUR)}$ mutation (A3243G)	143B	Influence of mitochondria metabolism in the epigenetic status in cells with different mtDNA mutation levels	(Kopinski, Janssen et al. 2019)
Encephalopathy disease	$tRNA^{Leu(UUR)}$ mutation (A3243T)	143B	Lower response in bystander non-irradiated cells after irradiation, using irradiated cells conditioned media from mutated mtNDA	(Miranda, Correia et al. 2020)

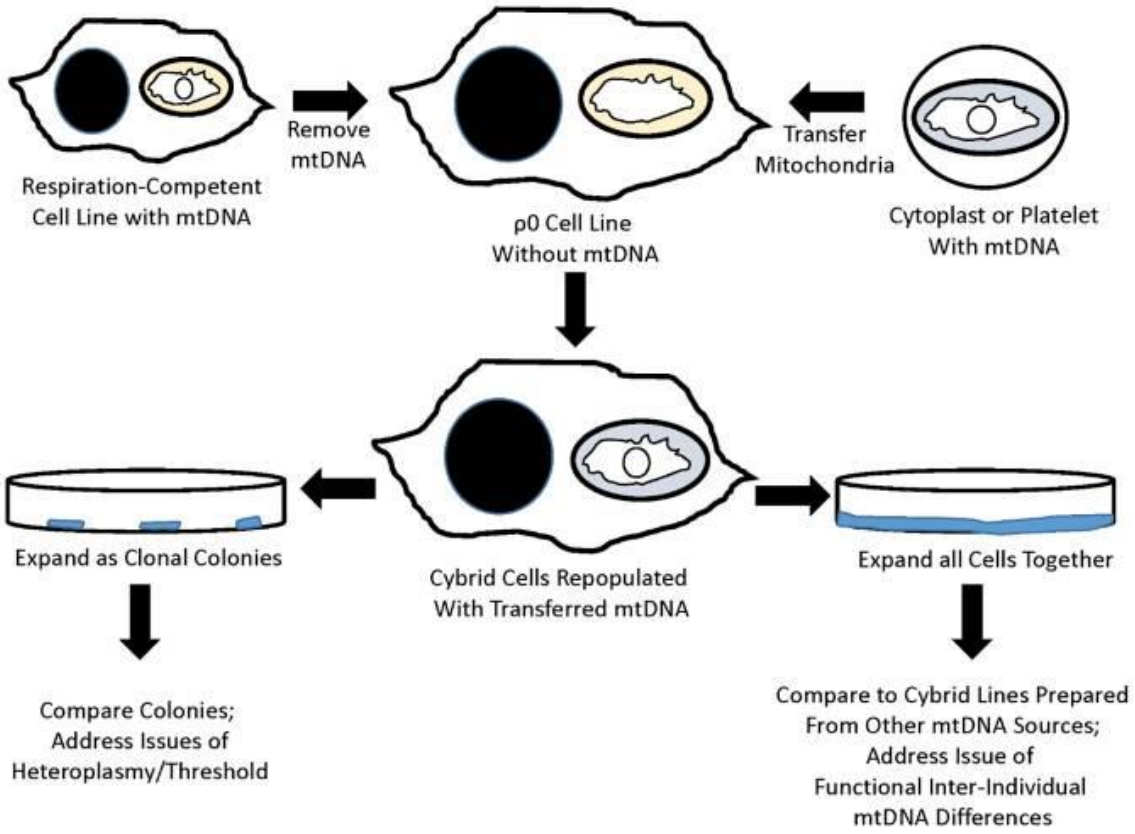

Figure 10 - Transmitochondrial cytoplasmic hybrid (cybrid) cell model. A parental cell line is subjected to depletion of mitochondrial DNA (mtDNA) generating ρ0 cell line. Cytoplasm or platelets from patients or cell lines carrying mtDNA mutation of interest (cell donor) is transfer to the ρ0 cell line, resulting in cybrid cells. (Swerdlow, Koppel et al. 2017)

Keeping in mind all the knowledge described above, between the relationship of mitochondrial function and intracellular levels of metabolites and between them and the activity of the cell chromatin-modifying enzymes, it seems rational to us to suggest, that mitochondrial activity might have an impact on the regulation of chromatin-modifying enzymes, consequently modulating cell gene transcriptional state. Thus, we hypothesize that pathogenic mutations in mtDNA (and in nDNA that encodes mitochondrial proteins) lead to modifications in the cellular epigenome and consequently in the cell transcriptome.

OBJECTIVES

It is established that epigenetic modifications are essential to gene transcription activation or repression. Epigenetic enzymes require metabolites from different pathways of mitochondria metabolism for maintenance or *de novo* formation of epigenetic marks. It is also known that mitochondrial dysfunction causes OXPHOS impairment, which affects energy and ROS production. Moreover, this dysfunction is also associated to alterations in mitochondria metabolism flux. In fact, mitochondrial dysfunction could be related to alterations in TCA cycle, one-carbon cycle, long-chain fatty acid beta-oxidation, amino acid metabolism and urea cycle. Changes in TCA flux and one-carbon cycle are associated with alteration in epigenetic enzymes activity since these cycles are crucial for production of co-factors or substrates essential for epigenetic modifications. Additionally, different mtDNA haplogroups present dissimilar metabolites concentrations, because of alterations in mitochondria function. However, a connection between the presence of mtDNA mutations and/or mtDNA variants and gene expression it was not yet completely established. Thus, and accordingly to our hypothesis "*pathogenic mutations in mtDNA (and in nDNA that encodes mitochondrial proteins) lead to modifications in the cellular epigenome and consequently in the cell transcriptome*", we aim to verify if different mtDNA mutations (and variants) could affect gene/protein expression, through regulating the activity of chromatin-modifying enzymes and epigenetic signatures.

For this work, we will use transmitochondrial cytoplasmic hybrid cells (cybrid cells), as study models. Cybrid cells are a valuable *in vitro* model to study the influence of mtDNA upon molecular and phenotypic changes since these cells use the same nuclear background. We will use 143B ρ0 (143B cells depleted of the mtDNA) cell lines and cybrid cell lines derived from the fusion of 143B ρ0 cells with mitochondria harboring distinct mutations [Ins3571C (ND1] and A3243T (MT-TL1)] and mitochondria harboring wildtype (WT) mtDNA from haplogroup H and J.

SPECIFIC AIMS

In order to verify our hypothesis, we intend to:

- Characterize each cell line, verifying the clonogenic potential and cell proliferation.
- Evaluate if different mtDNA variants alter global DNA methylation and hydroxymethylation.
- Verify specific post-translational modifications, such as histone acetylation and methylation marks.
- Analyze H3K27ac mark genome-wide distribution in cells with mtDNA mutation.
- Evaluate the expression of cell cycle regulators, specifically proteins involved in the p53 pathway.

MATERIALS AND METHODS

CELL CULTURE

The cybrid cell lines, carrying distinct WT mtDNA variants, belonging to different haplogroups, or distinct mtDNA mutations, used in this work were previously established in our laboratory using the 143B ρ^0 cell line as receptor of exogenous mtDNA as described in Table 4. This cell line was a kind gift from Dr. Keshav Singh (Roswell Park Cancer Institute, Buffalo USA). Briefly, 143B ρ^0 cell line is a mtDNA depleted cell line (ρ^0) derived from the parental osteosarcoma 143B cell line. The mtDNA depletion was achieved through the transient expression of UL12.5 Herpes simplex protein of the parental cell line, which led to the mtDNA degradation. As consequence, 143B ρ^0 cells possess mitochondria but not mtDNA. Thus, the 143B ρ^0 cell line and all the cybrid cell line derived from it have the same nuclear DNA genetic background of the 143B cell line.

Except when otherwise stated, all cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) High glucose (4.5 g/l) with Stable Glutamine and Sodium Pyruvate (Capricorn Scientific GmbH) supplemented with 10 % (v/v) inactivated fetal bovine serum (FBS) (GIBCO, Thermo Fisher Scientific), 1 % (v/v) penicillin/streptomycin (Pen/Strep) (Biowest) and 50 µg/ml uridine (Sigma Aldrich). Cells were routinely passed every three days and were maintained at 37 °C, 5 % CO2 in a humidified incubator and cultured as a monolayer.

Table 4 - Description of mtDNA depleted cell line (ρ0) and cybrid cell lines.

Cell line	mtDNA Source	Haplogroup	mtDNA alterations	Altered gene(s)
143B ρ0	-	-	-	All
Cy WT H	Enucleated XTC.UC1 oncocytic thyroid cancer ells	H	Wild Type	-
Cy CA 80%	Enucleated XTC.UC1 oncocytic thyroid cancer cells	H	Ins3571C	ND1
Cy CA 60%	Enucleated XTC.UC1 oncocytic thyroid cancer cells	H	Ins3571C	ND1
Cy MELAS	Platelets from a patient diagnosed with encephalopathy disease	H	A3243T	MT-TL1
Cy WT J	Platelets from a healthy individual	J	Wild Type	-

DNA EXTRACTION

Cell lines were plated in 6-well plates (TPP, Techno Plastic Products GmbH) at a determined density (Table 5) in order to get 60-70 % of confluence after 48 hours. After 24 hours, media was changed to ensure that there is no media exhaustion, and that all nutrients

are available. At 48 h, cells were washed with phosphate buffered saline 1x (PBS 1x) and trypsinized (Tryple Express Reagent; Thermo Fisher Scientific). After detachment, cells were collected and centrifuged (1200 rpm, 5 minutes). Total cell DNA was extracted using a commercial kit - "GRS Genomic DNA Kit - Blood & Cultured Cells" (GRISP) accordingly to the manufacturer's instructions.

DNA concentration was quantified using Nanodrop N-1000 spectrophotometer (Thermo Fisher Scientific) and its quality was obtained through the analysis of 260/280 nm and 260/230 nm ratios.

Table 5 - Number of cells of each cell line for DNA extraction, using a 6-well plate.

Cell line	Number of cells per well (6-well plate)
143B ρ0	120 000
Cy WT H	100 000
Cy CA 80%	100 000
Cy CA 60%	75 000
Cy MELAS	75 000
Cy WT J	75 000

POLYMERASE CHAIN REACTION (PCR)

Amplification of mtDNA fragements carrying the mutation location in cybrid cell lines was done by Polymerase chain reaction (PCR), using the primers described in Table 6.

For Ins3571C mutation we used the MyTaq™ HS Mix (Bioline). For the amplification, we used 50-25 ng of DNA, 0.12 µM of each Forward (FW) and Reverse (RV) primers and 1x MyTaq™ HS Mix in a 10 µL total reaction volume.

For A3243T mutation we used the GoTaq® Flexi DNA Polymerase kit (Promega). For the amplification, we used 25 ng of DNA, 0.1 µM of each Forward (FW) and Reverse (RV) primers, 1x PCR Buffer (5x GoTaqFlexi Buffer, Promega), 1.5 mM of Magnesium Chloride ($MgCl_2$) solution (Promega), 40 mM deoxyribonucleotide triphosphate (dNTPs) mix (Bioron GmbH), and 0.5 U of GoTAq DNA polymerase (Promega) in 12.5 µL total reaction volume.

Both reactions were performed in MyCycle™ termal cycler (Bio-Rad). For Ins3571C mutation, we performed a Touchdown (TD) PCR (PCR with higher specificity and sensitivity (Korbie and Mattick 2008)): initial denaturation of 2 minutes at 95 °C; 30 seconds of denaturation at 95 °C, 30 seconds for annealing at 68 °C (annealing temperature decreased by 1 °C/cycle - TD) and 30 seconds for elongation at 70 °C (10 cycles); followed by for 40 cycles of 30 seconds at 95 °C for denaturation, 30 seconds at 58 °C for annealing and 30 seconds at 72 °C

for elongation; with a final elongation of 1 minute at 72°C. For Ins3571C mutation we performed a normal PCR: initial denaturation of 5 minutes at 95 °C; 30 seconds for denaturation at 95 °C, 30 seconds for annealing at 55 °C and 30 seconds for elongation at 72 °C (40 cycles); with a final elongation of 5 minute at 72 °C.

Table 6 - Sequences of primers used for amplifications of mtDNA mutation by PCR.

Mutation	Primer Forward (FW)	Primer Reverse (RV)
ND1 (Ins3571C)	ACACCCACCCAAGAACAGGGTTT	GTAGAATGATGGCTAGGGTACT
$tRNA^{Leu(UUR}$ (A3243T)	AGCCGCTATTAAAGGTTCGTTTGTT	TGGGGCCTTTGCGTAGTTGTAT

AGAROSE GEL ELECTROPHORESIS

PCR amplification was confirmed through electrophoresis in 2 % agarose (Lonza) in 1x SGTB (Grisp) gels at 120 V. The gels were placed in a Sub-Cell GT Electrophoresis Cell (Bio-Rad) with running buffer – 1X SGTB. One kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific) were used to verify DNA fragments size. To run the gel, DNA fragments of each cell line were mixed with Gel Red (Biotium Inc) and loading buffer.

PCR products were evaluated in the ChemiDoc™ XRS Imaging system (Bio-Rad) using Quantity One – version 4.6.9 software (Bio-Rad).

SANGER SEQUENCING

After PCR validation, PCR products were incubated with 1 U/µl exonuclease I (Fermentas, Thermo Fisher Scientific) and 0.05 U/µl shrimp alkaline phosphatase (Fermentas, Thermo Fisher Scientific) at 37 °C for 20 minutes for purification. Then, these enzymes were inactivated at 80 °C for 15 minutes.

Purified PCR products were sequenced using 0.5 µL of Big Dye and 3.5 µL of 5x Sequence buffer (ABI Prism BigDye Terminator Kit (Perkin-Elmer)) with 0.3 µl of the correspondent primer 10 mM, 2.7 µL DNase and RNase free water and 3 µL of the purified PCR product in a total reaction volume 10 µl. The mix was amplified using MyCycler™ thermal cycle (Bio-Rad): initial denaturation of 10 seconds at 94 °C; denaturation of 10 seconds at 94 °C, annealing of 30 seconds at 56 °C and elongation of 2 minutes at 60 °C (35 cycles); with a final elongation step of 10 minutes at 60 °C. The sequencing products were purified by using Sephadex columns (Sephadex™ G-50 Fine, GE Healthcare) - accordingly to the manufacturer's instructions. After purification, 15 µl of formaldehyde was added in each sample and analyzed by an automated sequencer - ABI prism 3100 Genetic Analyzer (Perkin-Elmer).

VIABILITY ASSAY – PRESTO BLUE

Each cell line was plated at an initial density of 1000 cells per well in distinct 96 well plates for each time point: 24 h, 48 h, 72 h and 96 h. 8 wells were plated for each cell line to consider 4 replicates to each condition. As a negative control, media without cells was added to additional 8 wells.

Half of the 8 wells of each cell line were incubated with DMEM High glucose (4.5 g/l) with Stable Glutamine supplemented with 10 % (v/v) inactivated fetal bovine serum (FBS) (GIBCO, Thermo Fisher Scientific), 1 % (v/v) penicillin/streptomycin (Pen/Strep) (Biowest), 10 mM sodium pyruvate (Capricorn Scientific GmbH) and 50 µg/ml uridine (Sigma Aldrich). The remaining 4 wells were incubated with similar media without sodium pyruvate and uridine supplementation. Additionally, the negative control wells were incubated with the respective media (4 wells with media supplemented and the other 4 without sodium pyruvate and uridine supplementation) without cells. The negative control was used to remove the background fluorescence.

At each time point, the respective plate was gently washed with plain media (DMEM High Glucose (4.5 g/l), with L-Glutamine) and incubated with 10 % PrestoBlue® (PB) Reagent (Invitrogen, Thermo Fisher Scientific) diluted in complete media for 45 minutes.

Afterwards, fluorescence was quantified in a microplate reader - Synergy™ 4 Multi-Mode Microplate Reader (BioTeck® - Instruments Inc.) at 560-590 nm. Viable cells reduce the resazurin-based solution into a red color that can be measured fluorometrically. Differences in fluorescence allow to indirectly measure cell proliferation.

COLONY FORMATION ASSAY (CFA)

Each cell line was plated at an initial density of 100 cells per well in a 6-well plate (TPP Techno Plastic Products GmbH) for the period of 11 days in order to get cell colonies. Cell colonies were washed with PBS 1x and were incubated for 1 hour with 100 % methanol at -20 °C. Methanol were discarded and cell colonies were left to dry at room temperature (RT) for 3 hours to overnight. Cell colonies were marked by 0.1 % Sulforhodamine B during 30 minutes on RT. Sulforhodamine B were discarded, and cell colonies were washed with 1 % glacial acetic acid to clear unbound dye. Cell plates were incubated at RT and cell colonies and colony areas were counted in order to analyze the proliferation capability clonogenic potential and proliferation capability of all cybrid cell lines, respectively.

EVALUATION OF GLOBAL DNA METHYLATION STATUS

Global DNA methylation levels were evaluated through the commercially available Methylated DNA Quantification Kit (Fluorometric) (ab117129 – Abcam) accordingly to the manufacturer's instructions. This assay harbors a 96-well plate with high DNA affinity which enables DNA binding and one capture and one detection antibodies which attach to the methylated portion of DNA.

Methylated DNA were quantified by reading the relative fluorescence units (RFU) using a fluorescence spectrophotometer - Synergy™ 4 Multi-Mode Microplate Reader (BioTeck® - Instruments Inc.) - at Ex/Em= 530/590 nm.

EVALUATION OF GLOBAL DNA HYDROXYMETHYLATION STATUS

Global DNA hydroxymethylation levels were quantified through the commercially available Hydroxymethylated DNA Quantification Kit (Fluorometric) (ab117131 – Abcam) accordingly to the manufacturer's instructions. This assay harbors a 96-well plate with high DNA affinity which allows DNA binding and one capture and one detection antibodies which attach to the hydroxymethylated section of DNA.

Hydroxymethylated DNA were quantified by reading the RFU using a fluorescence spectrophotometer - Synergy™ 4 Multi-Mode Microplate Reader (BioTeck® - Instruments Inc) - at Ex/Em= 530/590 nm.

TOTAL CELL PROTEIN EXTRACTION

Cybrid cell lines were plated in 6-well plates (TPP, Techno Plastic Products GmbH) at a determined density (Table 7) in order to get 60-70 % of confluence after 48 hours. The cells were washed three times with PBS 1x. For cell lysis, a lysis solution composed by RIPA buffer (50 mM Tris-HCl, 1 % NP-40, 150 mM NaCl and 2 mM EDTA, pH 7.5), supplemented with phosphatase (Sigma-Aldrich) and protease inhibitors (Roche Applied Science). Cells were incubated with the lysis solution on ice for 15 minutes. Later, wells were scraped, and the lysate was collected. Each lysate was sonicated using Bioruptor Plus (Diagenode) and cell debris were pelleted by centrifugation (16000 xg, 15 minutes at 4 °C). The supernatant containing the total cell protein was collected to a new tube and stored at -20 °C. The protein amount of each condition was quantified by the DC™ Protein Assay (Bio-Rad), using Bovine Serum Albumin (BSA) (AppliChem) as protein concentration standard (250, 500, 750, 1000, 1500 and 3000 µg/mL). Absorbance was acquired in a microplate reader - Synergy™ 4 Multi-Mode Microplate Reader (BioTeck® - Instruments Inc.) at 655 nm. The protein amount was determined by the comparison of the

respective absorbance compared to a linear trend line obtained from the protein concentration standard controls.

Table 7 - Number of cells of each cell line for total cell protein extraction, using a 6-well plate.

Cell line	Number of cells per well (6-well plate)
143B ρ0	120 000
Cy WT H	100 000
Cy CA 80%	100 000
Cy CA 60%	75 000
Cy MELAS	75 000
Cy WT J	75 000

HISTONE EXTRACTION

Cybrid cell lines were plated in 55 cm^2 dishes (TPP, Techno Plastic Products GmbH) at a determined density (Table 8) in order to get 60-70 % of confluence after 48 hours. After 24 hours, media was changed to ensure that there is no media exhaustion, and all nutrients are available. After 48 h, cells were washed with PBS 1x, trypsinized and pelleted (1200 rpm, 5 minutes). Cells were washed with PBS 1x and pelleted once again (1200 rpm, 5 minutes). Cell pellets were re-suspended with hypotonic lysis buffer (10 mM Tris-HCl (pH=8), 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM 1,4-dithiothreitol (DTT), 0.011 mM Sodium Butyrate, 4 % protease inhibitor and 1 % phosphatase inhibitor), transferred to a 1.5 mL tube and incubated for 1 – 1.5 hours at 4 °C in a rotator to promote cell lysis. After, a centrifugation was carried (10000 xg, 10 minutes, 4 °C) in order to get cells nuclei (pellet). The pellet was re-suspended with 0.4 N sulfuric acid (H_2SO_4) (Sigma Aldrich) and incubated at 4 °C in a rotator overnight. This incubation allowed the splitting histones from the DNA. The samples were centrifuged (16 000 xg, 10 minutes, 4°C) and supernatant containing the histones was transferred to a new 1.5 mL tube. Histone precipitation was achieved by adding, drop by drop trichloroacetic acid (Sigma Aldrich) to a final concentration of 33 %. The samples were incubated on ice for at least 30 minutes until the samples appear milky. After a new centrifugation (16000 xg, 10 minutes, 4 °C), the pellets containing histones were washed twice with ice-cold acetone, to remove the remaining acid solutions without dissolving the protein pellets and centrifuged (16000 xg, 5 minutes, 4 °C). Later, the histone pellets were air-dried for 20 minutes at RT, re-suspended in ddH_2O. Histones were then transferred to a new 1.5 ml tube and stored at -20 °C. The histones amount of each condition was quantified by the DC™ Protein Assay (Bio-Rad), using Bovine Serum Albumin (BSA) (AppliChem) as protein concentration standard (250, 500, 750, 1000, 1500 and 3000 µg/mL).

Absorbance was acquired in a microplate reader - Synergy™ 4 Multi-Mode Microplate Reader (BioTeck® - Instruments Inc.) at 655 nm.

Table 8 - Number of cells of each cell line histone extraction, using a 55 cm^2 dishes.

Cell line	Number of cells per dish (55 cm^2 dishes)
143B ρ0	725 000
Cy WT H	580 000
Cy CA 80%	580 000
Cy CA 60%	435 000
Cy MELAS	435 000
Cy WT J	435 000

WESTERN BLOT

After protein quantification, 20/30 µg of total protein per sample and 2.5/5 µg of histones were used. Before loading Western blot gels, proteins were denatured with loading buffer (90 % laemmli 4x (Bio-Rad), 5 % β-mercaptoethanol (Sigma) and 5 % of bromothymol blue (Sigma)) at 95 °C for 5 minutes. Proteins were initially run in the stacking gel 4 % (v/v) bisacrylamide 29:1 (Bio-Rad) sodium dodecyl sulphate (SDS)-PAGE at 80 V during 30-45 minutes (until proteins reach the resolving gel part). Then, proteins were resolved in the 15 % (v/v) bisacrylamide 29:1 (Bio-Rad) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V during 1 h – 1 h 30 m. Precision Plus Protein™ Dual Color Standards (Bio-Rad) was also used to verify the proteins weight. The proteins were transferred using iBlot 2 Dry Blotting System (Invitrogen, Thermo Fisher Scientific) from the SDS-PAGE gel onto nitrocellulose (NC) membranes (GE Healthcare) using the recommended method P0, accordingly to the manufacturer protocol. The effectiveness of the transference was verified by staining of Ponceau S dye (Sigma-Aldrich) in the NC membranes. After transfer validation, the NC membranes were blocked using PBS 1x containing 0.1 % (v/v) OmniPur® Polyoxyethylene (20) Sorbitan Monooleate (Calbiochem) (PBS-T) and 5 % (w/v) low-fat dry milk at RT for 1 h. The blocked membranes were incubated with primary antibody of interest as shown in Table 9. Then, membranes were washed with PBS-T (five times, 5 minutes) and were incubated with anti-mouse (GE Healthcare) or anti-rabbit (Cell Signaling) horseradish peroxidase (HRP) secondary antibody diluted 1:3000 in PBS-T at RT for 1 h. After secondary antibody, membranes were washed with PBS-T (five times, 5 minutes) and chemiluminescence detection were obtained using Western Lightning Plus-Enhanced Chemiluminescence Substrate (ECL) (PerkinElmer) and Amersham Hyperfilm ECL (GE Healthcare).

Proteins expression was quantified using Image Lab Software (Bio-Rad) and normalized to the related loading control (Actin or H3 Total). Acetyl-p53 was also normalized to total p53.

Table 9 - Primary antibodies used for western-blot staining.

Protein	Antibody name	Company	Dilution	Incubation	Animal origin	Molecular Weight (kDa)
H3K9ac	Acetyl-Histone H3 (Lys9) (C5B11)	Cell Signaling	1:1000 Bovine serum albumin 5 %	4 °C, ON	Rabbit	17
H3K14ac	Acetyl-Histone H3 (Lys14) (D4B9)	Cell Signaling	1:1000 Bovine serum albumin 5 %	4 °C, ON	Rabbit	17
H3K18ac	Acetyl-Histone H3 (Lys18)	Cell Signaling	1:1000 Bovine serum albumin 5 %	4 °C, ON	Rabbit	17
H3K27ac	Acetyl-Histone H3 (Lys27) (D5E4) XP®	Cell Signaling	1:1000 Bovine serum albumin 5 %	4 °C, ON	Rabbit	17
H3K56ac	Acetyl-Histone H3 (Lys56)	Cell Signaling	1:1000 Bovine serum albumin 5 %	4 °C, ON	Rabbit	17
H3K9me3	Tri-Methyl-Histone H3 (Lys9) (D4W1U)	Cell Signaling	1:1000 Bovine serum albumin 5 %	4 °C, ON	Rabbit	17
H3K27me3	Tri-Methyl-Histone H3 (Lys27) (C36B11)	Cell Signaling	1:1000 Bovine serum albumin 5 %	4 °C, ON	Rabbit	17
H3K79me3	Tri-Methyl-Histone H3 (Lys79)	Cell Signaling	1:1000 Bovine serum albumin 5 %	4 °C, ON	Rabbit	17
Total H3	Histone H3 (D1H2) XP®	Cell Signaling	1:2000 Low fat milk 5 %	4 °C, ON	Rabbit	17
Acetyl p53	Anti-acetyl-p53 Antibody (Lys373, Lys382)	Sigma-Aldrich	1:750 Low fat milk 5 %	4 °C, ON	Rabbit	53
p53	NCL-L-p53-DO7	Leica	1:3000 Low fat milk 5 %	4 °C, ON	Mouse	53
p27	p27 Kip1 (D69C12) XP®	Cell Signaling	1:1000 Bovine serum albumin 5 %	4 °C, ON	Rabbit	27
COX II	Anti-MTCO2 antibody [12C4F12]	Abcam	1:1000 Low fat milk 5 %	4 °C, ON	Mouse	26
p21	p21 Waf1/Cip1 (12D1)	Cell Signaling	1:1000 Bovine serum albumin 5 %	4 °C, ON	Rabbit	21
Actin	beta-Actin Antibody (AC-15)	Novus Biologicals	1:3000 Low fat milk 5 %	4 °C, ON	Mouse	42

CHROMATIN IMMUNOPRECIPITATION FOLLOWED BY NEXT GENERATION SEQUENCING (CHIP-SEQ)

CELL CROSSLINKING AND LYSIS

Cy WT H and Cy CA 80% cell lines were washed with PBS 1x, trypsinized and pelleted (1200 rpm, 5 minutes). Cells were washed with ice-cold PBS 1x and crosslinked with 2 % of paraformaldehyde in PBS at RT for 10 minutes on a rotating wheel. Then, PFA was quenched using 0.125 M of Glycine at RT for 5 minutes on a rotating wheel. Crosslinked cells were pelleted (300 xg, 5 minutes, RT) and washed with ice-cold PBS 1x. For the following steps, lysis buffer (both cell and nuclei) and IP dilution buffer were freshly supplemented with protease inhibitors cocktail (complete, EDTA free tablets, Roche). Pellet cells were gently resuspended in cell lysis

buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 0.3 % NP-40) (1 mL per $3x10^6$ cells) and incubated on ice for 5 minutes. Cells were centrifuged (1200 xg, 5 minutes, 4 °C), pellets were gently resuspended in 400 µL nuclear lysis buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0 and 1 % SDS) (500 µL per 2 $x10^7$ cells) and incubated on ice for 10 minutes. After nuclear lysis, 800 µL of dilution Buffer (16.7 mM Tris-HCl pH 8.0, 1.2 mM EDTA pH 8.0, 167 mM NaCl, 0.01 % SDS and 1 % Triton X-100) were added. 5 % of total chromatin was removed as non-sonicated control, while the rest of the samples were processed for sonication.

CHROMATIN SONICATION AND REVERSE CROSSLINKING

Chromatin were sonicated using Bioruptor Plus (Diagenode) at HIGH settings, for 15 cycles of 30 seconds ON and 30 seconds OFF. After sonication, samples were centrifuged (16000 xg, 10 minutes at 4 °C) and the supernatant was transferred to a new tube. 5 % of sonicated chromatin was removed to validate the chromatin shearing. For this evaluation, the two 5 % aliquots (before and after sonication) were diluted with 10 mM TE until 50 µl total volume and were incubated with 1 µl of RNase A (10 mg/ml) at 37 °C for 30 minutes. After RNase treatment, samples underwent reverse crosslinking by incubating with 0,3 M NaCl and 2 µl proteinase K 10 mg/ml at 65 °C, shaking at 900 rpm, overnight. The following day, samples were purified using column-based DNA purification kit - NZYGelPure kit (NZY) – accordingly to the manufacturer's instruction.

Chromatin fragments size were evaluated in an agarose gel electrophoresis in a 2 % agarose gel, using GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) and NZYDNA Ladder VI (NZYtech) as ladders. DNA fragments size should be around 300 bp to be considered an efficient sonication.

From the sonicated sample, 1 % aliquot was stored as input control for Real-Time PCR (qPCR) at -20 °C. Of the rest of sonicated chromatin, 400 µl were processed for immunoprecipitation by overnight incubation with 1.5 ug of antibody for H3K27ac (ab4729 - Abcam) on a rotating wheel at 4 °C.

CHROMATIN IMMUNOPRECIPITATION (CHIP)

The following day, 20 µl of protein G Dynabeads for each IP (Invitrogen, Thermo Fisher Scientific) were twice washed with dilution buffer; buffer was removed using a DynaMag - Spin Magnet (Invitrogen, Thermo Fisher Scientific). After washing steps, Dynabeads were resuspended in 50 µl of dilution buffer and transferred to the chromatin-antibody samples and incubated for 1 – 1.5 hours on a rotating wheel at 4 °C. IP samples with beads were washed twice with wash buffer 1 (20 mN Tris-HCl pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.1 % SDS and 1 %

Triton X-100), wash buffer 2 (20 mN Tris-HCl pH 7.5, 2 mM EDTA, 500 mM NaCl, 0.1 % SDS and 1 % Triton X-100) and followed by wash buffer 3 (10 mN Tris-HCl pH 7.5, 1 mM EDTA, 250 mM LiCl, 1 % NP-40 and 1 % Na-deoxycholate), each step for 5 minutes. To remove each washing buffer, samples were placed in the magnetic rack for 1 minute, then buffer was removed; after that samples were removed from the magnet, new buffer was added, and samples were again placed on the rotating wheel. Then, samples were washed with 10 mM Tris-HCl pH 8.0 for 5 minutes, resuspended with the same solution and transfer to a new 1.5 ml tube in order to reduce tagmentation of unspecific fragments. Beads-bound chromatin samples were incubated with 50 µl VDR Tagmentation Buffer (50 mM TAPS NaOH, 25 mM $MgCl_2$, 50 % DMF and 43,5 µl of nuclease-free H_2O) and 2 µl of Tn5 transposase for 10 minutes at 37 °C. After incubation, samples were placed in the magnetic rack to remove and therefore block the transposase reaction, and subsequently washed 5 minutes twice with wash buffer 1, followed by twice with TE. During the last washes, 10 mM TE was added until total volume reaches 50 µl in the input, followed by treatment with 1 µl RNase for 30 minutes at 37 °C. Beads-bound chromatin samples were incubated twice with 150 µl of elution buffer (50 mM $NaHCO_3$ pH 8.8 and SDS 1 %) for 15 minutes on a rotating wheel at RT. After each incubation step, tubes were placed in the magnetic rack and the supernatant was collected and transferred to a new tube. During this time, elution buffer is also added to the inputs control. Afterwards, IP samples and inputs were incubated with 0,3 M NaCl and 2 µl proteinase K 10 mg/ml at 65 °C and with agitation (900 rpm) overnight. DNA fragments were purified using MinElute PCR Purification Kit (Qiagen) accordingly to manufacturer's instruction with minor modifications. Instead of pH indicator, we used 10 µl of NaAc 3 M pH 5.2.

H3K27AC ENRICHMENT BY REAL-TIME POLYMERASE CHAIN REACTION (QPCR)

qPCR reactions were performed using the primers for forkhead box P3 (FOXP3) and actin (housekeeping gene).

For the reaction we used we used 1 µl of ChIP material or input control, 0.5 µM of each Forward (FW) and Reverse (RV) primers and 2x iTaq Universal SYBR Green Suoermix (Bio-Rad) in 10 µL final reaction volume. qPCR reactions were performed in CFX96 Touch Real-Time PCR Detection System (Bio-Rad): initial denaturation of 30 seconds at 95 °C; 5 seconds for denaturation at 95 °C and 30 seconds at 60 °C for annealing/elongation (40 cycles) followed by standard melting curve step.

Data were analyzed by the ΔCt method, comparing the quantity of immunoprecipitated material relative to the initial amount of chromatin (input) at the selected promoter genes.

CHROMATIN QUANTIFICATION AND LIBRARY GENERATION

To quantify the IP chromatin, we used 1 µl of fragment DNA, 0.25 µM of each Forward (FW) and Reverse (RV) Illumina primers, 1x SYBR Green I (Thermo Fisher Scientific) and 5 µl of KAPA HiFi HotStart ReadyMix (Roche) in 10 µL final volume. qPCR reactions were performed in CFX96 Touch Real-Time PCR Detection System (Bio-Rad): HotStart activation of 45 seconds at 95 °C; pre-qPCR hold of 5 minutes at 72 °C; initial denaturation of 30 seconds at 98 °C; 10 seconds for denaturation at 98 °C, 30 seconds at 63 °C for annealing and 30 seconds at 72 °C for elongation (30 cycles) followed by final elongation at 72 °C for 1 minute.

The qPCR reaction was analyzed by CFX manager software (Bio-Rad) to retrieve the *Ct* value (cycle quantification). Number of cycles for library generation were performed as described by Schmidl *et al.* – N cycles + 1 (N= equal to the rounded-up *Ct* value verified in the previous qPCR reaction (Schmidl, Rendeiro et al. 2015). The library generation was performed by using the rest of the IP samples (18 µl), 25 µl of KAPA HiFi HotStart ReadyMix, 1 µM of each Forward (FW) and Reverse (RV) Illumina primers and complete with 5 µl of nuclease-free water to reach 50 µl final volume. PCR will be performed in a thermocycler with the following protocol: 45 seconds at 95 °C; pre-qPCR hold of 5 minutes at 72 °C; initial denaturation of 30 seconds at 98 °C; 10 seconds for denaturation at 98 °C, 30 seconds at 63 °C for annealing and 30 seconds at 72 °C for elongation for the calculated number of cycles. PCR reactions were purified by column-based DNA purification kit (QIAquick PCR purification kit – Qiagen) according to manufacturer's instructions. Libraries were eluted in 20 µl of elution buffer of the kit.

Evaluation of libraries size were performed using 2200 TapeStation (Agilent).

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism version 7.04 (GraphPad Software, Prism). Results were analyzed using Two-way ANOVA for viability test – presto blue with posterior Bonferroni post-test, and T-Test (comparison between two samples). P value ≤0.05 were considered statistically significant.

RESULTS

MODEL VALIDATION

In our study, we used cybrid cell lines that were previously established in our laboratory. Cybrids were derived from the parental cell line 143B ρ0 (osteosarcoma cell line with mtDNA depletion) in which exogenous mtDNA was inserted.

Firstly, we validated the presence of the mtDNA mutations in each cybrid cell line. As shown in Figures 11A and 12A, the control cybrid carries no mtDNA mutations in ND1 or $tRNA^{Leu\ (URR)}$ mtDNA genes.

Regarding the ND1 gene, we validated the presence of Ins3571C mutation in Cy CA 80% and Cy CA 60% cell lines (Figure 11). Additionaly, we observed that both cybrid cell lines harbor more than 50% of mutation (Figure 11B and 11C). Although we cannot quantify the mutational loading by Sanger sequencing, the size of the curves in the mutation sequence site indicate that Cy CA 80% has higher mutation levels than Cy CA 60% (Figure 11B and 11C). These results are compatible with previous results achieved in our laboratory, which showed that Cy CA 80% and Cy CA 60% harbor 80% and 60% of Ins3571C mutation, respectively. MtDNA of Cy MELAS was also sequenced for the ND1 gene and no mutation was found, as expected (data not shown).

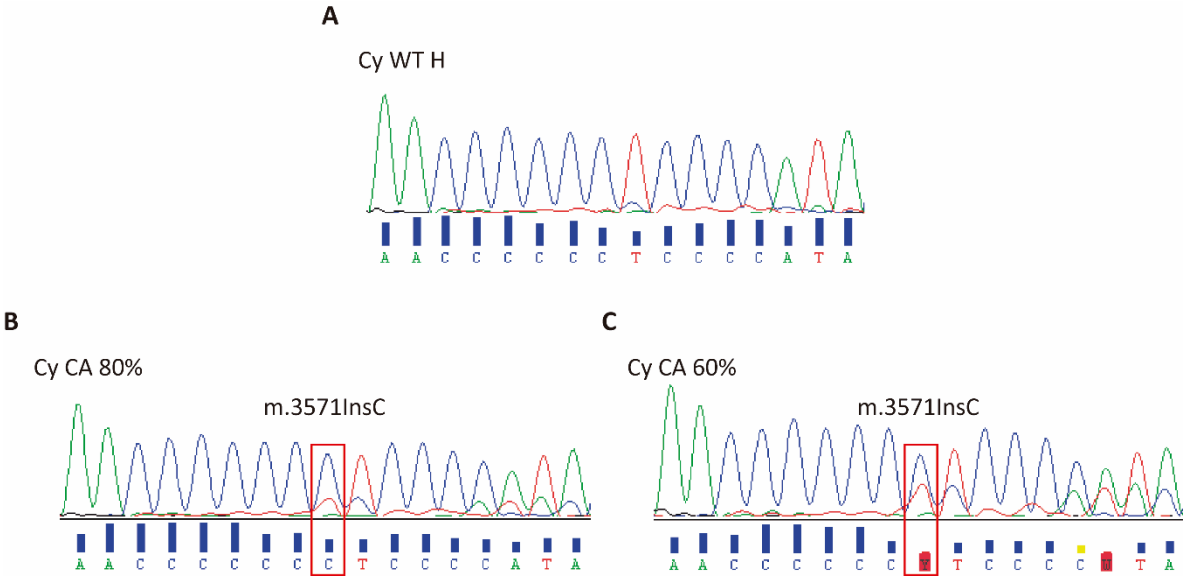

Figure 11 - Sequencing chromatograms of mtDNA ND1 gene with a cytosine insertion at the nucleotide 3571, representing the Cy CA 80% and Cy CA 60% cell lines. **A –** Sequencing chromatogram of Cy WT H cell line, which represent the normal sequence of mtDNA ND1 gene (Red line). **B –** Sequencing chromatogram of Cy CA 80% cell line, exhibiting the cytosine insertion in the 3571 position (Blue line), confirming the mutation of mtDNA ND1 gene. Cy CA 80% present higher heteroplasmy that 50%. **C –** Sequencing chromatogram of Cy CA 60% cell line, showing the cytosine insertion in the 3571 position (Blue line), confirming the mutation of mtDNA ND1 gene. Cy CA 60% present higher heteroplasmy that 50%.

We also confimed the presence of the A3243T mutation in the $tRNA^{Leu\ (URR)}$ gene in the Cy MELAS cell line (Figure 12). This cell line demonstrated a mutation heteroplasmy higher than

50% (Figure 12B). It is in acordance to previous results from our laboratory, in which there was seen that Cy MELAS cell line exhibit aproximally 60% of heteroplasmy.

MtDNA of Cy CA 80% and Cy CA 60% was also sequenced for the tRNA$^{Leu\ (URR)}$ gene and no mutations were found (data not shown).

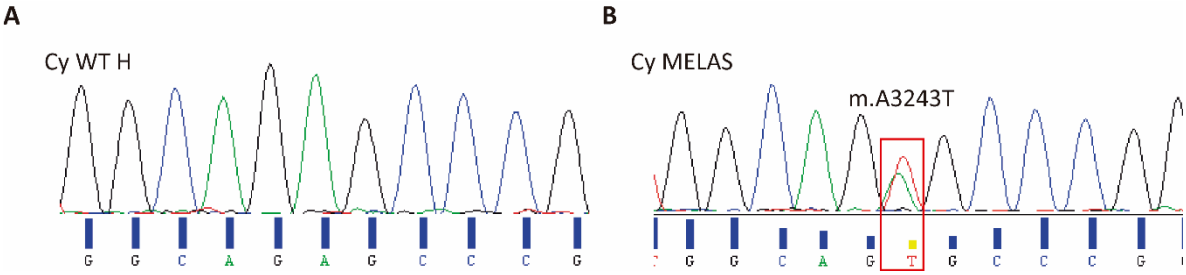

Figure 12 - Sequencing chromatograms of mtDNA tRNALeu (URR) gene with a A>G mutation in the 3243 position, representing the Cy MELAS cell line. **A –** Sequencing chromatogram of Cy WT H cell line, which represent the normal sequence of mtDNA tRNA$^{Leu\ (URR)}$ gene (Green line). **B –** Sequencing chromatogram of Cy MELAs cell line, exhibiting the A>G mutation in the 3243 position (Red line), confirming the mutation of mtDNA tRNALeu (URR) gene. Cy MELAS present higher heteroplasmy that 50%.

In the case of 143B ρ0 cell line, the validation was not possible using sequencing chromatograms, since when we attempt to sequence we obtain a chromatogram with several alterations in all sequence. It can be result of the presence of nuclear mitochondrial pseudogenes (NUMTS), which can explain the amplification (Parr, Maki et al. 2006) (data not shown). Thus, the end-up validation for the absence of mtDNA can be the analysis of COX II expression, a protein that is enconded by mtDNA. As expected, 143B ρ0 cell line reveled no COX II expression, by Western blot, indicating that 143B ρ0 cells does not express this mtDNA-encoded protein (Figure 13).

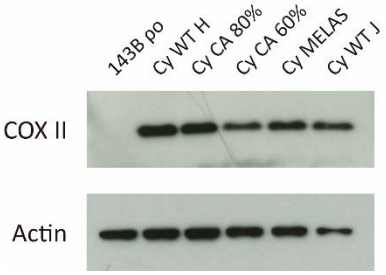

Figure 13 - Representation of western blot detection of cytochrome oxidase subunit 2 (COXII) and Actin, as the loading control, for the cell lines 143B ρ0, Cy WT H, Cy CA 80%, Cy CA 60%, Cy MELAS and Cy WT J, revealing no COX II expression in 143B ρ0 cell line.

Furthermore, ρ0 are known to be uridin and sodium pyruvate dependent to grow in culture. Thus, and as an alternative method of validation of the absence of mtDNA in this cell line, we performed cell viability assay of all cell lines growing in media with and without uridine and sodium piruvate. 143B ρ0 cell line demonstrated no cellular growth when incubated with media without uridine and sodium pyruvate supplementation (Figure 14, red arrow). This result

demonstrates that 143B ρ0 cell line is dependent of uridine and sodium pyruvate (Figure 14), as expected for mtDNA depleted cells (Grégoire, Morais et al. 1984, King and Attardi 1989). Additionally, Cy CA 60% cell line also exhibited significantly lower cell proliferation after 96h when incubated in media without supplementation [Two-away ANOVA p=0.0221] (Figure 14). Cy WT H, Cy CA 80%, Cy MELAS and Cy WT J cell lines demonstrated no statistically differences in media without uridine and sodium pyruvate supplementation (Figure 14). For a detailed analysis of each cell line, see Suplementary Figure 1.

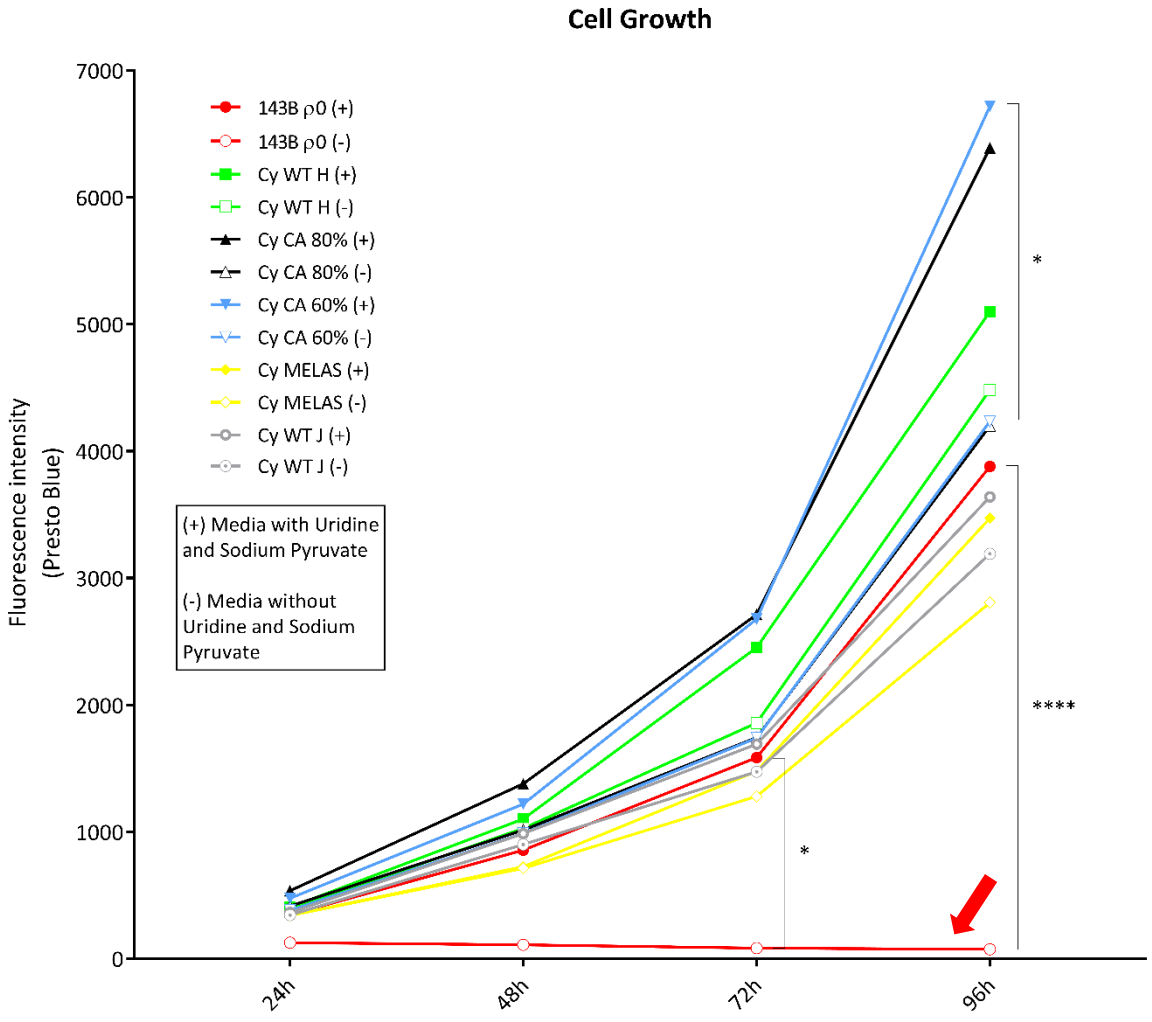

Figure 14 - Quantification of cell growth by presto blue assay of 143B ρ0, Cy WT H, Cy CA 80%, Cy CA 60%, Cy MELAS and Cy WT J cell lines in media with uridine and sodium pyruvate (+) and without supplementation (-). 143B ρ0 cell line showed significant lower cell growth after 72h and 96h in media without supplementation, compering to media with uridine and sodium pyruvate (Two-way ANOVA *p=0.0468, ****p<0.0001, respectively). Cy CA 60% demonstrated significant lower cell growth after 96h between the media (Two-way ANOVA *p=0.0221). No significant differences were found in the Cy WT H, Cy CA 80%, Cy MELAS and Cy WT J cell lines. The results are from three independent experiments (N=3). Results correspond to mean. Note: Individual graphs of each cell line with the mean and standard deviation are represented in Supplementary Figure 1.

CLONOGENIC POTENTIAL AND CELL PROLIFERATION

In order to characterize each cell line, we decided to evaluate the capacity of our cell lines grow individually and form colonies when plated at very low density through CFA assay. We determined the number and area of colonies formed for each cell line, which can characterize the single cell clonogenic potential and cell proliferation, respectively (Figure 15)

Regarding the number of colonies formed, 143B ρ0 showed significant lower clonogenic capability compared with Cy WT H cell line (control) [T-test p=0.0042] (Figure 15B). In contrast, Cy WT J cell lines showed increased clonogenic potential [T-test p=0.0149] (Figure 15B). No statistical differences were observed in Cy CA 80%, Cy CA 60% and Cy MELAS cell lines, compared with Cy WT H (Figure 15B)

Furthermore, 143B ρ0, Cy CA 80% and Cy CA 60% cell lines exhibited lower colony area compared with Cy WT H [T-test p<0.0001, p=0.0260 and p=<0.0001, respectively] (Figure 15C). In particular, 143B ρ0 and Cy CA 60% cell lines demonstrated lowest colony area (Figure 15C). Cy MELAs and CY WT J showed similar colony area compared to Cy WT H (Figure 15C).

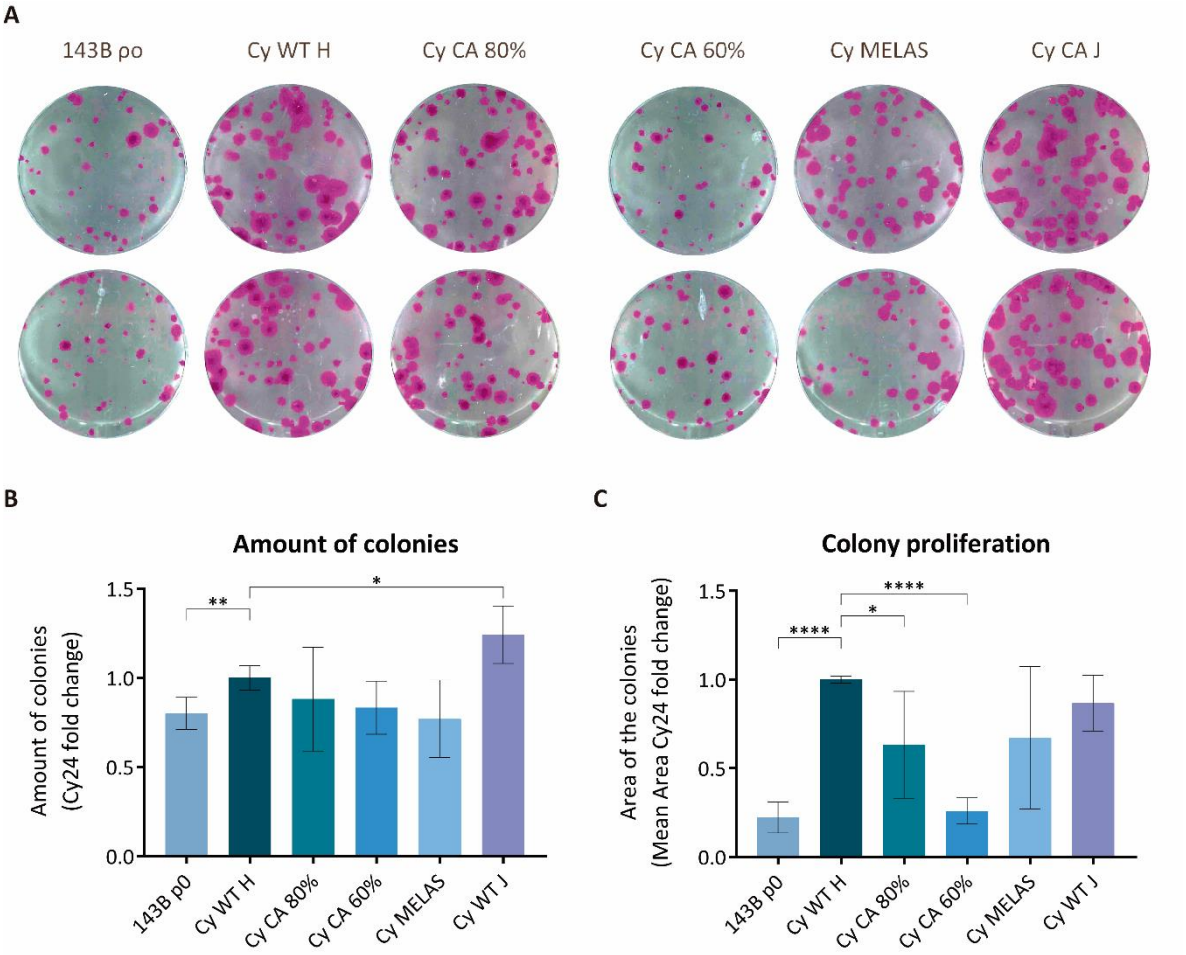

Figure 15 - Colony formation assay of 143B ρ0, Cy WT H, Cy CA 80%, Cy CA 60%, Cy MELAS and Cy WT J cell lines, which characterize clonogenic potential and cell proliferation. **A –** Representation of colony formation assay results of all cell lines after 11 days. **B –** Quantification of number of colonies formed by all cell lines from the colony formation assay after 11 day. The results were normalized to Cy WT H (control). Compared to Cy WT H, 143B ρ0 showed lower clonogenic potential (T-test **p=0.0042), whereas Cy WT J exhibited higher clonogenic potential (T-test *p=0.0149). Cy CA 80%, Cy CA 60% and Cy MELAS did not show any significant differences, compared to the control. The results are from three independent experiments. **C –** Quantification of area of the colonies formed after 11 days, which represent cell proliferation. The results were normalized to Cy WT H (control). Compared with Cy WT H, 143B ρ0, Cy CA 80% and Cy CA 60% demonstrated lower cell proliferation (T-test ****p<0.0001, *p=0.0260 and ****p=<0.0001, respectively), in particular 143B ρ0 and Cy CA 60% cell lines. No significant differences were found to Cy MELAS and Cy WT J, compared to Cy WT H. The results are from three independent experiments. Bars correspond to mean ± standard deviation.

GLOBAL DNA METHYLATION AND HYDROXYMETHYLATION

To detemrine if the presence of specific mtDNA mutations could affect the global levels of 5mC and 5hmC, we used commercially avalilable kits to evaluate the total cell DNA methylation and hydroxymethylation levels (Figure 16).

After determination of the total DNA methylation in our cell lines, we verified that 143B ρ0, Cy CA 80% and Cy CA 60% cell lines exhibited lower DNA methylation compared to the Cy Wt H, even though with no statistically significant differences (Figure 16A). In particular, Cy CA 80% cell line showed the lowest global DNA methylation (Figure 16A). Adittionaly, Cy MELAS and Cy WT J showed higher DNA methylation compared with CY WT H. From those, CY WT J

demonstrated higher DNA methylation, but no statistically significant difirences were found (Figure 16A).

In case of global DNA hydroxymethylation, we oberved lower DNA hydroxymethylation levels in 143 ρ0 and Cy MELAS cell lines compared to Cy WT H (Figure 16B). In particular, 143 ρ0 demonstrated the lowest global DNA hydroxymethylation, however with no statistical significant differences (Figure 16B). On the other hand, Cy CA 80% cell line showed higher DNA hydroxymethylation levels compared to Cy WT H cell line, although with no significance (Figure 16B). Cy CA 60% cell line showed similar levels of global DNA hydroxymethylation compared with Cy WT H (Figure 16B).

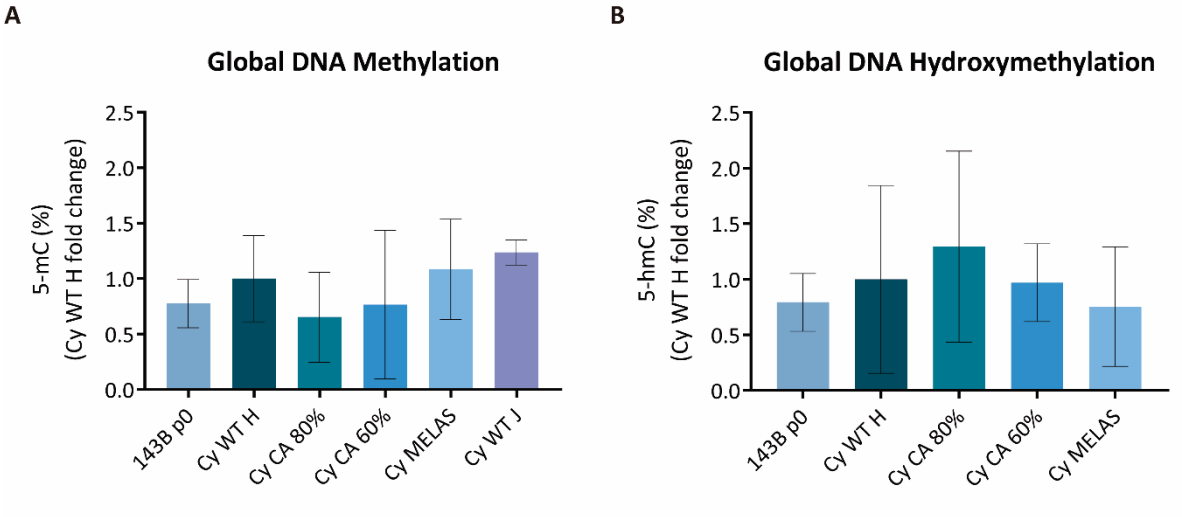

Figure 16 - Global DNA methylation and hydroxymethylation of 143B ρ0, Cy WT H, Cy CA 80%, Cy CA 60% and Cy MELAS cell lines, and global DNA methylation of Cy WT J cell line. **A –** Quantification of global DNA methylation of all cell lines. Results were normalized to Cy WT H. No significant differences were found. The results are from three independent experiments (N=3). Bars correspond to mean ± standard deviation **B –** Quantification of Global DNA hydroxymethylation of 143B ρ0, Cy WT H, Cy CA 80%, Cy CA 60% and Cy MELAS cell lines. Results were normalized to Cy WT H. No significant differences were found. The results are from three independent experiments (N=3). Bars correspond to mean ± standard deviation.

HISTONE POST-TRANSLATIONAL MODIFICATIONS (PTMS)

Since we found no statistical significant differences in the global DNA methylation and hydroxymathylation levels, we decided to evaluate the acetylation and methylation alterations in the histone H3 at specific residues, in particullar, H3K9ac, H3K14ac, H3K18ac, H3K27ac and H3K56ac (n=2) (Figure 17) and H3K9me3, H3K27me3 and H3K79me3 (N=1) (Figure 18).

To do this analysis, we initially tried to evaluate the levels of this marks using 30 micrograms of total protein extracts. However, we noticed that there were very slight signals or even there were no signals. Thereby, we proceed to the analysis of these marks in histone extracts from our cell lines to ensure that were was an sufficient enrichment in histones to get signals.

HISTONE 3 ACETYLATION

H3K9ac, H3K18ac and H3K27ac acetylation marks are linked to transcriptional activation and we decided to evaluate the levels of these marks in our cell lines by Western Blot.

There were some histone acetylation marks (H3K14ac and H3K56ac) to whose we could not get signals, which could be to the lack os antibody specificity or to the ansence of these marks in histones of our cell lines.

Regarding the ones we got signals, we verified that 143B ρ0 cell line has a tendency to have lower H3K27ac mark compared to Cy WT H cell line (Figure 17C). However, we did not oberve alterations in the levels of H3K9ac and H3K18ac marks in 143B ρ0 cell line, compared to Cy WT H cell line (Figure 17A and 17B). Moreover, Cy CA 80%, Cy CA 60% and Cy MELAS cell lines showed no difference in the acetylation epigenetic marks H3K9ac, H3K18ac and H3K27ac, compared to Cy WT H cell line (Figure 17).

Regarding the cybrid cell line carrying mtDNA from a different haplogroup, the Cy WT H, we observed lower levels in H3K27ac mark (Figure 17C). In this cybrid cell line we did not observe differences regarding the H3K9 and H3K18 acetylation marks compared with Cy WT H cell line (Figure 17A and 17B).

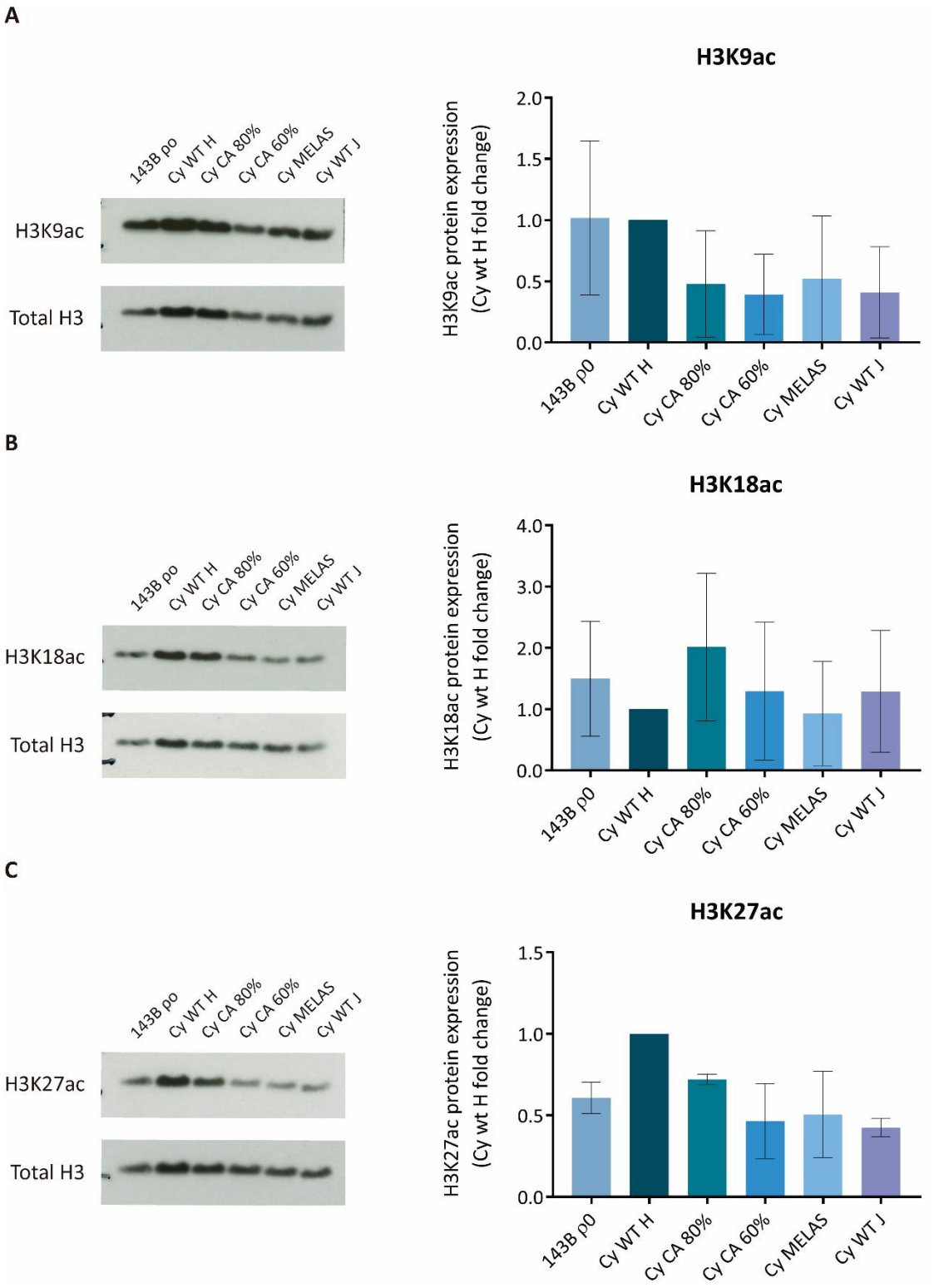

Figure 17 - Levels of acetylated histones at specific residues (H3K9ac, H3K18ac and H3K27ac) of 143B ρ0, Cy WT H, Cy CA 80%, Cy CA 60%, Cy MELAS and Cy WT J cell lines. Results were normalized to total H3 (loading control) and to Cy WT H (control). No statistic test was performed (n=2). Bars correspond to mean ± standard deviation **A –** Representation and quantification of H3K9ac levels in all cell lines. **B –** Representation and quantification of H3K18ac levels in all cell lines. **C –** Representation and quantification of H3K27ac levels in all cell lines.

H3K27ac Genome-wide enrichment

In order to evaluate H3K27ac (epigenetic mark for promoter and enhancer regions) genome-wide distribution, we intended to perform a ChIP-Seq analysis.

After cell lysis and sonication, we verified by an agarose gel electrophoresis that the size of the majority of DNA fragments was below 1000bp even if not optimal (200-500bp range) (Supplementary Figure 2).

Despite DNA fragments size, we verified the enrichment of H3K27ac in specific known regions of chromatin. We evaluated the H3K27ac enrichment at promoter level of beta-actin (a housekeeping gene) and FOXP3 (a gene not expressed in our cell line), and we verified the H3K27ac enrichment in the beta-actin promoter (positive control) but not in FOXP3 promoter (negative control) (Figure 18).

After verifying that the immunoprecipitation with H3K27ac antibody was successful, we evaluate the optimal number of cycles for generating a library for Next-Generation-Sequencing (NGS) (data not shown). This qPCR is an essential step to attach Illumina reading primers necessary for the sequencing and to determine the linear amplification range of the immunoprecipitated DNA.

After library generation, we verify our chromatin fragments size and conclude that our chromatin fragments were higher than 150-900 bp (Supplementary Figure 3). Overall, our results suggest that sonication step was not efficient enough. Thereby, sonication optimization is necessary.

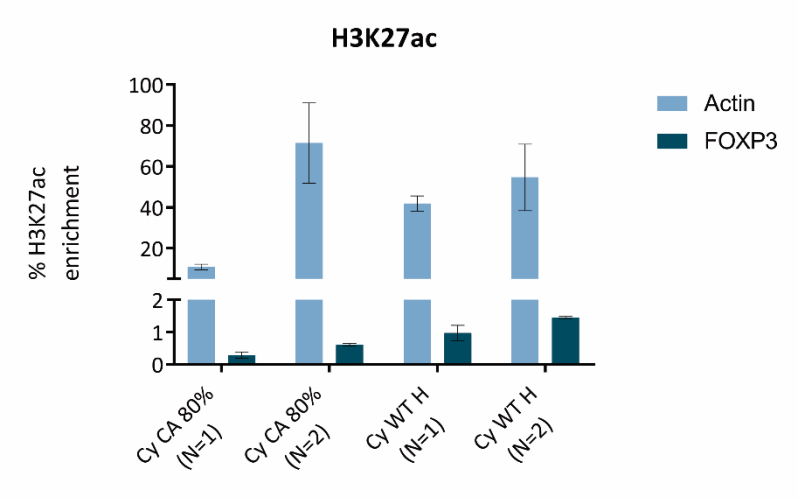

Figure 18 - Evaluation of efficiency in H3K27ac enrichment for Cy CA 80% and Cy WT H by qPCR, using actin (a housekeeping gene) and FOXP3 (a gene with lower enrichment), acting as a positive control and negative control, respectively). Enrichment was calculated as ΔCt relative to the input. Bars correspond to mean ± standard deviation.

HISTONE 3 TRIMETHYLATION

Besides to acetylation epigenetic marks in histones, methylation is other post-translation alteration that can affect gene transcription through chromatin structure and/or the accessibility of transcription factors to DNA. For instance, trimethylation marks in H3, in particularly H3K9 and H3K27 can act as repressor marks and trimethylation in H3K79 is correlated with transcription activation.

We observed no signal in H3K79me3 mark, which could be explained by lack of antibody specificity or anbsence of this marks in histones of our cell lines.

Regarding H3k9me3 marks, we observed that 143B ρ0, Cy CA 60%, Cy MELAS and Cy WT J cell lines showed a tendency for an increase in H3K9me3 levels compared with Cy WT H (Figure 19A). From those, the 143B ρ0 cell line exhibited higher H3K9me3 levels (Figure 19A). In case of Cy CA 80% cell line, we detect no apparent difference compared with Cy WT H cell line (Figure 19A).

Concerning H3K27me3 mark, Cy CA 60% and Cy MELAS cell lines demonstrated higher H3K27me3 levels, compared with Cy WT H (Figure 19B). Cy 80% cell line showed similar H3K27me3 levels whereas, 143B ρ0 and Cy WT J cell lines exhibited lower H3K27me3 levels, compared with Cy WT H (no statistic test was performed due to the number of experiments) (Figure 19B).

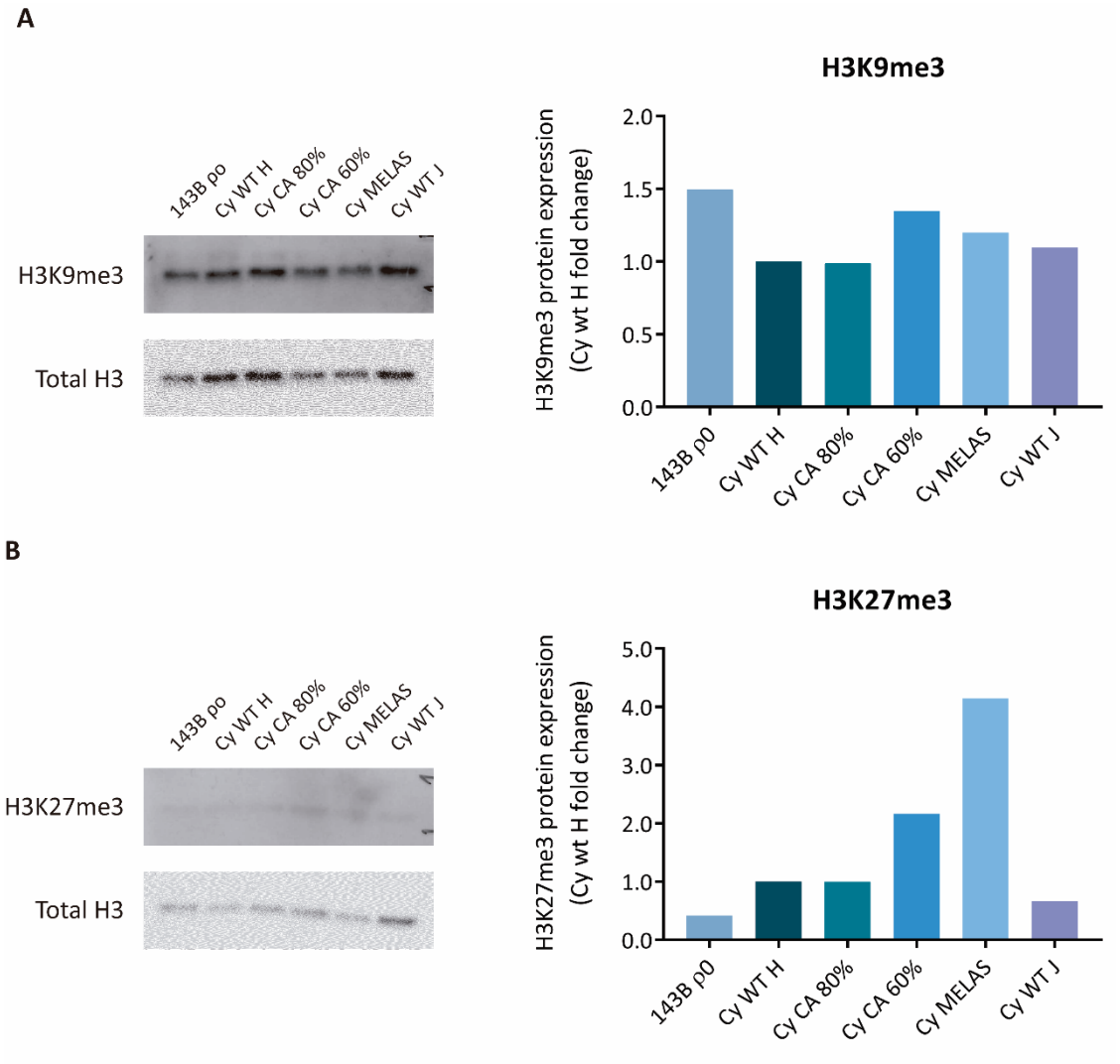

Figure 19 - Levels of trimethylated histones at specific residues (H3K9me3 and H3K27me3) of 143B ρ0, Cy WT H, Cy CA 80%, Cy CA 60%, Cy MELAS and Cy WT J cell lines. Results were normalized to total H3 (loading control) and to Cy WT H (control). No statistic test was performed (n=1). Bars correspond to mean. **A –** Representation and quantification of H3K9me3 expression in all cell lines. **B –** Representation and quantification of H3K27me3 levels in all cell lines

CELL CYCLE REGULATORS – P53 PATHWAY

Since we verified that clonogenic potential and cell proliferation were different in some cell lines, we evaluated protein expression of cell cycle regulators belonging to the p53 pathway (Figure 20). Moreover, activity of epigenetic enzymes could also interact with non-histone proteins, which could affect protein activity and response.

Concerning p53, we observed no differences in expression in protein expression (Figure 20). However, when we analyzed the p53 acetylation levels (in the Lysine residues 372 and 382), we observed that Cy MELAS and Cy WT J did not show p53 acetylation (Figure 20). In contrast, 143B ρ0, Cy CA 80% and Cy CA 60% cell lines showed a similar p53 acetylation levels, compared to Cy WT H (Figure 20).

Regarding the expression of the cell cycle regulator p27, we observed similar expression in all cell lines compared to Cy WT H (Figure 20).

In the case of other cell cycle regulator, the p21, we observed that Cy MELAS and Cy WT J cell lines exhibited lower expression levels compared to Cy WT H [T-test p=0.0013 and p=0.0087, respectively] (Figure 20). No statistically differences were observed in 143B ρ0, Cy CA 80% and Cy CA 60% cell lines, compared with Cy WT H (Figure 20).

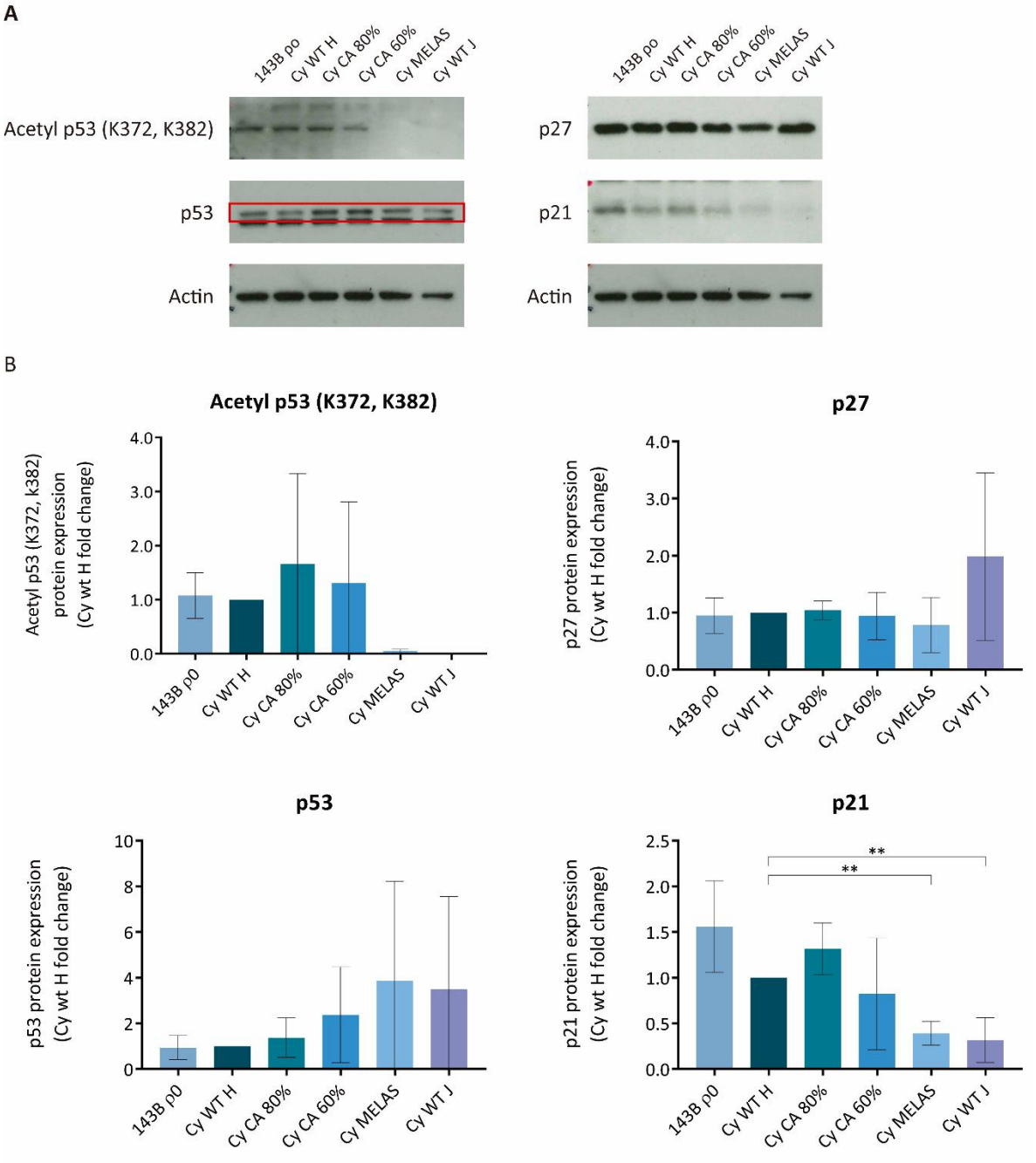

Figure 20 - Levels of cell cycle regulators that belong to the p53 pathway (acetyl p53 (K372, K382), p53, p27 and p21) of 143B ρ0, Cy WT H, Cy CA 80%, Cy CA 60%, Cy MELAS and Cy WT J cell lines. **A -** Representation of western blot results of acetyl p53 (K372, K382), p27 and p21 levels and the loading control (actin) of all cell lines. Since this staining was performed in the same membrane, the loading control is duplicated. **B –** Quantification of western blot result of acetyl p53 (K372, K382), p53, p27 and p21 expression of all cell lines which were normalized to the loading control (actin) and to Cy WT H cell line (control). Acetyl p53 (K372, K382) expression was also normalized to p53 and statistic test was not performed (n=2). Bars correspond to mean ± standard deviation.

DISCUSSION

CLONOGENIC POTENTIAL AND CELL PROLIFERATION

Mitochondrial functions, as the mitochondrial metabolism and the generation of ROS control cell proliferation, differentiation, and adaptation to stress and aging. Thus, mitochondrial dysfunction could affect cell proliferation.

Impairment of mitochondrial membrane potential (MMP) is linked to a decrease in cell proliferation (Martínez-Reyes, Diebold et al. 2016). Martínez-Reyes *et al.* generated cell lines from human embryonic kidney 293 (HEK293) with progressive mtDNA depletion and ectopic expression of 2 nonmammalian proteins—NADH dehydrogenase-like 1 (NDI1) and alternative oxidase (AOX) – that restore NAD^+ and FAD and kept the TCA flux without restoring the mitochondrial membrane potential (Martínez-Reyes, Diebold et al. 2016). Using these cell lines, they described a decrease in cell proliferation and ROS levels and increase of AMP-activated protein kinase (AMPK) levels (a regulator of cellular energy homeostasis), as a consequence of decrease of ATP production (Martínez-Reyes, Diebold et al. 2016). Martinez-Reyes *et al.* reported that the reestablishment of MMP induced ROS production and partially reestablished cell proliferation (Martínez-Reyes, Diebold et al. 2016). However, increase of ROS levels did not rescue the total proliferative capability of the cell line, indicating that AMPK could also regulate cell proliferation (Martínez-Reyes, Diebold et al. 2016). In fact, we observed a significant decrease in cell proliferation in 143B ρ0 cell line. However, we also observed a significant decrease in the clonogenic potential of these cells. These results suggest that impairment of mitochondrial potential and increase of AMPK levels could also affect cell capacity to survive as single cells and form new colonies. Previous results in our lab demonstrated that 143B ρ0 cells have lower MMP levels (data not shown) and also possess reduced levels of ROS (Miranda, Correia et al. 2020).

Concerning Cy CA 80% and Cy CA 60% cell lines, Ins3571C mutation affect the complex I of the OXPHOS system, which is linked to regulation of NAD+/NADH ratio and ROS production. The ND1 is a key player in the maintenance of complex I structure and function, a complex that belongs to the OXPHOS system (Iommarini, Ghelli et al. 2018). Iommarini *et al.* indicated a threshold between 85%-93% for Ins3571C mutation (the same mutation carrying the Cy CA 80% and Cy CA 60%), would affect complex I and energy production (Iommarini, Ghelli et al. 2018). However, we verified significant differences in cell proliferation, compared to Cy WT H cell line. Unexpectedly, Cy CA 60% cell line exhibited the lowest cell proliferation during colony formation and growth. We hypothesize that Cy CA 60% induce initial cellular compensation mechanisms to the presence and consequences of the mutation levels, before reaching mitochondrial dysfunction, as a way to maintain cell homeostasis. In line with this, Lozoya *et al.* verified that

an initial response is triggered in a cell culture system with progressive mtDNA depletion in the early stages of mtDNA depletion (cells with, approximately, 30% of mtDNA) (Lozoya, Martinez-Reyes et al. 2018). Interestingly, the same authors suggested that cells in early stages of progressive mtDNA depletion adjust their metabolism in order to cells adapt nucleic acid metabolism (transcription, DNA repair, and replication), cell cycle, protein translation, methylation reactions, and redox homeostasis. (Lozoya, Martinez-Reyes et al. 2018). In line with this, we suggest that compensatory mechanism in Cy CA 80% and, particularly in Cy CA 60% could be responsible for differences in cell proliferation, despite both cell lines present mutation levels below the threshold considered by Iommarini *et al.* (Iommarini, Ghelli et al. 2018)

Regarding the mtDNA variants, it was previously described that cells carrying haplogroup J seems to produce less ROS comparing to haplogroup H (Bellizzi, D'Aquila et al. 2012). Cy WT J did not show any significant differences in colony proliferation but showed increased clonogenic capacity. We suggest that the clonogenic potential in this cybrid could be linked to lower production of ROS (Bellizzi, D'Aquila et al. 2012).

GLOBAL DNA METHYLATION AND HYDROXYMETHYLATION

Mitochondria are key players in the cellular epigenetic reactions since they supply a variety of cofactors or substrates necessary by the activity of enzymes involved in cell epigenetic regulation. One of those examples is SAM, which is a methyl group donor crucial for DNA methylation provided by mitochondria, through the methionine cycle.

Accordingly to our hypothesis, mtDNA mutations can promote the dysfunction of mitochondrial activity, resulting in variations in the concentration of intracellular metabolites. In its turn, mitochondrial dysfunction can result in alterations in the activity of several epigenetic enzymes and consequently in the epigenetic status of cells, including DNA methylation. In fact, it was shown by Smiraglia *et al.* that, as a result of depletion of mtDNA, the levels of DNA methylation can increase, as a consequence of mitochondrial dysfunction (Smiraglia, Kulawiec et al. 2008). A recent study involving a cell culture system with progressive mtDNA depletion suggested that depletion of mtDNA induces an activation of serine biosynthesis and remodeling of folate and one carbon metabolism towards transsulfuration, which affects the methionine cycle (Lozoya, Martinez-Reyes et al. 2018). Consequently, there is an induction in generation of SAM and, therefore, increase in the global DNA methylation (Lozoya, Martinez-Reyes et al. 2018). In contrast to these reports, our results indicated that global DNA methylation in 143B ρ0 cell line was not significantly different compared to Cy WT H cell line (control). Lozola *et al.* described an increase of SAM and methylthioadenosine (MTA) levels when cells have significant mtDNA depletion (cells with approximately 10% of mtDNA); but when cell achieved total mtDNA

depletion, SAM and MTA levels decreased (Lozoya, Martinez-Reyes et al. 2018). Moreover, the authors detected maximal DNA hypermethylation when cell have significant mtDNA depletion, which decreased with total mtDNA depletion (Lozoya, Martinez-Reyes et al. 2018). In line with these reports, it is possible that 143B ρ0 could reached a level of homeostasis when grown in this condition (lack of DNA) in culture for long periods as is the case, in a way where the potential alterations we were expecting were lost. One experiment that could allow to verify this, would be the analysis of DNA methylation and hydroxymethylation upon the generation of new 143B ρ0 cells immediately after UL12.5 ectopic expression and validation of mtDNA elimination.

Other hypothesis to explain why we did not see any differences regarding DNA methylation can be a result of maintenance of NADH oxidation. Lozoya *et al.* described that maintenance in NADH oxidation in HEK293 cells total depleted of mtDNA through ectopically expression of NDI1 and AOX maintained NAD^+/NADH ratio and TCA flux (Lozoya, Martinez-Reyes et al. 2018). The authors also verified that this cell line maintained the levels of SAM, SAH and MTA using the same cell lines (Lozoya, Martinez-Reyes et al. 2018). They suggested that maintenance of oxidation of NADH and, subsequently maintenance of TCA flux, result in a compensatory mechanism to maintain the levels of metabolites essential for methionine cycle. This hypothesis is compatible with previous results obtained in our laboratory, which indicated that 143B ρ0 did not demonstrate alterations in the NAD^+ levels, despite lack of OXPHOS. This can be a result of the supplementation of the cell culture media used to maintain cell lines with pyruvate and uridine. Ly and Lawen demonstrated that the pyruvate/lactate ratio can be used to maintain NADH/NAD^+ ratio when OXPHOS is compromised (Ly and Lawen 2003).

It is therefore possible that in our study 143B ρ0 cells can have a balanced NAD^+/NADH, as a result of cell culture conditions and cellular adaptations. Thus, we suggest that NAD^+/NADH maintenance can contribute to keep the levels of cellular homeostasis, namely in the levels of DNA methylation. Previous results from our laboratory also showed no significant alterations in NAD^+ levels in the Cy CA 60% and Cy MELAS, which can also explain the lack of differences in these cells. Based on this explanation, we should test in further studies the methylation levels in cells growing in cell culture media with more restrictive levels of glucose and/or pyruvate.

In Cy CA 80% and Cy CA 60% cell lines, we did not observe significant differences in DNA methylation and hydroxymethylation. These results supports the hypothesis that these cell lines do not have sufficient heteroplasmy levels to impair complex I and to affect DNA methylation.

We also did not observe in Cy MELAS cell line significant differences in DNA methylation. Kopinsi *et al.* described that 143B cells harboring 60% of A3243G mutation have an induction of NAD^+ synthesis, in order to balance NAD^+/NADH ratio and stimulate OXPHOS (Kopinski, Janssen et al. 2019). Is it then tempting to speculate that in Cy MELAS, the lack of differences in DNA

methylation can be due to the maintenance of the NAD^+/NADH levels, in a similar fashion as described in this report.

Other possibility to these results can be a decrease in DNA cytosine hydroxymethylation and maintenance of the levels of cytosine methylation (Figure 4). In fact, alteration in TCA cycle could result in a decrease of α-KG (a co-factor of TET enzymes) and increase of succinate, fumarate and 2-HG levels, which are known to inhibit TET enzymes through competition of the active site (Lozoya, Martinez-Reyes et al. 2018).

However, we demonstrated no significant decrease in DNA hydroxymethylation in all cell lines, even in 143B ρ0 cell line. In line with our results, Lozoya *et al.* observed no alterations in the levels of α-KG and neither in succinate/α-KG or fumarate/α-KG ratios in total mtDNA depleted cells (Lozoya, Martinez-Reyes et al. 2018), which can explain the lack of differences in DNA hydroxymethylation in 143B ρ0 cell line.

Regarding Cy MELAS cell line, Kopinsky *et al.* described that 143B cells harboring 60% of A3243G mutation demonstrated an increase of α-KG levels (Kopinski, Janssen et al. 2019). However, levels of DNA hydroxymethylation were not significantly different, compared with Cy WT H cell line. It is important to note that the cybrid cell lines from our study carry the A3243T mutation, while the cybrids from Kopinsky *et al.* carry a mutation in the same nucleotide position but to a Guanine (m.A3243G). Both mutations are characteristic of metabolic syndromes, we may not exclude that heteroplasmy levels of these distinct mutations could not induce different cellular alterations or different degrees of those alterations.

Furthermore, it has been suggested that different haplogroups have a different impact on mitochondria metabolism, particular in OXPHOS efficiency and in the TCA cycle (Moreno-Loshuertos, Acín-Pérez et al. 2006, Bellizzi, D'Aquila et al. 2012). If these variations affect the levels of the metabolites necessary by the epigenetic enzymes, the cellular DNA methylation status can be different between cells carrying different mtDNA haplogroups. Bellizzi *et al*. demonstrated that a 143B-derived cybrid cell line with the haplogroup J show significant higher DNA methylation levels compared to a 143B-derived cybrid cell line with haplogroup H, as a result of overexpression of MAT1A, which is linked to SAM production (Bellizzi, D'Aquila et al. 2012). However, our results demonstrate no differences in DNA methylation in Cy WT J cell line compared to Cy WT H cell line. In the future, it would be important to evaluate the levels of MAT1A in our cells to ascertain if there are or there are not differences in the expression of this protein in the cybrid cell lines from our study carrying WT mtDNA from different haplogroups.

HISTONE POST-TRANSLATIONAL MODIFICATIONS (PTMS)

Mitochondria dysfunction is linked to alterations in the cell epigenetic status, in particular histone acetylation and methylation, by changing the production of specific metabolites. In fact, acetyl-CoA and α-KG are two substrates essential to histone acetylation and histone demethylation reactions, respectively. Other example is SAM is used as a substrate of the enzymes responsible for nDNA methylation but also for histone methylation. Therefore, we hypothesize that deficiency in mitochondrial metabolism can provoke alterations in the levels of histone epigenetic marks.

Histone acetylation

We evaluate the acetylation marks in H3K9, H3K14, H3K18, H3K27 and H3K56 in all cell lines, which are epigenetic marks that leads to transcriptional activation. We could detect the acetylation in the histone 3 residues H3K9, H3K18, H3K27.

It has been shown in HEK293 cells that total loss of mtDNA leads to decrease in the acetylation of certain histone lysines (H3K9, H3K18 and H3K27), as a result of TCA cycle impairment (Martínez-Reyes, Diebold et al. 2016). However, we observed that 143B ρ0 cell line did not show differences in the studied acetylation marks, except for H3K27ac. For this acetylation mark, we observed lower levels compared to Cy WT H. Martínez-Reyes *et al.* described that in cells with total mtDNA depletion where NDI1 and AOX were ectopically expressed demonstrated that acetylation marks in H3K9, H3K18 and H3K27 is counteracted as a result of maintenance of TCA cycle (Martínez-Reyes, Diebold et al. 2016). As referred before, 143B ρ0 cell line seems to preserve the TCA flux because of maintenance of NAD^+/NADH ratio. In our study, 143B ρ0 cell culture media was supplemented with pyruvate, which can be used to generate nuclear acetyl-CoA and be responsible for maintenance of H3 acetylation marks. In 2014, it was demonstrated that the pyruvate dehydrogenase complex can be present in the nucleus and generate or maintain a certain pool of acetyl-CoA levels in this organelle and regulate histone acetylation levels (Sutendra, Kinnaird et al. 2014). However, Martínez-Reyes *et al.* did not observe H3 acetylation marks restauration of inducible ρ0 cells with the supplementation of acetate and pyruvate, suggesting that other TCA metabolites might be necessary (Sutendra, Kinnaird et al. 2014, Shi and Tu 2015). This way, the similar levels seen in the acetylation marks in our mtDNA depeleted cells remais to be clarified. Based on the previous reports, it would be interesting to incubate these cells with cell culture media where restricted pyruvate/acetate (and/or other metabolites that fuel the TCA cycle) and study the effects in histone acetylation levels.

Concerning Cy CA 80% and Cy CA 60% cell lines, we observed no differences comparing to Cy WT H cell line. It can be explained by the fact that the heteroplasmy levels are not sufficient to impair complex I and, consequently affect histone acetylation.

In case of Cy MELAS cell line, we also observed no differences in histone acetylation marks, comparing to Cy WT H cell line. Kopinski *et al.* described that 143B cells harboring 60% of m.A3243G mutation maintain histone acetylation levels, as well as the acetyl-CoA levels (Kopinski, Janssen et al. 2019), which is in line with our results.

Regarding the mtDNA variants, we were expecting significant modifications on histone acetylation levels in Cy WT J cell line, comparing with Cy WT H cell line, since different ROS production is linked to changes in TCA flux. However, we only observed a tendency in H3K27ac levels (lower) comparing with Cy WT J, whereas H3K9ac and H3K18ac levels seemed similar. In next experiments, we should evaluate the ROS levels in our cell lines in order to understand if ROS levels can justify some of our results.

Histone methylation

Concerning histone methylation, we observed in all cell lines trimethylation marks in H3K9 and H3K27, which act as repressor marks.

We observed that 143B ρ0 tend to have higher trimethylation in H3K9 levels and lower trimethylation in H3K27 levels. Martínez-Reyes *et al.* demonstrated that cells with total mtDNA depletion exhibited no significant differences in global histone methylation (Martínez-Reyes, Diebold et al. 2016). In line with this, the authors did not observed alterations in the α-KG levels (Martínez-Reyes, Diebold et al. 2016). The maintenance of α-KG levels, as well as the maintenance of SAM and NAD^+/NADH ratio in mtDNA depleted cells can contribute to the preservation of global histone methylation levels (Lozoya, Martinez-Reyes et al. 2018; Martínez-Reyes, Diebold et al. 2016). However, we analysed the methylation levels of two specific lysine residues (Lysines 9 and 27), which do not represent the global histone methylation levels (all lysine residues) as these reports did. It would be important to check the global histone methylation levels to verify if our cells demonstrate similar results to the previous reports.

Concerning the Ins3571C mutation, Cy CA 80% cell line demonstrated maintenance in the H3K9me3 and H3K27me3 marks, whereas Cy CA 60% cell line exhibited a tendency to be higher in trimethylation levels in H3K9 and H3K27, compared to Cy WT H cell line. These results could support our hypothesis, which suggest initial cellular compensation mechanisms, before achieving mitochondrial dysfunction.

Our results also showed an increase of the epigenetic marks H3K9me3 and H3K27me3 in Cy MELAS compared with Cy WT H cell line. These outcomes were unexpected, since Kopinski

et al. described that cells with 60% of m.A3243G mutation showed an increase in α-KG levels (Kopinski, Janssen et al. 2019).

We were expecting an increase in the histone trimethylation marks in Cy WT J cell line, based on the descriptions of Bellizzi *et al.* that described that cells with haplogroup H overexpress MAT1A, comparing with haplogroup H (Bellizzi, D'Aquila et al. 2012). However, our results demonstrated similar levels between both cybrid cells carrying these different mtDNA haplogroups. We suggest that the preservation of histone trimethylation levels could be related with lower ROS production, since ROS could inhibit alpha-ketoglutarate dehydrogenase (α-KGDH), resulting in inhibition of histone demethylase. Once again, it appears to be important to determine the ROS levels in our cybrid cell, in order to infer the role of ROS in the epigenetic alterations (or lack of them).

CELL CYCLE REGULATORS

Some reports observed that epigenetic modifications are also important for the regulation of cell cycle regulators, specifically p53 and p53 target genes. Indeed, we observed differences in proliferative capacities and cell proliferation of the cybrid cell lines in the colony formation assay.

We observed similar p53 levels in all cell lines. However, we observed that there is no p53 acetylation in Cy MELAS and Cy WT H cells (residues K373 and K382). Acetylation in p53 is performed by HATs and is crucial for p53 activity. p53 acetylation can regulate cell fate in response to DNA damage and activate p53-target genes transcription. The p53 acetylation in K320, K373, K381 and K382 residues results in recruitment of the coactivators CPB (a histone acetyltransferase) and TRRAP (possesses HAT subunits), resulting in an increase in histone H3 and H4 acetylation in the promoter region of p21 (CIP1/WAF1/CDKN1A), increasing its expression (Barlev, Liu et al. 2001). p21 is a cyclin-dependent kinase inhibitor which regulates cell proliferation. In accordance with this, Cy MELAS and Cy WT J (cell lines without p53 acetylation) showed significant decrease in p21 levels.

CONCLUSION AND FUTURE PERSPECTIVES

Overall, mtDNA could be important for regulating gene/protein expression. Some essential regulators of gene/protein expression are also crucial regulators of cell epigenetic landscape. We suggest that different mtDNA mutations and haplogroups could alter gene/protein expression through variations in epigenetic modification in proteins that can control expression in specific genes. However, we notice maintenance in cell epigenetic status, particularly DNA methylation and hydroxymethylation, and histone acetylation. We suggest that different mtDNA variants (and mutations) preserve the levels of key metabolites crucial for epigenetic activity, which maintain cellular homeostasis and cell epigenetic landscape. In case of Ins3571C mutation, we suggest that the mechanism for maintenance of cell epigenetic status is reached by an initial compensatory response in order to maintain the levels of crucial metabolites, before cells reach mitochondrial dysfunction. However, we propose that this compensatory mechanism is not sufficient to maintain all the levels of epigenetic status, in particular histone methylation.

Nevertheless, previous result from our lab indicated that the parental cell line 143B and 143B ρ0 exhibited alterations in methylation in specific nDNA genes. In line with these results, we suggest that methylation and hydroxymethylation in specific nDNA genes are alter, despite of maintenance of global DNA methylation and hydroxymethylation.

Similar to DNA methylation and hydroxymethylation, we expect to verify significant differences in the outcomes of the ChIP-Seq on H3K27ac, comparing Cy WT H with Cy CA 80%.

In order to better understand our results, we believe that it would be important to increase the number of experiments and study the:

- different epigenetic mechanisms in cells lines in cell culture media restricted of pyruvate/acetate and other metabolites from the TCA cycle;
- levels of metabolites crucial for epigenetic activity, as well as ATP and ROS levels;
- global levels of histone methylation and acetylation;
- levels of epigenetic enzymes;
- methylation/hydroxymethylation in specific nDNA genes in all cell lines and verify if it associates with changes in mRNA expression.

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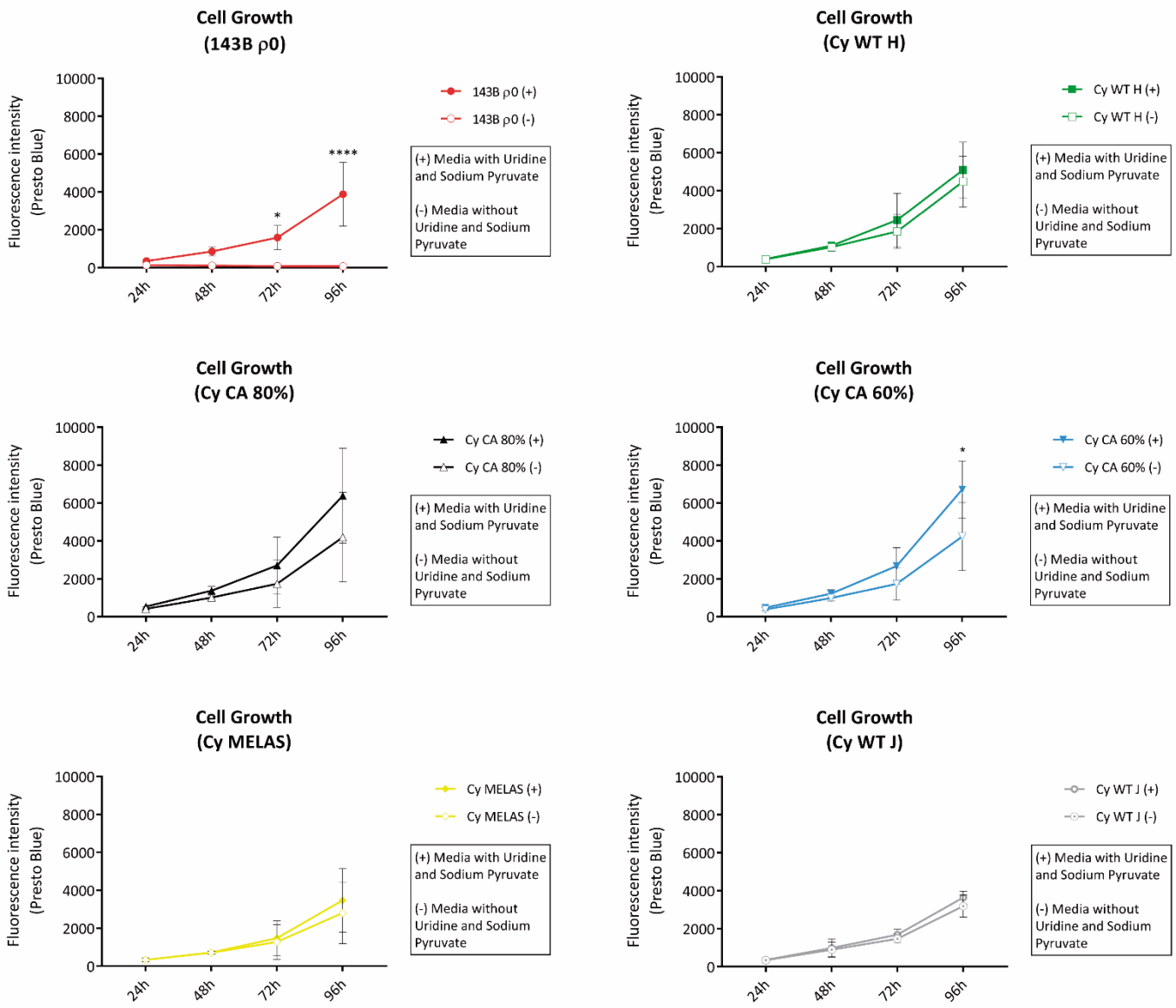
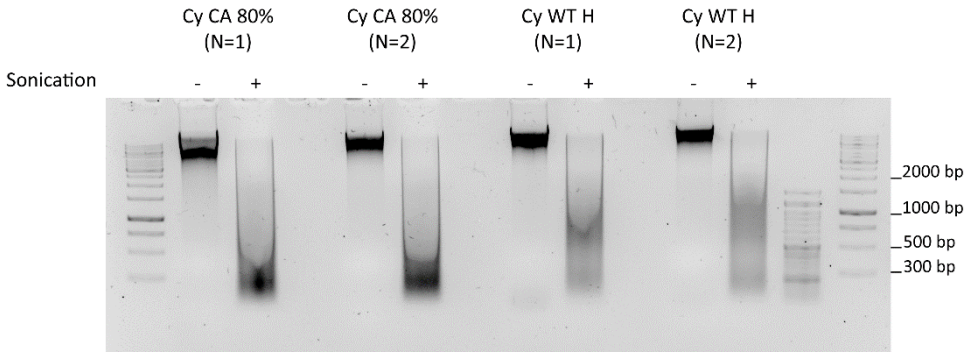
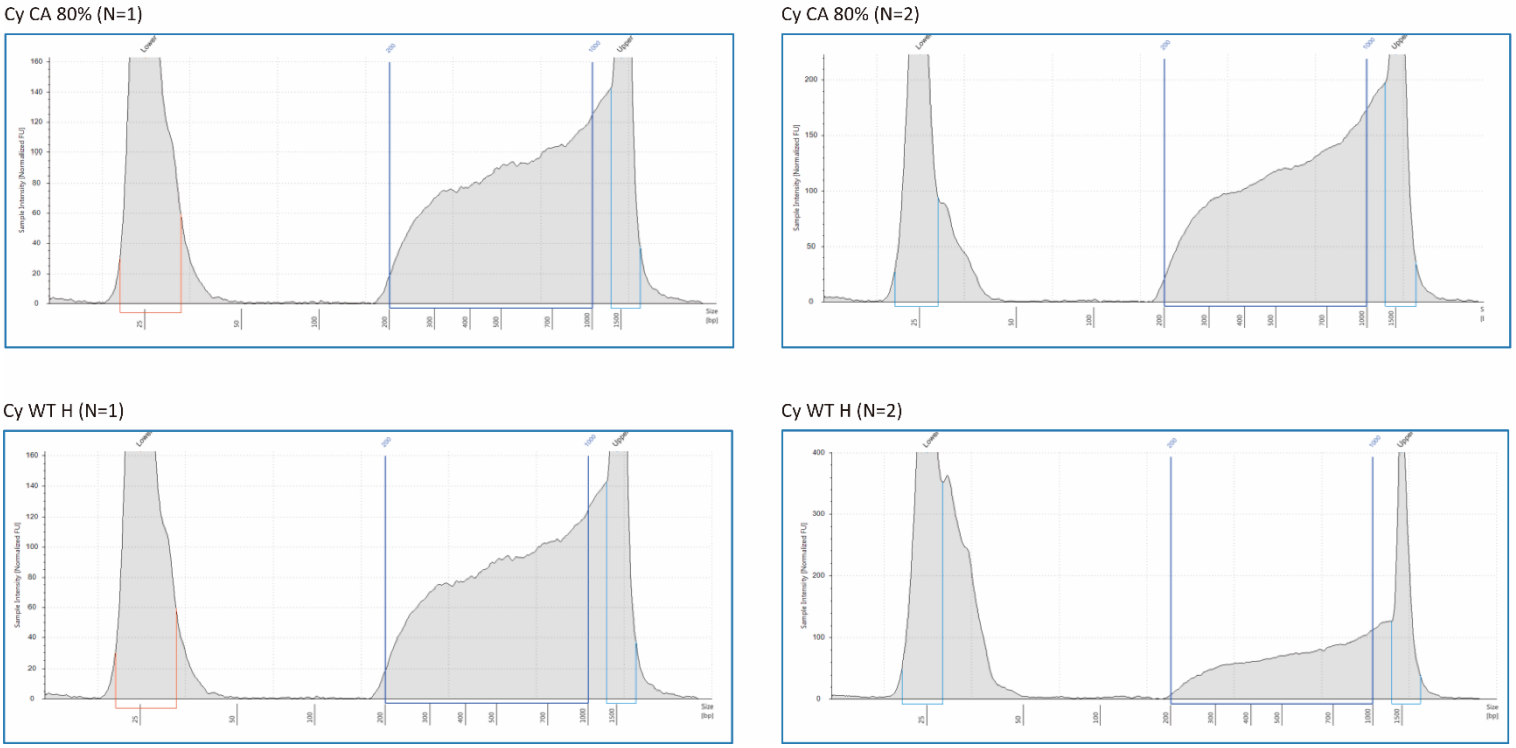
SUPPLEMENTARY DATA



Supplementary Figure 1 - Quantification of cell growth by presto blue assay of 143B ρ0, Cy WT H, Cy CA 80%, Cy CA 60%, Cy MELAS and Cy WT J cell lines in media with uridine and sodium pyruvate (+) and without supplementation (-). 143B ρ0 cell line showed significant lower cell growth after 72h and 96h in media without supplementation, compering to media with uridine and sodium pyruvate (Two-way ANOVA *p=0.0468, ****p<0.0001, respectively). Cy CA 60% demonstrated significant lower cell growth after 96h between the media (Two-way ANOVA *p=0.0221). No significant differences were found in the Cy WT H, Cy CA 80%, Cy MELAS and Cy WT J cell lines. The results are from three independent experiments (N=3). Results correspond to mean ± standard deviation.



Supplementary Figure 2 - Analysis of DNA fragments size of Cy CA 80% and Cy WT H by agarose gel electrophoresis. As control for initial chromatin integrity, we used DNA fragments that did not undergo sonication (-). DNA Fragments that undergo sonication (+).



Supplementary Figure 3 - Evaluation of chromatin size after library generation in Cy CA 80% and Cy WT H by 2200 TapeStation.

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