Declaration

This thesis has been entirely composed by me and I have carried out all experiments, except where otherwise stated. This work has not been submitted for any other degree or professional qualification.

All experimental activities here presented were performed at the Institute of Genetics and Molecular Medicine, Edinburgh Cancer Research UK Centre, Scotland.

João Pedro Santiago de Jesus, June 2015

“Either write something worth reading or do something worth writing.”

-Benjamin Franklin

Abstract

Cellular senescence is defined as a state of stable exit from the cell cycle caused from a variety of stresses. Even though they remain metabolically active, senescent cells cannot proliferate. As a consequence, it is commonly viewed as a tumour suppressor mechanism.

Lipid peroxidation, a process that arises during reactive oxygen species (ROS) production, may play an important role in modulating cellular signalling under senescence stress. 4-Hydroxy-2-nonenal (4-HNE) is a highly diffusible and reactive molecule produced during lipid peroxidation. This electrophilic aldehyde forms covalent adducts with proteins, altering their activity and function.

From this report, it has been confirmed that Histone H3 is one of the proteins that bounds to 4-HNE, as human fibroblasts (IMR90) treated with 4-HNE and oncogene-induced senescent cells have presented this interaction. Remarkably, IMR90 treated with 4-HNE changed they histone post-translational modifications (PTMs) when compared with the control cells, a feature never reported before. Furthermore, the changes promoted by 4-HNE are the same as the ones that have been reported as fundamental changes for induction and maintenance of a senescence phenotype, cases in point: trimethylation of histone 3 on both lysine 9 and lysine 27 (H3K9me3 and H3K27me3), and loss of methylation in histone 3 lysine 4 (H3K4me3). In fact, we were able to see these repressive marks in high levels on 4-HNE treated IMR90 and IMR90 cells expressing a chimeric fusion protein with Ras (IMR90:ER) - oncogene-induced senescent cells.

Following the epigenetic changes, 4-HNE cells also showed absence of proliferative markers - 5-bromo-2'-deoxyuridine (BrdU) incorporation - an essential characteristic for senescence cells. On top of that, an upregulation of the tumour suppressor

protein p21 was also seen in this cells, which reinforces the idea that HNE treated cells present a senescence-like phenotype.

Lastly, IMR90 treated with 4-HNE also presented a global and drastic change in nuclear architecture. These cells presented a highly compacted chromatin full of repressive markers (heterochromatin), this is marker usually seen in senescent cells known as Senescence associated heterochromatin foci.

Further understanding on the relation of 4-HNE, histone post-translational modifications and senescence can have a great impact in new tumour suppressive therapies.

KEY WORDS: 4-hydroxy-2-nonenal; Senescence; Lipid peroxidation; Epigenetics; Histone post-translational modifications;

Resumo

Senescência celular é definida como um processo de saída estável do ciclo celular quando promovido por uma grande variedade de stresses. Apesar de células senescentes continuarem metabolicamente ativas, estas não têm a capacidade de proliferar. Como consequência, este processo é muitas vezes visto como um mecanismo supressor de tumores.

Peroxidação lipídica, é um processo que resulta da produção de espécies reativas de oxigénio (ROS), este pode ter um papel importante na modulação do *stress* celular que leva à senescência. *4-Hydroxy-2-nonenal* (4-HNE) é uma molécula reativa que se passa facilmente por membranas e é produzida durante a peroxidação lipídica. Este aldeído electrofílico forma adutos covalentes com proteínas, alterando a sua estrutura.

Neste trabalho foi confirmado que a histona H3 é uma das proteínas que se liga à 4-HNE, esta interação foi apresentada em fibroblastos humanos (IMR90) tratados com 4-HNE e fibroblastos induzidos oncogenicamente à senescência. Invulgarmente, quando comparado com células controlo, IMR90 que sofreram tratamento com 4-HNE mudaram as suas marcas pós-traducionais das histonas (PTMs), um acontecimento nunca reportado antes com 4-HNE. Esta caracteristica tornou-se ainda mais interessante quando observamos que, as mudanças produzidas por esta molécula são as mesmas que são aceites globalmente como fundamentais para a indução e manutenção de um fenótipo de senescência, casos em questão: tri-metilação na lisina 9 e lisina 27 na histona 3 (H3K9me3 and H3K27me3), perda da tri-metilação na lisina 4 histona 3 (H3K4me3). De facto, conseguimos observar estas marcas repressivas em IMR90 tratadas com 4-HNE e IMR90 células que expressam uma proteína de fusão quimérica com Ras (IMR90:ER) – células senescentes por indução oncogénica.

Na sequência das alterações epigenéticas, células tratadas com 4-HNE também mostraram ausência de marcadores de proliferação – incorporação de *5-bromo-2'-deoxyuridine* (BrdU) – uma característica essencial para células senescentes. Reforçando a ideia que 4-HNE promove um fenótipo semelhante ao da senescência, também foi observado uma regulação positiva da proteína supressora de tumor p21.

Por último, células tratadas com 4-HNE também apresentaram uma alteração global e drástica na arquitetura nuclear. Estas células apresentaram uma cromatina muito compactada e cheia de marcas repressivas (heterocromatina), este é uma característica geralmente observada em células senescentes e conhecida por heterocromatina *foci* associada à senescência (SAHF).

Uma maior compreensão sobre a relação entre 4-HNE, modificações pós-translacionais em histonas e senescência pode ter um grande impacto em novas terapias para cancro.

Palavras-chave: 4-hydroxy-2-nonenal; Senescência; peroxidação lipídica; Epigenética; Modificação pós-traducionais das histonas;

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Abbreviations

4-HNE	4-Hydroxynonenal, or 4-hydroxy-2-nonenal
^{o}C	degrees centigrade
BSA	bovine serum albumin
CDK	Cyclin-dependent kinase
CDKi	Cyclin-dependent kinase inhibitor protein
ChIP	Chromatin immunoprecipitation
DAPI	4', 6-diamidino-2-phnylindole
dH_2O	distilled water
DNA	deoxyribonucleotide
HCl	hydrochloric acid
H3K4	Histone 3 lysine 4
H3K4me3	Histone 3 lysine 4 trimethylated
H3K4ac	Histone 3 lysine 4 acetylated
H3K9	Histone 3 lysine 9
H3K9me3	Histone 3 lysine 9 trimethylated
H3K9ac	Histone 3 lysine 9 acetylated
H3K27	Histone 3 lysine 27
H3K27me3	Histone 3 lysine 27 trimethylated
HAT	Histone acetyltransferases
HDAC	Histone deacetylase
KDa	Kilo Daltons
L	litre
$MgCl_2$	magnesium chloride
µm	micrometre
µg	microgram
µM	micromolar
mL	millilitre
µL	microlitre
nM	nanomolar
mM	millimolar
M	molar
NaCl	sodium chloride
nM	nanomolar

OIS	oncogene induced senescence
PBS	phosphate buffered saline
PFA	paraformaldehyde
PTM	post translational modification
Rb	retinoblastoma protein
pRb	phosphorylated retinoblastoma protein
ROS	reactive oxygen species
SAHF	senescence associated heterochromatin foci
SASP	senescence associated secretory phenotype
TBS	Tris-buffered saline
TBST	TBS with tween

CHAPTER 1: Introduction

1.1. Cellular Senescence: a tumour suppression mechanism

1.1.1 Senescence

In the year 1965, Hayflick, was confronted with the dogma that individual human cells were immortal, proving that after a finite number of cellular divisions, normal human fibroblasts eventually entered a state of permanent growth arrest (Hayflick & Moorhead 1961). This evidence opens a door to a new state - Cellular senescence. There are two major circumstances which can promote an end in replication. The first invokes the existence of a molecular clock (telomere shortening) which keeps track of cell divisions and eventually stop them (Hayflick & Moorhead 1961). The second is often mention as premature cellular senescence and can be achieved in the absence of telomere dysfunction (Hanahan & Weinberg 2011).

Cellular senescence is confined to mitotic cells, and it can be characterized as a state of stable exit from the cell cycle, even though these cells remain metabolically active. It differs from other non-dividing cell processes, such as quiescent or terminally differentiated cells, by several markers and morphological alterations. These features include senescence-associated β-galactosidase (SAβ-gal) activity, cell cycle inhibitors (CDKIs), expression of tumour suppressors and the absence of proliferative markers. DNA damage response (DDR) markers, nuclear foci of constitutive heterochromatin, reactive oxygen species (ROS) and prominent secretion of signalling molecules are not characteristics always present in senescence cells, although you may also observe them in a large percentage of these cells. A complete list of features can be seen in table 1. It should be highlighted that, in isolation, none of these markers can unquestionably identify senescent cells, whether in vitro or in vivo.

Senescence is particularly relevant in cancer and ageing, both processes are characterized by a severe accumulation of cellular damage and dysfunctions. Senescence becomes a crucial barrier against cancer progression and senescent cells

accumulate during ageing. The growth arrest (typically occurs in the G1 phase of the cell cycle) prevents the spread of damage to the next cell generation avoiding potential malignant transformation, and it also gives a chance to rectify or clear the damage cells.

Like apoptosis, senescence is an extreme response to cellular stress and is an important tumour- suppressive mechanism. In contrast to apoptosis, though, in which the cytotoxic signals converge to a common mechanism, senescence is typically a delayed stress response involving multiple effector mechanisms. It is not clear what determines whether cells undergo one way or another. However, it is clear that one determining factor is cell type, cases in point, damaged fibroblasts and epithelial cells tend to senesce, whereas damaged lymphocytes tend to undergo apoptosis (Campisi & d'Adda di Fagagna 2007). The nature and intensity of the damage or stress may also be major players. However, it is clear that senescence and apoptosis regulatory systems communicate between them, using p53 tumour suppressor protein as a common regulator of both pathways. This is the reason why p53 may perhaps be considered the biggest regulator of the cell fate (Li et al. 2012).

Table 1: Morphological changes and markers of senescence

Morphological changes	Lack of cell proliferation
	Lack of response for grow factors
	Large, flat and vacuolized morphology*
	SAβ-gal activity
	SASP
	SAHF
	Telomere dysfunction-induced foci
	Occasionally multinucleated*
Molecular Markers	Upregulation of Cyclin dependent Kinase (CDK) examples: p16, p21, p27 ect
	Absence of proliferative markers, e.g. 5-bromodeoxyrudine (BrdU)
	Upregulation of p53
	Upregulation of ARF
	Release of SASP (IL6, IL8, IL1)
	Heterocromatin markers (H3K27me3, H3K9me3, H3.3, ect)
	Levels of ROS increased
	DNA damage response (DDR) markers

*_These are specific in vitro features, in vivo_ senescent cells retain the normal morphology

Senescence, as all the biological processes, has beneficial and detrimental roles. In general, transient induction of senescence followed by tissue remodelling is beneficial because it contributes to the elimination of damaged cells. On the other hand, persistent senescence or the inability to eliminate senescent cells is detrimental.

1.2. Molecular and morphological alterations of senescence

1.2.1 Morphology of senescent cells

Cellular senescence is usually accompanied by morphological changes - in a nutshell, cells become large, flat, vacuolized and, occasionally, multinucleated (Cho et al. 2004). These changes are visible in *in vitro* culture, although, *in vivo* senescent cells retain some of the normal morphology dictated by tissue architecture (Muñoz-Espín & Serrano 2014). The mechanisms underlying such senescence-related morphological alterations are not completely understood, making this phenotype one of those situations where the phenomenon has become generally accepted.

1.2.2 Senescence-associated β-galactosidase

Senescence-associated β-galactosidase activity is based on the increased lysosomal content of senescent cells. This reflects the enlargement of the lysosomal compartment in senescent cells combined with an increase in autophagy (Kurz et al. 2000). Acid b-D-galactosidase (EC 3.2.1.23) is a eukaryotic hydrolase localized in the lysosome when active, this enzyme is enabled for detection by in situ staining. For most of the mammalian cells this detection is made at pH 4.0, for senescence though, it can only be sensed at pH around 6.0 (Lee et al. 2006). This exposure is specific for senescent cells as quiescent or terminally differentiated cells (also cells with no replication capacity) do not have the same distinctiveness. Reasons why SA-β-gal activity has been the most extensively utilized biomarker for senescence not only for the simplicity of the assay but also, its apparent specificity for senescent cells.

Autophagy is the process responsible for the eradication of cellular proteins and damaged or superfluous organelles thus limiting any cell growth instability. This evolutionarily conserved process is characterized by the formation of a double membrane cytosolic vesicles, known as an autophagosome, which sequester

cytoplasmic content and deliver it to the lysosomes (Kurz et al. 2000). Autophagy as an effector mechanism of senescence, is important for the hasty protein remodelling required to make the efficient transition from a proliferative to a senescent state. In order to do this, autophagy must not be disrupted.

In spite of its popularity, SA-β-gal should not be regarded as a unique identifier for senescent cells.

1.2.3 Absence of proliferative markers

Cellular senescence is based on stable cell cycle arrest, the absence of proliferative markers, such as 5-bromodeoxyuridine (BrdU) incorporation is an essential condition to certificate the state of senescence (Campisi & d'Adda di Fagagna 2007). BrdU is a synthetic nucleoside, and a structural analogue of thymidine. Seen by the cells as thymidine, BrdU is going to be incorporated as part of DNA synthesis (during the S phase of the cell cycle). Cells proliferating are going to incorporate more BrdU than cells that have no proliferation rate, for the simple fact that they need to produce more DNA. By detecting the incorporated chemical, we can measure the rate of proliferation. Another method to measure proliferation is by immunostaining of proteins such as proliferating cell nuclear antigen (PCNA) and Ki-67.

These are not a specific markers for senescence, they do not distinguish between senescent cells, quiescent or terminally differentiated cells. Although their absence does not confirm senescence by itself, senescent cannot be characterize without it.

1.2.4 Cyclin-dependent kinases

Progression through the cell-division cycle is regulated by the harmonized activities of cyclin-dependent kinases (CDK) complexes. They are serine/threonine kinases which are modulated by interactions with cyclins and CDK inhibitors (CKIs). In mammalian cells, a well-established cooperation between this trio (CDK, cyclins and CDKIs) plays an indispensable role determining whether cells will continue proliferating or will cease dividing.

Cyclin-dependent kinases can be seen as surveillance checkpoints monitoring the cell cycle and halt its progression if necessary. Which makes it important in processes such as transcription, epigenetic regulation, metabolism, stem cell self-renewal, neuronal functions and spermatogenesis. Currently, there are more than 20, well described, members of the CDK family. All of this members contains a conserved catalytic core made up of an ATP-binding pocket, a PSTAIRE-like cyclin-binding domain and an activating T-loop motif.

In contrast with the CDK family, cyclins belong to a remarkably diverse group of proteins classified exclusively on the ability to couple with the CDK. To achieve CDK activation, a cyclin has to adjoin with the PSTAIRE helix, this will displace the T-loop and expose the substrate-binding interface. The second phase comprises a readjust in critical residues within the active site, briefing it for the phosphorylation.

Whereas most cyclins promote CDK activity, CDKIs restrain CDK activity. CDKIs are comprehended into two classes based on their structure and CDK specificity. The Ink4 family members, which comes down to $p16^{INK4a}$ (also known as CDKn2a), $p15^{INK4b}$ (CDKn2b), $p18^{INK4c}$ (CDKn2c) and $p19^{INK4d}$ (CDKn2d), binds to Cdk4 and Cdk6 inhibiting their kinase activities. This is achieved by interfering with their association with D-type cyclins. On the other hand, the Cip/Kip family members, $p21^{Cip1}$ (CDKn1a), $p27^{Kip1}$

(CDKn1b) and $p57^{Kip2}$ (CDKn1c), have a more broadly spectrum by interfering with the activities of cyclin D-, E-, A- and B-dependent kinase complexes.

The involvement of cell cycle regulators in transcription remains intimately linked to retinoblastoma (Rb)/E2F pathway. In the hypophosphorylated state retinoblastoma protein binds to members of the E2F family, sequestering their transcription factors. Cdk4/6 and Cdk2, in association with their respective catalytic partners D- and E-type cyclins, are responsible for successive phosphorylation of Rb, therefore not allowing Rb to seize E2F transcription factors. By easing its inhibition on E2F, CDK4/6 and CDK2 are authorising the activation of necessary genes to stimulate an S phase entry and a DNA synthesis. However, one upper level of regulation for these cyclin-CDK complexes is achieved by the binding of the CDK inhibitors mentioned above.

As exposed directly above, the kinase-dependent transcriptional control of G1/S transition is well accepted, corresponding events mediating the shift from G2 into M phase are still to be fully understood. FOxM1 is a member of the forkhead box (Fox) superfamily of transcription factors target genes of which include essential regulators of mitosis and components of the spindle assembly checkpoint.(Denicourt & Dowdy 2004). The transcriptional function of FoxM1 is kept silent during most phases of the cell cycle through an auto-inhibition, although, during the G2 phase of the cell cycle, this auto-inhibition is released through CDK2-cyclinA-dependent hyperphosphorylation. In addition, CDK-dependent phosphorylation serves to recruit transcriptional co-activators such as the histone deacetylase p300/CREB binding protein. In the presence of appropriate mitogenic signals involving the Raf/MEK/MAPK signaling pathway, this complex promotes the expression of genes responsible for driving mitotic entry. By proving the impact of phosphorylated Rb and FoxM1 in gene expression patterns, it is easy to conclude that cell cycle regulators can post-translationally modify components of the transcriptional machinery in an effective way so they can control the transitions of the cell cycle, namely G1/S and G2/M (Ogryzko et al. 1996).

While blocking the cell cycle, CDK inhibitors do not deactivate growth-promoting pathways such as mammalian target of rapamycin (mTOR) or Mitogen-activated protein kinases (MAPK). The simplest way to describe it is, while cell cycle is blocked, growth may be stimulated. We may so define CDKIs as common mediators of senescence, comprehending $p16^{INK4a}$, $p15^{INK4b}$, $p21^{Cip1}$, $p27^{Kip1}$, as well as their effectors hypophosphorylated Rb, FoxM1 and p53 (Di et al. 1994). DNA damaging agents as radiation, mitogenic stimulus, oncogenes and tumor suppressors, all induce CDK inhibitors, thus blocking the cell cycle despite continuous growth stimulation (Serrano et al. 1997) (Banks et al. 2014).

1.2.5 DNA damage response

What tells cells to stop dividing?

In 1971, Alexei Olovnikov was thinking about DNA molecules – while he was waiting for the train in Moscow subway station, he was imaging the DNA polymerase to be the train moving along the tunnel and the trails being the double strain DNA. He realises there was a “dead zone” (word use by himself) between the front end of a subway (the engine) and the beginning of the track (Gerontology et al. 1996). Translating that thought to what happens in DNA replication, he recognises that the first bit of DNA would not be replicated as it would be underneath the “engine”.

One year after, James D. Watson was thinking about it as well while preparing one of his lectures for the biochemistry course at Harvard (Greider 1998). He knew that DNA polymerases only assembles nucleotides in a 5’ to 3’ direction and requires an RNA primer to do it. Therefore, when the polymerase reaches the end of a linear DNA molecule, there is a problem in completing replication. He realised that and explained that linear phage genomes can avoid this due to the capacity of join multiple genomes before replication, reducing the number of non-replicative ends, the reason why they can have more division cycles. Interestingly, although both models

recognized the same problem - named the end-replication problem, Olovnikov predicted at the beginning of DNA replication, whereas Watson predicted it at the end. Both models are discussing the telomere shortening.

Telomeres are made of simple repeated DNA sequence ('-TTAGGG-3' in vertebrates) associated with a protein complex called "shelterin" that cap the ends (Salama et al. 2014). This motif was conserved throughout evolution so it can protect the end of a chromosome from deterioration or from fusion with neighbouring chromosomes. Telomeres are subject to attrition though, due to the phenomenon explained above - cells lose among 50 to 200 base pairs of telomeric DNA during each S phase (Anon n.d.).

When they reach a critical minimal length, their protective structure is disrupted. This triggers a DNA damage response. Communication between DNA damage response-associated factors and the cell cycle machinery is carried out by DNA damage Kinases: ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related protein kinase (ATR) and cell cycle checkpoint kinase 1 and 2 (CHK1 and CHK2) (Campisi & d'Adda di Fagagna 2007). The activation of this DNA damage Kinases leads to phosphorylation and activation of several cell cycle proteins, including p53. This would result in an inhibition of some CDK complexes and consequent transient proliferation arrest (quiescence), which would allow the cells to repair their damage (Walter et al. 2015). However, if the DNA damage exceeds an undefined threshold, cells are destined to undergo either apoptosis or senescence. The threshold and the factors that define the different outcomes (apoptosis or senescence) are mainly elusive. Nonetheless, as described previously, the cell type, intensity, duration and nature of the damage are important in this point. But also, the activation of oncogenes or tumor suppressors, including p53 and the p16-Rb pathways, is the main disparity between the senescence, apoptosis or cancer (Pérez-Mancera et al. 2014). Activation of the DDR pathways is involved in both the induction and maintenance of senescence in many cases.

The end-replication problem can be attenuated by telomerase. This enzyme adds telomeric DNA repeats directly to chromosome ends. It contains a catalytic protein component (telomerase reverse transcriptase) and a template RNA component, which helps to prevent the triggering of a DNA-damage response (Collins & Mitchell 2002). In contrast with cells not completed differentiated, somatic cells do not express telomerase, so they are unable to maintain telomeres over time.

In addition to replicative exhaustion there are other ways to achieve DNA Damage Response. Many chemotherapeutic drugs cause severe DNA damage and, as expected, such drugs induce senescence in normal cells. Oncogene activation also causes DDR activation and cellular senescence. Oncogene-induced DNA damage is initiated by alterations in DNA replication (Di Micco et al. 2011). Strong activation of some oncogenes (such as Ras or Raf) induces senescence (Serrano et al. 1997) in a DNA damage response way, and this process appears to be telomere-independent (Jones 2000). Both DNA damage- and telomere-initiated senescence depend strongly on p53 and are usually accompanied by an expression of p21. However, in many cells, DNA damage and dysfunctional telomeres also induce p16 expression.

1.2.6 Oncogene-induced senescence

Earlier studies done in mutant HRAS ($HRAS^{V12}$), accomplish the purpose of immortalizing mammalian cell lines. However, this oncogenitically transformed primary cells are induced in cell cycle arrest, with a phenotype similar to cells in replicative senescence(Serrano et al. 1997). This phenomenon has been come to be known as oncogene-induced senescence (OIS).

As shown before, there are several ways to induce senescence, one of them consist on overexpression of oncogenes. Senescent cells, when expressing high levels of oncogenes, rather than increasing their proliferation, they stop dividing and suffer morphological and molecular changes.

Oncogenes arise from mutant forms of proto-oncogenes, these are harmless genes needed for cell replication and differentiation. They grow into oncogenes due to a mutation, promoting an increase in their expression.

Oncogenic signalling is transmitted through a complex interconnected string of cascades that have ample breaks for self-attenuation through negative-feedback regulation. These negative-feedback is the reason why the activation of an endogenous oncogene does not necessarily translate into the full activation of its downstream effectors.

It is nearly impossible to replicate truthfully *in vitro* what really happens *in vivo*. *In vivo* the influence of a particular oncogene on its downstream effectors increases progressively during tumour development, this is achieved due to the cumulative loss of negative-feedback regulators. As an example, mitogen-activated protein kinase (MAPK) phosphatases, also known as dual-specificity phosphatases, dephosphorylate tyrosine and threonine motifs of MAPK (Toh et al. 2004), and by doing so, they work as negative regulators of MAPK signalling, therefore opposing oncogenic effect of Ras activity.

It has been propose that oncogenic stress increases progressively during tumour development and that senescence is triggered at a point when tumours have already been initiated but have not reached a fully malignant phenotype (Collado 2006). It is important to bear in mind that most studies of OIS have relied on the overexpression of activated oncogenes, although this does not correspond to what happens *in vivo* (Pérez-Mancera et al. 2014). It has been proven that endogenous levels of an

activated oncogene is not only insufficient to trigger senescence but is also extremely inefficient in the generation of tumours. This is the main gap between senescence in preneoplasia and therapy-induced senescence done in laboratories.

1.2.7 Tumour suppressors

The senescent cell is driven to cycle by the "stuck accelerator pedal" (oncogenes) but is blocked by the "powerful brakes" (tumour suppressors) (Blagosklonny 2011). Following the oncogene stimulation, there is activation of tumour suppressors in order to trigger a senescence phenotype.

The p53 and $p16^{INK4a}$-Rb signal transduction cascades are usually activated in senescence. Reason why they also have been used as biomarkers to classify senescent cells.

It is well known that p53 (encoded by TP53 in humans and by Trp53 in mice) plays a major role as a tumour suppressor to the point of being considered the "cellular gatekeeper for growth and division" (Levine 1997). Essentially all of the mechanisms converge to its activation, putting p53 as the major node in all the processes mediating pro-senescence signals. It emerges from unscheduled oncogene activation, telomere dysfunction, DNA damage, and reactive oxygen species. It is no surprise that p53 levels and activity increase during senescence (Serrano et al. 1997). It is an important tumour suppressor that acts by restricting proliferation induction through endorsing various cell cycle checkpoints, and leading the cell to apoptosis or cellular senescence. However, in human cells, $p16^{INK4a}$-Rb signal seems to have a more prominent role than p53.

In human fibroblasts undergoing replicative or premature senescence, Rb accumulates in its hypophosphorylated form (the active conformation of Rb) (Lin et al. 1998). It has been proven that a disruption in the gene encoding the $p16^{INK4a}$ gene

(INK4/ARF locus) predisposes mice and humans to tumour genesis (Qiu et al. 2011). The role of p16 is to inhibit CDK4 and CDK6 (explained in Cyclin-dependent kinases chapter), bringing to a halt the cell cycle division.

Other proteins in the $p16^{INK4a}$-Rb and p53 pathways, especially p21 and $p15^{INK4b}$, are also often accumulated in senescent cells, and have been used as markers to demonstrate the activation of the two major tumour suppressor pathways in senescence.

1.2.8 The senescence-associated heterochromatic foci (SAHF)

Senescent is also associated with a global and drastic change in chromatin. This changes in the chromatin structure endorse altered expression of proteins that affect the chromatin structure. The senescence-associated heterochromatic foci (SAHF) appears to be dependent on p16/Rb pathway activation (Narita et al. 2003).

SAHF represents a highly compacted and heterochromatic form of individual chromosomes. The main objective of SAHFs is contribute to silencing of proliferation-promoting genes in senescent cell, but it has been also linked to the restrain of DNA Damage Response, suggesting that the role of SAHFs is not limited to gene regulation. SAHFs have been strongly correlated with the irreversibility of senescence arrest (Narita et al. 2003), and might be the mechanism which defines the point of "not going back" in terms of growth arrest. Therefore, we can also identifiy senescence by the cytological markers of Senescence-associated heterochromatin foci (SAHFs) and senescence-associated DNA-damage foci (SDFs). They are detected by the auspicious binding of DNA dyes, such as 4',6-diamidino-2-phenylindole (DAPI).

SAHFs are enriched with repressive epigenetic marks, such as histone three Lysine nine trimethylated (H3K9me3) (Narita et al. 2003) and histone three Lysine twenty seven trimethylated (H3K27me3) (Chandra & Narita 2013) and lack of histone three

lysine nine acetylated (H3K9ac) and lysine four trimethylated (H3K4me3) (Narita et al. 2003). SAHF also has proteins that affect the chromatin structure like heterochromatin like protein 1 (HP1) and it is also characterized by the accumulation of HMGA proteins (non-histone chromatin architectural proteins). HP1 is an evolutionary conserved protein with three isoforms in mammals: HP1α, HP1β and HP1γ. They interact with chromatin by binding to H3K9me3 through their chromodomain, it has also been recently reported that HP1 molecules may be directly recruited to DNA through interactions with non-coding satellite RNAs.

SAHFs consists in a multi-layered structure. As you can see in figure 1, in their core SAHFs are enriched with H3K9me3, a constitutive heterochromatin mark. This core is bordered by a ring enriched of H3K27me3, a facultative heterochromatin mark (Chandra & Narita 2013). Interestingly, it has been proposed that SAHFs is likely to be achieved mainly through the spatial rearrangement of pre-existing repressing marks, rather than spreading of heterochromatin. From the general structure and the way it is obtained we may speculate that "global" gene alterations can be facilitated depending on their chromatin positions, but there must be room for some point "local" gene modifications (Di Micco et al. 2011).

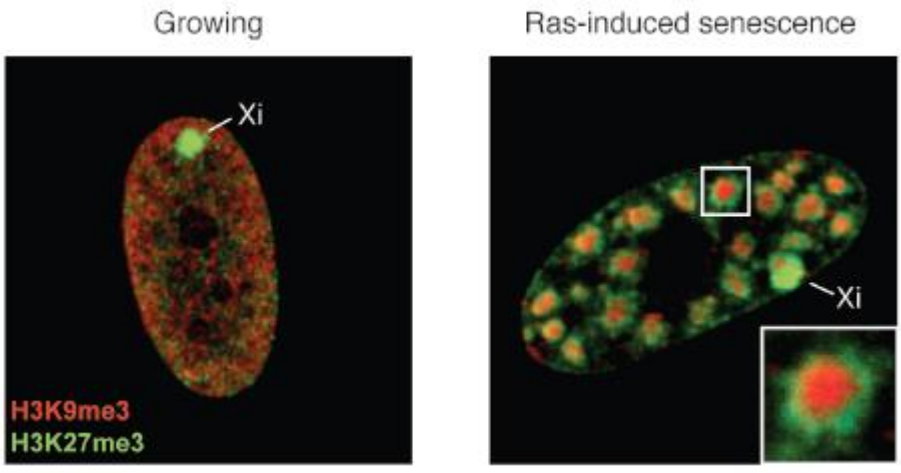


Figure 1: Image A represent a confocal image of a growing cell (on the left) and a Ras-induced senescent cell (on the right). On the left picture you can see the repressive marks (H3K9me3 and H3K27me3) dispersed but always close to the nucleus envelope. Xi represents inactive X chromosome. On the right we have the Ras-induced senescent cell. It is possible to see that the chromatin architecture is changed, forming now a red core (H3K9me3) surrounded by a green ring (H3K27me3). From (Chandra & Narita 2013)

It has been purpose that SAHFs enforces and maintains senescence by suppressing the transcription of proliferative E2F-target genes (Narita et al. 2003). Indeed the promoters of these genes are stably repressed in the senescence condition. However, further evidence is needed as cells with SAHF-like structure aren't able to repress the E2F promoters and their effectors (Fraga & Esteller 2007).

Lastly, it is worth mention that SAHF are preferentially formed during oncogene-induced senescence but not during replicative senescence or during ageing (Di Micco et al. 2011). Furthermore global heterochromatin induction is becoming to be considered a general hallmark of oncogene-induced stress.

1.2.9 Reactive oxygen species

Mitochondria, apart from providing the energy necessary for the survival of cells, is also the major source of reactive oxygen species (ROS) responsible for the damage of proteins, nucleic acids and lipids. Studies in senescent cells have shown mitochondrial changes, which leads to metabolic inefficiency (Passos et al. 2007), morphologic changes (Balaban et al. 2005) and ROS generation (Lee et al. 1999). The mitochondrial density increases in senescent cells (Passos et al. 2007) which evidence suggests is due to a rapid mitochondrial reorganization in response to oxidative stress or be also linked to the metabolism change needed in a senescence phenotype. For example, senescent cells shift they glucose metabolism. Moreover, the mitochondrial dysfunction can cause changes in gene expression via retrograde response pathway. This signalling pathway is triggered by the loss of mitochondrial membrane potential and culminates in the induction of a wide-ranging nuclear target genes (Butow & Avadhani 2004). One of the hallmarks of the retrograde response is its capacity to extend the replicative life span, has shown above in the DNA damage response

chapter, this can lead to senescence. It has been also demonstrated that treatment with antioxidants delays or prevents senescence (Haendeler et al. 2004). On the other hand, factors that lead to increase of ROS, for example ambient with high concentrations of oxygen, accelerate the induction of senescence (Skulachev et al. 2009). The opposite is also valid, as activations of key players of senescence, including loss of telomeric protective functions, DNA damage and oncogene activation, can lead to ROS formation.

Over-expression of activated RAS has been shown to induce senescence and increase ROS levels, as well as the over-expression of the tumour suppressor genes p53 and $p16^{INK4a}$-Rb (Itahana et al. 2003). Correspondingly, p53 transcriptional target, p21, when over-expressed can also induce senescence with a parallel rise in intracellular ROS, which, antioxidants, appear to prevent (Macip et al. 2002).

Although ROS undoubtedly plays an important role in senescence, the nature and mechanism of this contribution remains largely unclear. It seems to add an extra layer of complexity to the already elaborate senescence phenotype. Nonetheless, it might be that ROS is in fact the missing link between the secretory phenotype and its proposed role in the reinforcement of the senescent phenotype. However there are some questions to be answer, the major ones are: what are the cellular targets of ROS in senescent cells? How does the level of ROS increase in senescence?

1.3. Altered in gene expression during senescence due to epigenetic changes

1.3.1 Altered gene expression in senescence

The genetic path to senescence can be seen in a fairly straightforward way: mutation of tumour suppressors and/or oncogenes causes respectively loss or gain of function and abnormal expression (Campisi & d'Adda di Fagagna 2007).

Cells suffer striking changes in gene expression when shifting to senescence. Major changes comprise alterations in cell-cycle inhibitors or activators. Two cell-cycle inhibitors that are often expressed by senescent cells are the cyclin-dependent kinase inhibitors (CDKIs) $p21^{Cip1}$ and $p16^{INK4a}$. The $p21^{Cip1}$ and $p16^{INK4a}$ are components of tumour-suppressor pathways, which are governed by the p53 and retinoblastoma proteins, respectively. Both pathways can establish and maintain the growth arrest that is typical of senescence. They frequently become disrupted in cancer, and consequently the pathways they govern are also disrupted (Kubo & Kaye 2001) (Muller & Vousden 2013).

Senescent cells also repress genes that encode proteins responsible to stimulate or enable cell-cycle progression, cases in point: replication-dependent histones, c-FOS, cyclin A, cyclin B and proliferating cell nuclear antigen (PCNA). E2F is the transcription factor that induces some of the previous proteins, when it comes to senescence E2F is inactivated by a phosphorylated Rb (pRb), this causes an inhibition of the other proteins. Furthermore, in some senescent cells, E2F target genes are silenced by the reorganization of chromatin, also dependent of pRb, a phenomenon already discussed - senescence-associated heterochromatin foci.

Interestingly, many changes in gene expression are unrelated to growth arrest, some of them are related with the senescence-associated secretory phenotype. Many senescent cells overexpress genes that encode secreted proteins that can alter the

tissue microenvironment. The mechanisms responsible for this gene expression revolution are still unknown. Nonetheless these genetic alterations are important, specific and mostly conserved within individual cell types, as an example, it allows senescent fibroblasts to overexpress proteins that mediate local inflammation – interleukin 6 and 8 (IL6 and IL8).

1.3.2 Epigenetics

Cancer has conventionally been viewed as a set of diseases which are motivated by the accumulation of genetic mutations (Hanahan & Weinberg 2011). However, this hypothesis has recently been expanded to incorporate the disruption of epigenetic regulatory mechanisms that are prevalent in cancer (Baylin & Jones 2011) (Sandoval & Esteller 2012). We can affirm that genetic and epigenetic mechanisms are not separate events. For this reason, we need to combine both in order to study senescent phenotype.

Epigenetics is a difficult term to define, with a lot of discrepancy in the scientific community. Adrian Bird, a professor at the University of Edinburgh, who lead the research that described for the first time the CpG islands, said: "Epigenetics is a useful word if you don't know what's going on — if you do, use something else". Clearly there was a necessity for an agreement between the scientific community in order to define how to address at epigenetics. In the near end of 2008 there was a conference involving specialists to discuss, features of epigenetic control, and arrive in a consensus in the until then, a very controversy definition of epigenetics. The best definition was the follow: ‘‘an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence.’’. However, the use of the term ‘‘heritable’’ has been dropped in recent usage, allowing the term epigenetic to mean the information carried by the genome, for example information contained by the chromatin.

To further characterize and understand the mechanisms involved in epigenetics we need to define the three sources that endorse an epigenetic change: epigenator signal, epigenetic Initiator, and maintainers of the epigenetic process (Herceg & Vaissière 2011).

The epigenetic phenotype is triggered by changes in the environment of the cell. Everything occurring upstream of the first event on the chromosome it would be considered as an epigenator signal(Berger et al. 2009). The epigenator signal needs to be transient, remaining in the cell long enough to trigger the epigenetic phenotype but it will not stay for subsequent events. We can state that an epigenator**,** arises from the environment and triggers an intracellular pathway, culminating in the activation of the Epigenetic Initiator (Herceg & Vaissière 2011).

The epigenetic initiator defines the location on a chromosome where the epigenetic change is going to be established. It could be a DNA-binding protein, a noncoding RNA, or any other entity able to define the chromatin structure to be assembled. Distinct from the epigenator, the initiator may not dissipate after its action, but rather may stick it out with the Maintainer (Koerner & Barlow 2010). There are not many well-defined examples of epigenators but noncoding XistRNA, which is sufficient for silencing the mammalian X chromosome, and DNA-binding factors are some specimens of it (Koerner & Barlow 2010).

The epigenetic maintainer signal sustains the chromatin environment in the first and subsequent generations. (Berger et al. 2009). However, it is not sufficient to initiate an epigenetic change by itself. The signal may involve many different pathways, including DNA methylation, histone modifications, histone variants, nucleosome positioning, amongst others. Amongst the maintainers, there are histones deacetylated and DNA methylation at CpG islands.

The role of one particular class of potential maintenance signals - post-translational modifications of histone proteins - requires particular clarification. The term “epigenetic” is not always a correct term to define histone modifications. Many modifications play a role in more dynamic processes such as transcriptional induction and DNA repair.

1.3.2.1 Chromatin modifications

The nucleosome is the fundamental unit of chromatin and it is composed by an octamer of the four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped. On the opposite of what it was used to think some years ago, chromatin is not an inert structure. The chromatin state determines the extent to which genes are active (euchromatin) or silent (heterochromatin), this depends largely on histone modifications (for example, acetylation and methylation).

The histones are predominantly globular except for their (N)-terminal tails which are unstructured, and it is in this tails that most of the modifications are done, affecting inter-nucleosomal interactions. There are a large number of different histone post-translational modifications (PTMs) and it is clear that they perform a fundamental role in most biological processes which involve manipulation and expression of DNA.

Until the time this report was written, there were eight distinct types of modifications in histones: acetylation, lysine methylation, arginine methylation, phosphorylation, ubiquitylation, sumoylation, adenosina difosfato (ADP) ribosylation, deimination and proline isomerization. These modifications play important roles in DNA-dependent processes by regulating DNA transcription, replication and repair. In this report we will focus in acetylation and lysine methylation, all of the others, although interesting, are behind the scope of this thesis.

1.3.2.1.1 Histone acetylation

Histone acetylation is crucial for the control of chromatin structure and thus for the regulation of gene expression. It is also highly dynamic and tightly regulated by the opposing action of two families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs).

Acetylation is consistently associated with activation of transcription, although there are some exceptions. HATs are divided into three main families, GNAT, MYST, and CBP/p300 (Sterner & Berger 2000), with the assistance of acetyl-CoA as cofactor, this enzymes catalyse the transfer of an acetyl group to lysine. This reaction weakens the interaction between histones and DNA because it neutralizes the positive charge of lysine. With the DNA free it is more likely to happen its transcription.

Deacetylation is the reverse of acetylation, an action that restores the positive charge of the lysine, and by doing so it is correlated with transcriptional repression. This modification theoretically stabilizes the local chromatin architecture. There are three distinct families of histone deacetylases: the class I and class II histone deacetylases and the class III NAD-dependant enzymes of the Sir family.

1.3.2.1.2 Lysine Methylation

Unlike acetylation or even phosphorylation, histone methylation does not change the charge of the histone protein (lysine or arginine). It adds a methyl group to the amino acids of histones. To add an extra level of complexity, when considering this modification, bear in mind that lysines may be mono-, di- or tri-methylated.

Lysine methyltransferases have enormous specificity compared to acetyltransferases, meaning that each methyltransferase is correlated with a specific alteration. For example EZH2 is responsible for the methylation of lysine 27 in histone

3 (H3K27), the lysine 9 of histone 3 (H3K9) has a lot of methyltransferases capable of methylate it (SUV39H1, ESET, CLL8, SpClr4 …), but all of them are specific for H3K9 and are unable to methylate any other lysine. The same happens with lysine 4 of histone 3 (H3K4) (MLL1, MLL 2, MLL 3, MLL 4, MLL 5, SET1A, SET1B, …) and lysine 20 histone 3 (H4K20) (Pr-SET 7, Pr-SET 8, SUV4 20H1, …).

Three methylation sites on histones are implicated in activation of transcription - H3K4, lysine 36 of histone 3 (H3K36), and lysine 79 of histone 3 (H3K79). Two of these, H3K4me and H3K36me are correlated with transcriptional elongation. On the other hand, there are three lysine methylation sites connected to transcriptional repression: H3K9, H3K27 and H4K20.

The H3K9 repression involves not only the recruitment of methylating enzymes but also HP1 to the promoter of repressed genes (Muramatsu et al. 2013), both are mediated by Rb and Tripartite motif-containing 28 (Trim28). The H3K27 repression is tangled with HOX genes, which are important during embryo development (Agger et al. 2007), the inactivation of X chromosome and during genomic imprinting. A lot less is known about H4K20 repression, but some studies target this repression to DNA repair (Huyen et al. 2004).

For many years, histone methylation was considered a stable and static modification. But, in 2004, it was suggested demethylation as a potential mechanisms for both lysine and arginine (Shin & Janknecht 2007). Nowadays, it is clear that they will antagonize methylation when requested in the right place at the right time.

1.3.3 Chromatin modifications implicated in senescence

As shown before, histone modification patterns can be very dynamic and subjected to histone-modifying enzymes like the ones previously mention. Relatively little is known overall about the changes in chromatin structure during senescence, and which regulatory networks are implicated in these. The major insight into the contribution of chromatin modifications to senescence are the changes in INK4-ARF locus and the senescence-associated heterochromatin foci. Thus, chromatin modifications compromised in senescence is an emerging field that promises exciting revelations in the near future.

Interestingly, two apparently distinct mechanisms, cellular senescence and cancer have a lot in common when it comes to global epigenetic profiles. Changes in DNA methylation, global hypomethylation of repetitive DNA sequences and regional hypermethylation of some gene promoters and histone posttranslational modifications are very similar between both senescence and cancer.

When it comes to histones post-translational modifications between normal cells and senescent cells there is a different behaviour. The differences can be divided into two groups: global changes and particular changes. The last one consists almost entirely on changes in tumour suppressors.

As covered before, INK4 is a tumour suppressor gene and plays a major role in senescence. Upon replicative or oncogenic stress, the products of this locus accumulate, actively participating in the growth arrest. What happens at a chromatin level is the loss of repressive marks and promotion of acetylation culminating in activation of transcription (Agherbi et al. 2009). This is the procedure for practically all the tumour suppressors active during senescence.

At a molecular level, it has recently been shown that Polycomb proteins are bound to the INK4a/ARF locus and dissociated during senescence (Agherbi et al. 2009). In proliferating cells, INK4 locus is enriched with H3K27me3 marks, due to polycomb repressive complex two (PRC2) which restores the levels of the repressive mark, leading to the recruitment of polycomb repressive complex one (PRC1) to the INK4-ARF locus which consequently is kept repressed (Overhoff et al. 2014). When the switch to replicative senescence happens there is a downregulation of EZH2 and recruitment of JMJD3 (Agherbi et al. 2009), a histone lysine demethylase that catalyses the demethylation of H3K27. The removal of the H3K27me3 mark, and loss of PRC1 binding on INK4-ARF locus predisposes it to activation.

Additionally, MLL1 protein (an H3K4 specific methyltransferase) catalyse the trimethylation of H3K4, which is associated with active transcription. Patterns of methylation at lysine 4 and 27 of histone H3 have been associated with gene activation and repression that are tightly regulated and assumed to specific gene expression programs.

On a global level, the histone modifications relevant to cellular senescence are repressive marks. These include the increase of trimethylation of histone 3 on both lysine 9 and lysine 27 (H3K9me3 and H3K27me3), trimethylation of histone 4 on lysine 20 (H4K20) and loss of methylation in histone 3 lysine 4 and consequent acetylation (H3K4ac) (Decottignies & d'Adda di Fagagna 2011). Promoter CpG-island hypermethylation in cancer cells is also known to be associated with this particular combination of histone marks.

While histone acetylation is generally considered to be correlated with transcriptional activation, H3K9me3 and H3K27me3 are considered the two main silencing mechanisms in mammalian cells. By inhibiting the acetylation and endorsing the last two, the senescent cells are, in general, promoting a heterochromatin state. Do not forget that H3K9me3 and H3K27me3 play a major role in SAHFs and H3K9me3

is often accompanied by DNA methylation (discussed in senescence associated heterochromatin foci chapter). To date, there has been no report on the presence of H4K20me3 marks in SAHF.

The complexity of histone modifications and the role these play on the accessibility of the chromatin in senescence has become evident during the past few years, and it is a huge object of interest nowadays.

1.4. 4-hydroxy-2-nonenal a product of lipid peroxidation

Metabolic processes and environmental conditions cause the constant formation of oxidizing species over the lifetime of cells. In mammalian cells, they are produced intracellularly by several number of mechanisms, being the main one mitochondrial respiration (NADPH oxidase system). The excess generation of these reactive oxygen species has the ability, either directly or indirectly, damage biomolecules (proteins, DNA, lipids).

Oxidative stress is progressively being seen as a main upstream component in the signalling cascade involved in many cellular functions, such as cell proliferation, inflammatory responses, and stimulating adhesion molecule. ROS has been implicated in the activation of transcription factors causing chromatin remodelling and gene expression of pro-inflammatory mediators. Moreover, data have implicated ROS as being a common phenomenon in many senescent cells.

1.4.1 Formation and detoxification of 4-hydroxy-2-nonenal

Elevated rates of lipid peroxidation are usually observed along with increased ROS production (figure 2). ROS are highly reactive and when generated close to cell membranes, they oxidize membrane phospholipids (lipid peroxidation). This process leads to the generation and accumulation of lipid peroxidation products, such as 4-hydroxy-2-nonenal (4-HNE).

Under physiological conditions, cells have production of 4-HNE from different sources. However, the most common source is the endogenous one. Materialises through omega six (ω6) polyunsaturated fatty acids peroxidation, such as linoleic acid and arachidonic acid, with reactive oxygen species (ROS) produced by the mitochondrial electron transport chain (Siems & Grune). Other sources for HNE generation include peroxidation of plasma low-density lipoproteins (LDL), drugs or

exposure to environmental contaminants, such as ethanol or food processed. Because of the basal level of ROS inherent to life under aerobic conditions, there is a basal level of HNE in the cell. The HNE concentration in human blood and serum is estimated to fluctuate between 0.05–0.15 mM, but in pathological situations its concentration can be greatly increased (more than 100 mM) (Siems & Grune).

High concentrations of 4-HNE are prejudicial to the cell. In cells, 4-HNE accumulates in membranes at concentrations around 10µM, but it can be as great as 5mM in cells under oxidative perturbations (Uchida 2003). Mammalian cells have developed multiple enzymatic pathways for the detoxification of HNE. The best characterized of these enzymes include the glutathione S-transferases (GSTs), aldehyde dehydrogenase, and alcohol dehydrogenase. Cells behave differently regarding HNE detoxification. For instance, colonocytes metabolize 100% of 40 mM HNE in 90 minutes (Baradat et al. 2011), whereas hepatocytes are more efficient and metabolize 95% of 100 mM HNE in 3 minutes (Siems et al. 1997). However, there are also studies proving that depletion of HNE can affect gene expression of important genes to senescence, cases in point, Fas, Tp53, p21 and c-myc (Patrick et al. 2005). 4-HNE between a 1 to 10µM range is said to not exert toxic effects (Dalleau et al. 2013).

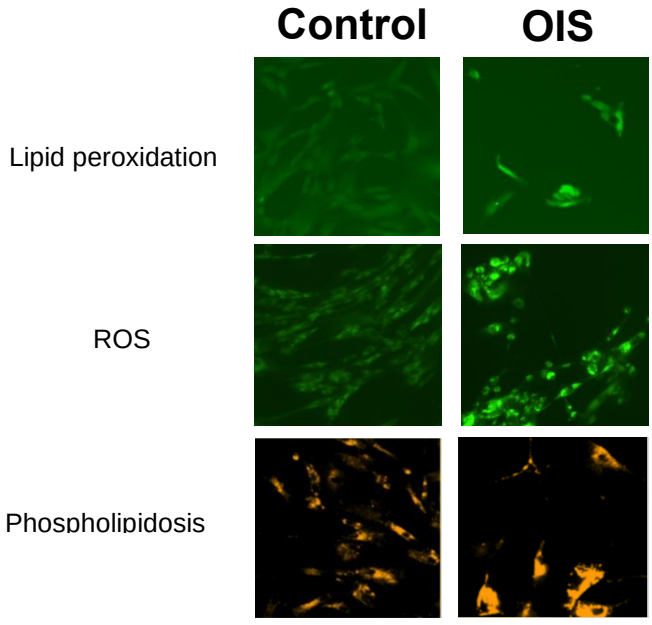


Figure 2: Levels of ROS, lipid peroxidation and phospholipidosis in control cells and oncogene induced senescence. Experiment done in the laboratory

1.4.2 4-hydroxy-2-nonenal structure and reactivity

Despite its relative stability compared with free radicals, the chemical structure of 4-HNE possesses three reactive groups: an aldehyde, a double-bond between carbon 2 and 3, and a hydroxyl group at carbon 4.

Due to its structure, 4-HNE is a highly diffusible molecule, and for that it can pass through membranes and spread beyond its initial production site. Bearing in mind its diffusion capacity, reactivity and longer half-life than ROS, it is not a surprise that this molecule is a good signal propagator. In fact, it has been suggested that 4-HNE can act as an autocrine and paracrine signal molecule (Dalleau et al. 2013).

Figure 3 is a schematic image that represents the structure of 4-HNE, its reactive groups and they main reactions. The carbonyl group of 4-HNE, conjugated with the double bound, attracts the electrons from carbon 4 and endorses the hydroxyl as a powerful withdrawing group. This moiety is labile and exhibits an innate reactivity with available nucleophiles such as sulfhydryl group of cysteine, the imidazole moiety of histidine and the e-amino group of lysine. When combining with HNE, these amino acids, generate a diversity of intra- and intermolecular covalent adducts called Michael-adducts. There is also the possibility to form a pyrrole-type adduct or Schiff base formation between these amino acids and the aldehyde group, but the kinetics are inherently slow and reversible, making Michael-adducts more predominant.

Because of its chemical reactivity, 4-HNE can exert pleiotropic effects. Clear evidence shows that 4-HNE can induce mitochondrial damage (Zhong & Yin 2015). Mitochondria has the capacity to assimilate intracellular stimulus for pro-apoptotic (Liu et al. 1999) and senescent signals. So, HNE can then induce a mitochondrial crisis which can directly affect cell divisions. It has also been shown that HNE can directly affect DNA (due to the double bound between Carbon 2 and 3) leading to the activation of p53 upon DNA damage, and if the stress or damage reach a certain

threshold, p53 can trigger senescence or cell death. Moreover, (Sharma et al. 2008) showed that exogenous HNE can promote the phosphorylation of p53, on serine 15, and its nuclear translocation. Microarrays show that HNE is able to down-regulate cell cycle promoting genes, cyclins (A2, B1, B2, D1, F and K), topoisomerase IIa and DNA polymerase δ (West & Marnett 2005) (Weigel et al. 2002). HNE also upregulates some cell cycle-arresting genes such as Gadd34 (growth arrest and DNA damage) or cyclin G2 (West & Marnett 2005). However, the specificity of HNE is highly dependent on its concentration, the duration of exposure and the cell type.

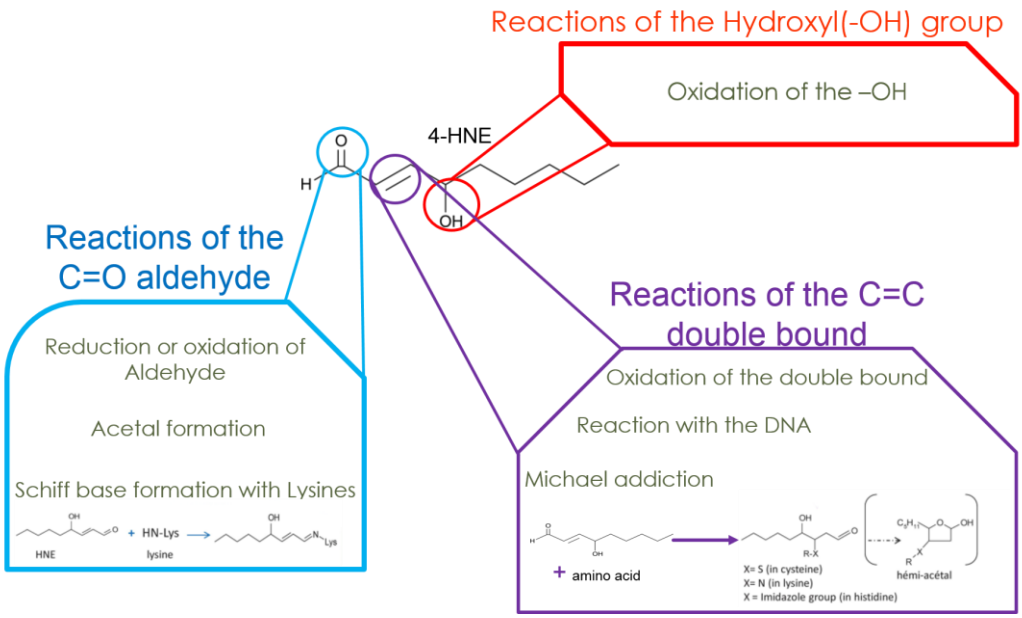


Figure 3: Structure and reactive groups of 4-Hydroxy-2-nonenal, as well as main reactions. The information needed to elaborate this figure was extracted from several papers

1.5. Scope and aims

The main goal of the present study was to research the role of 4-hydroxynonenal as a histone pattern modifier. For this purpose, we incubated 4-HNE in IMR90 with an optimal time and concentration. In addition, we wanted to compare the patterns with the ones obtain from senescent cells, which are well described by the literature, in order to do that we used IMR90 infected with ER:RAS. Lastly, we wanted to see if the effect could be reversed with the use of an HNE scavenger.

The specific aims of this project were:

a. To confirm that 4-hydroxy-2-nonenal strongly links with histone 3

b. To describe the histone patterns changes promoted in non-senescent cells treated with 4-hydroxynonenal;

c. To discern if the changes in histone patterns have a role in the promotion of senescence-associated heterochromatin foci;

d. To examine if the use of scavengers can reverse the 4-hydroxynonenal dependent senescence;

CHAPTER 2: Materials and Methods

2.1. Materials

2.1.1. Chemicals and Reagents

Histone purification kit:

Histone Purification Mini Kit from active motif™ # 400256;

Acetone, Amresco #E646;

Perchloric acid 70% solution in water, Acros Organics #223312500;

Hydrochloric acid 37% in water, Sigma #258148;

Histone Incubation:

Histone H3 Human, Recombinant New England BioLabs™ M2502S;

4-hydroxynonenal Millipore® #393204;

Bovine Serum Albumin, SIGMA™ A9418;

Cell culture:

Dulbecco's Modified Eagle Medium (DMEM), Gibco #41965-039;

0.05% Trypsin EDTA 1x (Trypsin) Gibco #26300054;

Fetal Bovine Serum (FBS) Hyclone #SV3018003;

Antibiotic-Antimycotic (anti-anti) 100x Gibco #1524062;

Cell culture dish 100mmx20mm, Gorning® #430167;

(Z)-4-Hydroxytamoxifen (4-OHT), Sigma™ H7904-SMG;

Western Blots:

Anti-Hidroxy nonenal (HNE J-2) abcam ab48506;

Anti-Trimethyl H3 Lys4 Rabbit (05-745R milipore™);

Anti-Trimethyl H3 Lys9 Rabbit (07-442 milipore™);

Anti-Trimethyl H3 Lys27 Rabbit (07-449 milipore™);

Anti-Histone H3 Rabbit abcam™ ab1971;

Complete Tablets, mini EDTA-free, EASYpack™ Roche 04693159001;

Enhanced chemiluminescence (ECL) Western blot detection, Amersham™ ECL Prime Western blotting Detection Reagent 28-9829-42AD;

Cell Lysis buffer 10x, Cell Signaling™ 9803S;

Sample buffer, laemmli 2x concentrated, Sigma™ S3401-1VL;

Precision Plus Protein™ Dual Xtra Standards, BIO-RAD 161-0377;

NuPAGE 4-12% Bis-Tris Gel, Novex® NP0322BOX;

NuPAGE 16% Bis-Tris Gel, Novex® NP0322BOX;

Pre-Diluted Protein Assay Standards Bovine Serum Albumin (BSA) set, Thermo Scientific™ 23208;

Dried Milk Marvel Original;

iBlot® Gel transfer stacks Nitrocellulose, Regular Life Technologies™;

Protein Assay Dye Reagent Concentrate, BIO-RAD™ 500-0006;

B-galactosidase:

Glutaraldehyde, Sigma™ G5882;

L-carnosine, Sigma C9625-5G (diluted in water);

Anti-BrdU BD Purified Mouse, pharmigen™ 555627;

Anti-P16 (M-156) Santa Cruz Biotechnology™ 62607;

Anti-21 produced in mouse Sigma® P1484;

Phosphate-buffered 4% paraformaldehyde (PFA): FD Neurotechnology (PF101);

Chromatin Immunoprecipitation:

Anti-Histone H3 antibody [mAbcam 10799];

Beads Dynabeads® Protein G, Life technologies™ Novex® 10004D;

IgG mouse sc-2025;

2.1.2. *Solutions*

Phosphate Buffered Saline (PBS): 8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, 0.24g of KH_2PO_4 and adjust volume to 1 litre with l distilled water (dH_2O);

4% PFA: 16% PFA with diluted in 9mL of PBS (Once made, keep at 4ºC and discard after 1 week);

10x TBS: 13.9 g of Tris, 87.66g of NaCl and make up to 1 litre with dH_2O**;**

1x TBS: 100 ml of 10x TBS with and adjust volume to 1 litre with dH_2O**;**

1x TBSTw: 1 ml of Tween-20 in 1Liter of 1x TBS;

Glutaraldehyde solution: 1mL of Glutaraldehyde in 49mL of PBS;

X-Gal staining solution: 9,3ml of PBS/$MgCl_2$ with 0,5ml of KC 20x and 500µL of X-Gal Solution;

Potassium cyanide (KC): 100mM $K_3Fe(CN)_6$ and 100mM $K_4Fe(CN)_6$ $3H_2O$ in PBS;

PBS/MgCl2 solution: 1mM of MgCl2 in PBS 1x, pH6;

DAPI solution: 1ug DAPI per 1ml of PBS;

Blocking solution: 1%BSA with 0.2% Gelatin Fish in PBS;

Triton solution: 0.2% Triton™ X-100 Sigma 1001325622 in PBS;

RIPA Lysis Buffer: 0.5 M EDTA, pH 8.0, 1 M Tris, pH 8.0 with 5 M NaCl, 1 mL of NP-40 (IGEPAL CA-630), 5 mL of 10% sodium deoxycholate, 1mL of 10% SDS and 84ml of dH2O;

RIPA Lysis Buffer with Inhibitors: 10 mL of Ice cold RIPA Lysis Buffer with 0.1 mL of 100X Halt Protease Inhibitor CockTail (make fresh and keep on ice);

Histone incubation buffer Tris–HCl 5 mM, pH 8.0;

2.1.3. Instruments

Incubator HERAcell 150i, Thermo Scientific™;

Odyssey-SA, Lycor™;

ChemiDOC MP Imaging system, BIO-RAD™;

iBlot® Invitrogen™;

Multilabel Counter, Victor™ #1420;

HCA ImageXpress® Micro XL;

2.2 Protocols

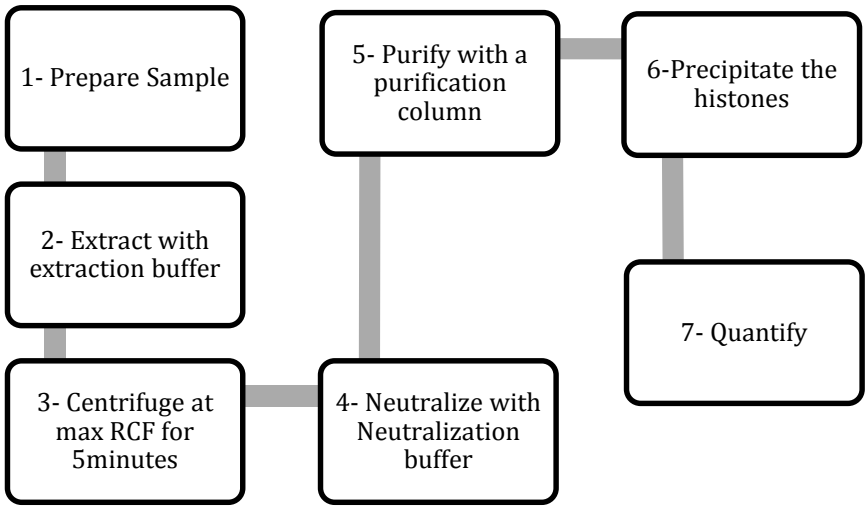
2.2.1 Cell Culture

The types of cells used were: IMR90 cells - human, *Homo sapiens* fibroblasts established from lung tissue. These cells exhibit fibroblast cellular morphology, and are suitable for transfection; and IMR90 ER:RAS - IMR90 cells expressing a chimeric fusion protein with Ras which activates on treatment with 4-hydroxytamoxifen (4-OHT). Cells were cultured at 37ºC 5% CO2 Incubator HERAcell 150i Thermo Scientific™. The medium used consisted on Dulbecco's Modified Eagle Medium (DMEM) from Gibco®, with 10% Fetal Bovine Serum (FBS) Hyclone® and 1% of Antibiotic-Antimycotic (anti-anti) 100x. For cells labelled as positive for tamoxifen it was added 1% of (Z)-4-Hydroxytamoxifen from Sigma™ every time we changed the medium. For cells said to be treated with 4-hydroxynonenal we had 10µM of 4-hydroxynonenal from Millipore®, once 2 hours before harvest.

2.2.2 Histone purification

This protocol is described by fluxogram 1, this will simplify the scrutiny of a result described ahead, in the segment: The histone purification protocol promotes loss of 4-hydroxy-2-nonenal. The cells that passed through the histone purification were: IMR90 cells treated with 4-OHT during 4days (IMR90-Stop), IMR90 cells treated with 4-OHT during 4 days and 4-HNE 2 hours before harvest (IMR90-HNE), IMR90:ER treated with 4-OHT during 4 days (IRAS 4Days) and IMR90:ER treated with 4-OHT during 8 days (IRAS 8Days).

The protocol used is Histone Purification Mini Kit from active motif™ # 400256



Fluxogram 1: Summary of Histone purification main steps.

2.2.3 Histone Incubation

This protocol was used when we wanted to incubate Histone H_3 Human, Recombinant (New England BioLabs™) or Bovine Serum Albumin (SIGMA™) with 4-hydroxynonenal Millipore®.

The histones and the BSA were mixed together with the histone incubation buffer for one hour. It was done as described previously on (Kurien et al. 2011)

2.2.4 Western Blot

Previously to a Western blot it is needed to quantify the samples, so it was used the Bradford technique with pre-Diluted Protein Assay Standards Bovine Serum Albumin (Thermo Scientific™) and Protein Assay Dye Reagent Concentrate, BIO-RAD™. The absorbance was read in a multilabel Counter (Victor®)

Quantified samples were mix with laemmli 2x concentrated (Sigma™) and dH_2O. Samples were loaded on the NuPAGE 4-12% or 16% Bis-Tris Gel (Novex®) and submerged in 3-(N-morpholino) propanesulfonic acid (MOPS). The transfer was done in iBlot® Gel transfer stacks Nitrocellulose (Life Technologies™) in a iBlot® (Invitrogen™) machine.

Membranes were block and incubate with the primary and antibody. The membranes were analysed with two different methods: by immunofluorescence and by highly sensitive chemiluminescent (ECL) system.

Comparing both, the fluorescent system offers an increased time saving over chemiluminescent while producing less background noise. However chemiluminescent is more sensitive and better for proteins hard to detect, like 4-HNE. The primary antibody is the same for both systems, but the secondary is different. The machine for detection was also different, Odyssey-SA (Lycor™) for immunofluorescence and ChemiDOC MP Imaging system from BIO-RAD™ for ECL system.

2.2.5 β-galactosidase for carnosine experiment

For the carnosine β-galactosidase experiment, it was plated half of the IMR90:ER cells with tamoxifen and the other half without. Carnosine treated cells had 20nM and 50nM of L-carnosine (Sigma™). The plate was fixed with glutaraldehyde solution for 15min and stain with X-Gal staining solution. Incubated at 37ºC until the positive control cells turn blue. It was counted the blue and total cells number on an optical microscope. The full extension protocol can be seen in (Bodnar et al. 1998)

2.2.6 Immunofluorescence protocol

For the immunofluorescence we used IMR90 cells treated with 4-OHT during 4days (IMR90-Stop), IMR90 cells treated with 4-OHT during 4 days and 4-HNE 2 hours before harvest (IMR90-HNE), IMR90:ER treated with 4-OHT during 4 days (IRAS 4Days) and IMR90:ER treated with 4-OHT during 8 days (IRAS 8Days).

After the incubation and treatment of the cells described above it was fixed and permeabilised with Phosphate-buffered 4% paraformaldehyde (PFA) and 0.2% Triton solution (sigma™).

Cells were blocked and incubated with different primary antibodies (anti-trimethyl H3 Lys4 rabbit, anti-trimethyl H3 Lys9 rabbit, anti-trimethyl H3 Lys27 Rabbit (all from milipore™), purified Mouse anti-BrdU (BD pharmigen™), anti-p16 and anti-p21 (both from Santa Cruz®)) and secondary antibodies (anti-rabbit alexa Fluor® 568 donkey and anti-mouse alexa fluor® 568 donkey). A nuclear staining was done with DAPI solution. Pictures were acquired and analyze on HCA ImageXpress® Micro XL

2.2.7 Chromatin Immunoprecipitation (ChIP)

The protocol was previously described on (oshizum et al 2007). We performed a chromatin immunoprecipitation for histone H3, for this experiment we used the follow cell types: we used IMR90 cells treated with 4-OHT during 4days (IMR90-Stop), IMR90 cells treated with 4-OHT during 4 days and 4-HNE 2 hours before harvest (IMR90-HNE) and IMR90:ER treated with 4-OHT during 8 days (IRAS 8Days). This protocol can be divided in 3 main steps: beads-Ab incubation, cell lysis and immunoprecipitation.

Beads-Ab incubation started by washes of Dynabeads Protein G (Life technologies™) with a RIPA lysis buffer. After the buffer has been removed the buffer it was

incubated our specific antibody (Anti-Histone H3 antibody from Abcam™) and IgG negative antibody with the clean beads.

Cell lyses began by adding RIPA lysis buffer containing fresh protease inhibitors to the cells. Then the lysates were incubated on ice for 10 to 20min, centrifugated and the supernatant was left shaking over night.

Afterwards it was mixed the beads with the lysates. This mix was incubated and, using a magnet to keep the beads attach to it, the lysates were washed. At the end, the beads were discarded, it was added laemmli to the purified lysates and a Western Blot was run. The final membranes were incubated with anti-histone H3 or anti-4-HNE.

2.2 Statistical analyses

Data is presented as Mean absolute difference (MD) when two or more independent experiments were performed. MD is equal to the absolute difference of two independent values, in this work we will always do MD comparing a cell of interest with our negative control (IMR90-Stop). Therefore, all the significant values (*) are always relating with IMR90-Stop cells. The statistical analyses were done in GraphPad, ImageJ for band quantification and immunofluorescence images and excel™ for support

CHAPTER 3: Results

3.1. Introduction

4-hydroxy-2-nonenal reactions with proteins happen towards cysteine, histidine or lysine. These are the amino acids with more affinity to 4-HNE, but even between them there is an order of preference. 4-HNE is considered a soft electrophile, according to Pearson concept it has more affinity to cysteine sulfhydryl groups (soft nucleophile) than histidine and Lysine nitrogen groups (harder nucleophiles). In fact, cysteine is 1000 more reactive toward 4-HNE than histidine or lysine. However, if cellular toxicant concentrations rise (low μM range) or the exposure durations increases, the available cysteine pool will diminish and adduct formation will shift to residues with lower reactivity, that is, Lysine.

So it does happen that histones are rich in lysine, and they are the main source of histone modifications. Until the time this report was written, to my knowledge, there was only one paper showing that HNE bounds to lysine-rich histones altering their conformation. (Drake et al. 2004). They found alterations in histones H_2A, subsequent to the addition of the lipid peroxidation product - 4-HNE.

3.2 4-hydroxy-2-nonenal bounds to recombinant histone H3

In view of this results, we started considering the contributions that HNE might add to the senescence phenotype. The involvement of 4-hydroxy-2-nonenal in histones and histone modifications was the approach explored.

In order to achieve that, it was planned a protocol involving cells IMR90-Stop (negative control), IMR90-Stop treated with 10µM of 4-HNE (sample of interest) and IMR90-RAS (positive control), all treated with 4 days of tamoxifen. It was also added IMR90-RAS cells treated with 8 days tamoxifen (4-OHT). An illustrative scheme can be seen in table 2, stating: cell type, Days of 4-OHT treatment, HNE treatment conditions and diminutive (easier classification, by which each cell is going to be treated for now on in this report). At the end of there are four different cell types to harvest and analyse.

After cell harvest and before analyse, a multiple day protocol for histone purification is needed. Once all the antibodies were set up and tried in the cell samples above described, the antibody anti-4-HNE did not show any signal in any of the samples. It was hypothesized that there could be some problem with the histone purification protocol.

Table 2. Schematic view of the diferent cell types, their treatment and diminutive by which they are call in this report.

Cell type	Days of 4-OHT treatment	HNE treatment		Diminutive
IMR90-Stop	4 days		Cell harvest and analysis	IMR90-Stop (negative control)
IMR90-Stop		10µM 2Hours before harvest		IMR90-HNE (sample of interest)
IMR90-RAS				IRAS 4days (positive control)
IMR90-RAS	8 days			IRAS 8days

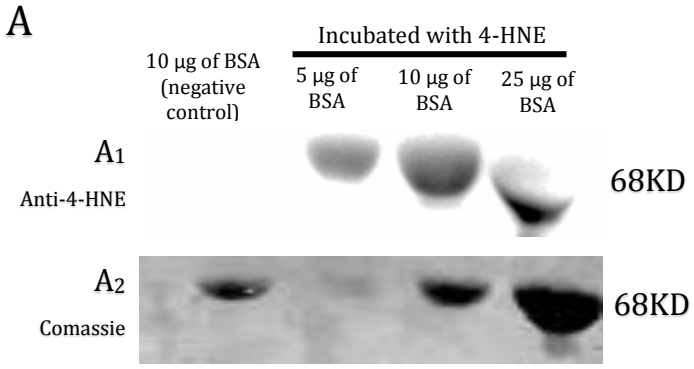
In order to rationalise what was happening it was performed a set of experiments. In figure 4 there are evidence that in truth 4-HNE bounds to histone H3, similar to what was reported by (Drake et al. 2004) with histone H_2a. We also prove that this bound is lost after the purification protocol.

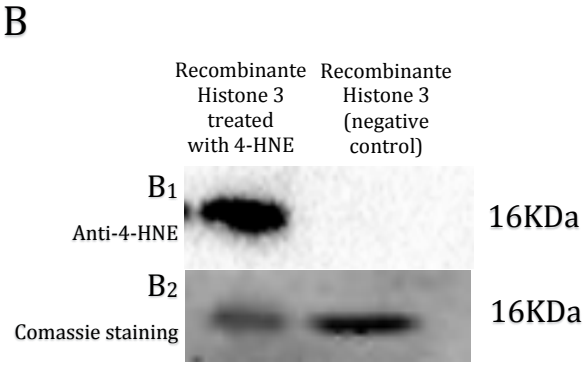
In figure 4 A it was used a wide range concentration of Bovine serum albumin (BSA) incubated with and without 4-HNE. The figure 4 A_1 corresponds to the membrane incubated against anti-4-HNE and figure 4 A_2 is the coomassie staining of the gel. In the first lane, there is 10µg of BSA without 4-HNE, and there was no signal for 4-HNE antibody. In the second, third and fourth lane there are 5µg, 10µg and 25µg of BSA, respectively, incubated with 4-HNE. The bands are at 68 kilo daltons (KDa) and the signal increases consistently as the concentration increases as well.

The evidence which proves that 4-HNE bounds to histone 3 is in figure 4 B. In this experiment it was realized the incubation of recombinant histone H3 with and without 4-HNE, to see if the antibody for 4-HNE could recognize a bound between the two of them. The first lane has 2µg of histones without 4-HNE, whilst the second has 2µg of histones with 4-HNE. A band at 16KDa in the second lane of figure 4 B_1 was recognized by anti-4-HNE antibody. As you can see from the coomassie staining in figure 4 B_2, there is more protein in the second lane, but it is not significant as the anti-HNE membrane does not have any band in that lane. In fact, it gives more strength to the precision of anti-4-HNE antibody.

Now, that it has been identified a bound between histones and 4-HNE, there is a need to justify why was not possible to see the same result when using the histone purification protocol. In figure 4 C it was used the same recombinant histone H3 incubation protocol with and without 4-HNE, but this time we had an extra lane with recombinant histone H3 incubated with 4-HNE that later passed throw histone

purification protocol. Both lane one (histone H3 incubated with 4-HNE that later proceed throw histone purification) and lane two (histone H3 incubated without 4-HNE, negative control) shown no band, whilst lane 3 (histone H3 incubated with 4-HNE) shows a band around 16KDa (figure 4 C_1). In figure 4 C_2 is possible to see that the amount of protein is essentially even between the lanes. Proven by coomassie staining.





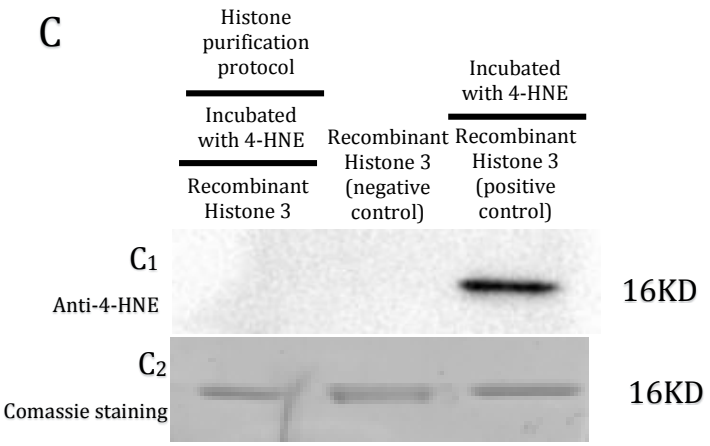


Figure 4. 4-hydroxy-2-nonenal bounds to recombinant histone H3 (**A**) (previous page) 4-HNE was detected by the antibody anti-HNE in a specific way. Different quantities of BSA (5µg, 10µg and 25µg were incubated with 4-HNE (lane 2, 3 and 4). 10µg of BSA were mixed with incubation buffer (lane 1). Membrane **A_1** was incubated with antibody anti-4-HNE, whilst **A_2** is the comassie staining of the gel (**B**) A band of recombinant histones incubated with 4-HNE was detected at 16KDa. Recombinant histone H3 (2µg) was incubated with 4-HNE (10 µg) (lane 1) and without HNE (lane 2) for the period of 1 hour. Membrane **B_1** was incubated with antibody anti-4-HNE, whilst **B_2** is the comassie staining of the gel. (**C**) The 4-HNE signal was lost during due to histone purification. Recombinant histone H3 was incubated with 4-HNE and then purified by the histone purification protocol (lane 1). Recombinant histone H3 was incubated without 4-HNE (lane 2) and with 4-HNE (lane 3)for the period of 1 hour . Membrane **C_1** was incubated with antibody anti-4-HNE, whilst **C_2** is the comassie staining of the gel.

3.3 The histone purification protocol promotes loss of 4-hydroxy-2-nonenal

Lastly, there was a try to understand what was happening in histone purification protocol, has it was not perceptive in what step it was losing 4-HNE. So it was decided to take samples of the histone purification protocol (described in methods) as it was developing.

First of all, it was done the incubation protocol for recombinant histone H3 with HNE, the sample taken from this incubation represents the input and our positive control (lane 1).

From the input we took two samples of 10µL and started the purification protocol with two different concentrations: sample 1 with 30µL of extraction buffer and 10µL of input (step 2 seen in the fluxogram 1) and sample 2 90µL of the same extraction buffer and 10µL of input (lane 2 and 3 respectably).

After adding the extraction buffer there is centrifugation (step 3 of fluxogram 1). Lanes 4 and 5 correspond to sample 1 and 2 pellets of this centrifugation respectably. The next important step is to add neutralization buffer to the supernatant (step 4 from fluxogram 1), after the neutralization buffer has been added for both sample 1 and 2 (lane 6 and 7 respectably) there was a total loss of signal. Lastly, we pass the samples throw the purification column (step 5). Lane 8 and 9 corresponds to samples 1 and 2 of the purification column, respectably. Whilst lane 10 corresponds to the input (control) without any purification step except for the purifying column.

The membrane with the anti-HNE is figure 5 A_1. Lane 1 to 3 have a distinct band at 16KDa, whilst lanes 4 and 5 have a fainted one at the same weight. From lane 6 to 9 there are no bands, whereas lane 10 as a fainted band as well. Figure 5 A_2 represents a membrane staining with an anti-histone H3 antibody, it was done in a different blot with the same quantities as the anti-HNE one. The purpose

was to see if there was lost of histone H3 in the process. All the samples were quantified so 2µg of protein was added. The pellets were small and they were resuspended in laemmli 1x, which made it impossible for quantification. All the pellet sample was loaded in the anti-HNE membrane.

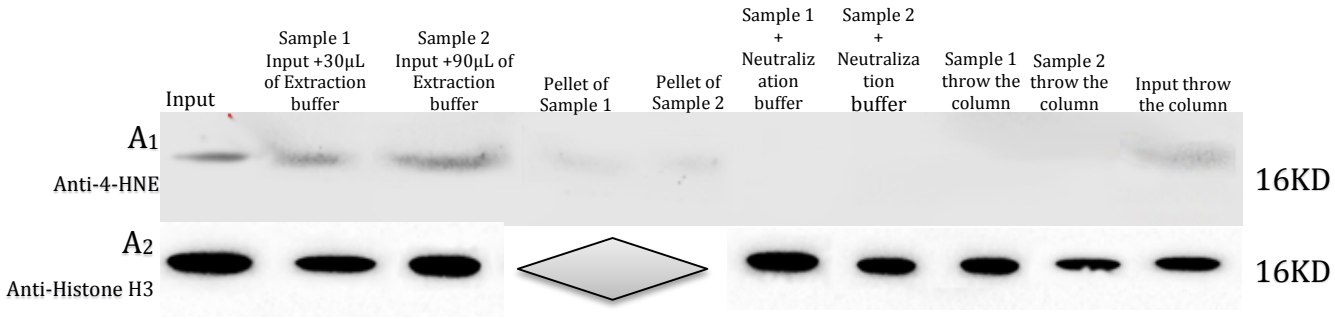


Figure 5. The histone purification protocol promotes loss of 4-hydroxy-2-nonenal Samples from the different histone purification protocol were taken: Input (lane 1); Input plus extraction buffer (lane 2 and 3); Pellets (lane 4 and 5); Input plus extraction buffer plus neutralization buffer (lane 6 and 7) purify through a column (lane 8, 9 and 10). Membrane **A_1** was incubated with antibody anti-4-HNE, whilst **A_2** is incubated with an anti-Histone H3 antibody

3.4 Histones from cells incubated with 4-hydroxynonenal and from senescent cells bind to 4-HNE. On the other hand, non-senescent cells do not show this interaction

When confronted with the results presented directly above, it was necessary to demonstrate the bound between histones H3 and 4-HNE with a different approach. The best way to do it was by a chromatin immunoprecipitation (ChIP). The ChIP was prepared with histone H3 antibody and an IgG negative for control, so everything that is associated with histone H3 would be pull down and detected by Western blot.

The chromatin immunoprecipitation protocol was done for IMR90-Stop, IMR90-HNE and IRAS 8days cells, according to the protocol described in methods.

The lanes are intercalated with IgG and histone H3 pull down. In lane 1 is IMR90-Stop immunoprecipitated with IgG negative. Lane number 2 is IMR90-Stop immunoprecipitated with histone H3. The same happens with IMR90-HNE cells in lane 3 and 4 and IRAS 8days in lane 5 and 6. In figure 6 A_1 the membrane is against histone H3. There are no bands in the IgG lanes (1,3 and 5) at 16KDa which proves that histone H3 was not pull down in this samples. In the anti-HNE membrane (figure 6 A_2) only have intense bands at 16KDa in IMR90-HNE pulled down by histone H3 (lane 4) and IRAS-8Days also pulled down by histone H3 (lane 6)

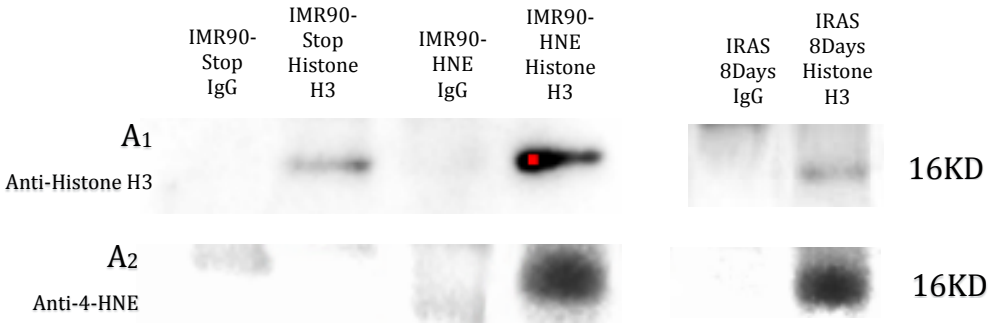


Figure 6. Histones from senescent cells and from cells incubated with 4-HNE bind to 4-hydroxynonenal. On the other hand, normal cells do not have this reaction. After a chromatin immunoprecipitation, it was preform a western blot. Samples from chromatin immunoprecipitation were divided in IgG pull down and Histone H3 pull down. All types of cells has a sample of each intercalated: IMR90-Stop pull down by IgG (lane 1); IMR90-Stop pull down by histone H3 (lane 2); IMR90-4-HNE pull down by IgG (lane 3) IMR90-4-HNE pull down by histone H3 (lane 4); IRAS 8days pull down by IgG (lane 5); IRAS 8 days pull down by histone H3 (lane 6); Membrane **A_1** was incubated with antibody anti-histone H3, whilst **A_2** is incubated with an anti-4-HNE

3.5 4-hydroxynonenal is a histone H3 pattern modifier. Histone H3 lysine 4, 9 and 27 change their methylation pattern due to 4-hydroxynonenal

After it was confirmed the ability of 4-HNE to bound with histone H3, the attention was directed to how this interaction may affect the histone patterns, particularly the ones related to senescence. As explained before in chromatin modifications implicated in senescence chapter, changes in methylation of Lysine 4, 9 and 27 are the most important ones for senescence phenotype. For that reason, they were chosen for our study. This experiment was repeated several times, most of the times with the same result. The graphs X, Y and Z represent the results all of them.

The experiment started with lysis and histone purification of our samples (IMR90-Stop, IMR90-HNE, IRAS 4days and IRAS 8days).

The membrane anti-Histone H3 (figure 7 D) has bands with similar intensity in all the lanes, and the same happens in the coomassie staining (figure 7 E). It is also visible from the staining that the signal is not saturated. In figure 7 A we have a membrane marked against histone 3 lysine 4 trimethylated (H3K4me3) and it is clear that the signal decreases progressively from lane 1 to lane 4. The next image (figure 7 B) represents a membrane stained with anti-histone 3 lysine 9 trimethylated (H3K9me3). In this membrane the first lane (IMR90-Stop) has a fainted band and, although the difference between the three others is not accentuated, the dissimilarity between these ones and the first is noteworthy. Lastly there is figure 7 C, which represents the anti-histone 3 lysine 27 trimethylated (H3K27me3) membrane. In this one there is a similarity between the intensity of lane 1 and 3, fainted bands, and between lane 2 and 4, more intense bands.

The graphs 1 A, B and C show the statistical analyse of different western blots. All the histone modifications were normalized with histone H3. Important to remember that all the significant values (*) are always relating with IMR90-Stop cells.

The graphs show similar results to the ones we can see in the images. Case in point, IMR90-Stop has the highest values in lysine 4 trimethylated and IMR90-HNE although it has less (Mean absolute difference (MD) = 0.46) it does not come close to IRAS 4Days (MD = 0.78) and IRAS 8Days (MD = 0.81), both with two degrees of significance(*). The values in lysine 9 trimethylated are also similar to what is grasp from membranes. IMR90-HNE and IRAS 4Days have close values, with MDs of 0.73 and 0.85 respectively. IRAS 8Days has a bigger difference to IMR90-Stop achieving an MD of 1.1, and two degrees of significance. At last there is lysine 27 trimethylated and the biggest MD is the 1.0 of IMR90-HNE, follow closely by IRAS 8Days (MD = 0.9). IRAS 4Days did not have a significant difference to IMR90-Stop, having an MD of 0.25.

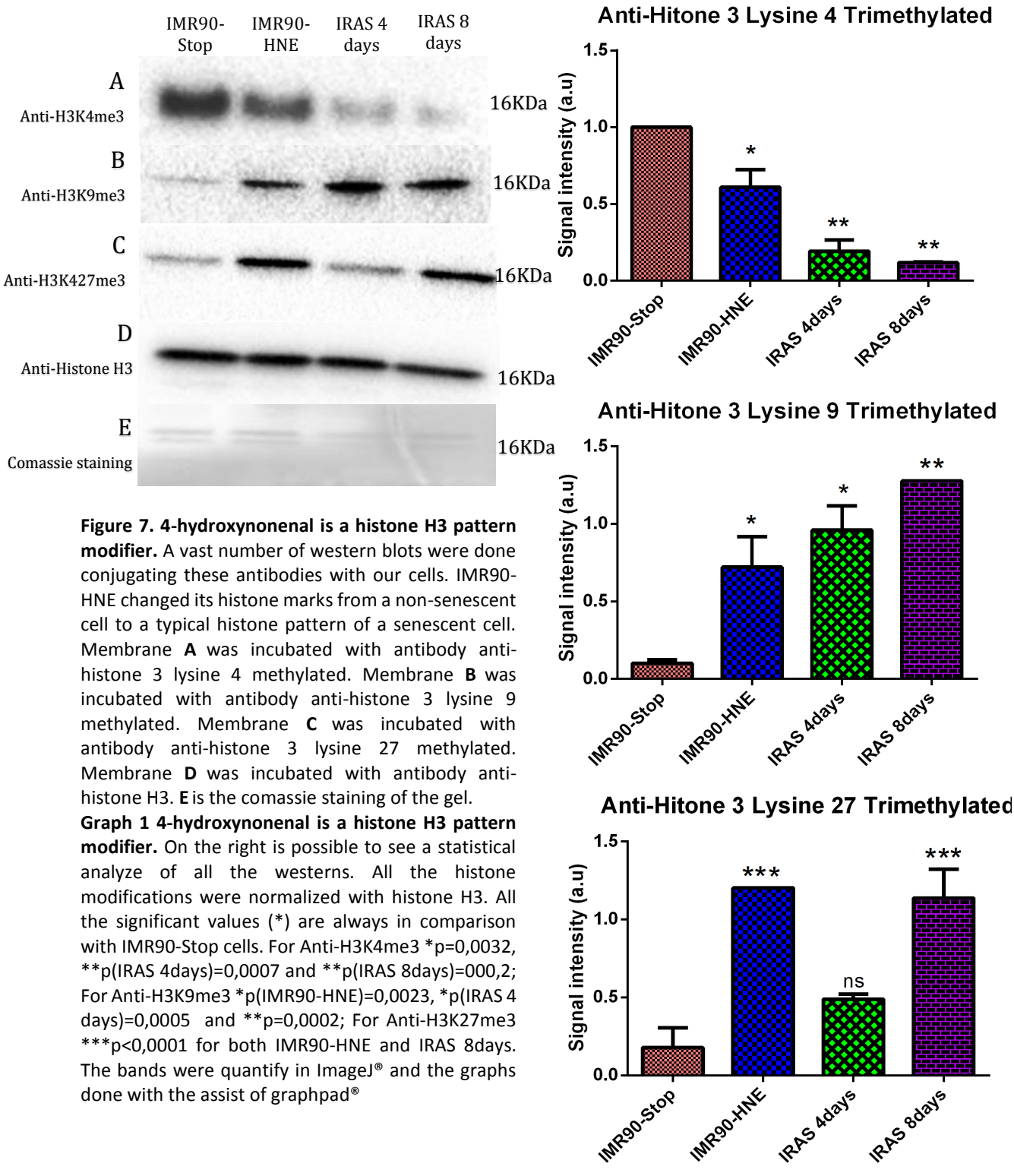


Figure 7. 4-hydroxynonenal is a histone H3 pattern modifier. A vast number of western blots were done conjugating these antibodies with our cells. IMR90-HNE changed its histone marks from a non-senescent cell to a typical histone pattern of a senescent cell. Membrane **A** was incubated with antibody anti-histone 3 lysine 4 methylated. Membrane **B** was incubated with antibody anti-histone 3 lysine 9 methylated. Membrane **C** was incubated with antibody anti-histone 3 lysine 27 methylated. Membrane **D** was incubated with antibody anti-histone H3. **E** is the comassie staining of the gel.

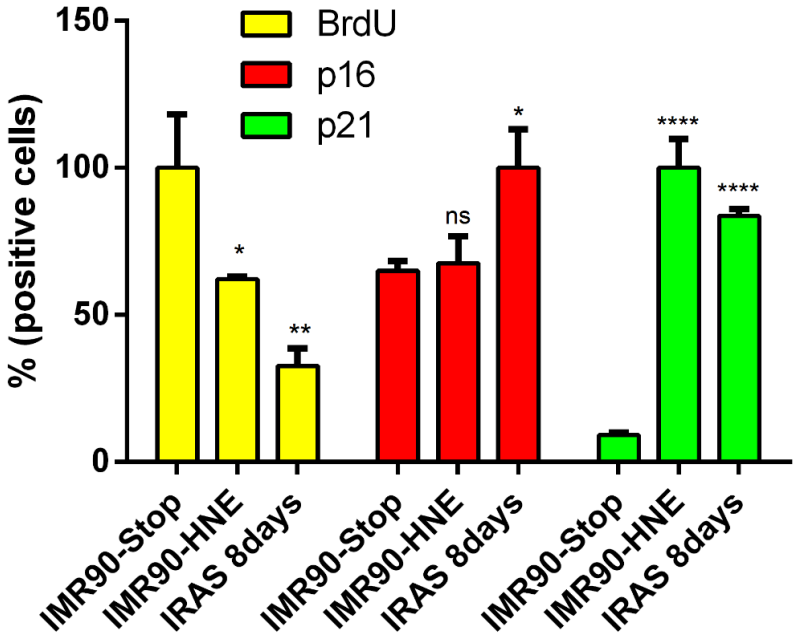
Graph 1 4-hydroxynonenal is a histone H3 pattern modifier. On the right is possible to see a statistical analyze of all the westerns. All the histone modifications were normalized with histone H3. All the significant values (*) are always in comparison with IMR90-Stop cells. For Anti-H3K4me3 *p=0,0032, **p(IRAS 4days)=0,0007 and **p(IRAS 8days)=000,2; For Anti-H3K9me3 *p(IMR90-HNE)=0,0023, *p(IRAS 4 days)=0,0005 and **p=0,0002; For Anti-H3K27me3 ***p<0,0001 for both IMR90-HNE and IRAS 8days. The bands were quantify in ImageJ® and the graphs done with the assist of graphpad®

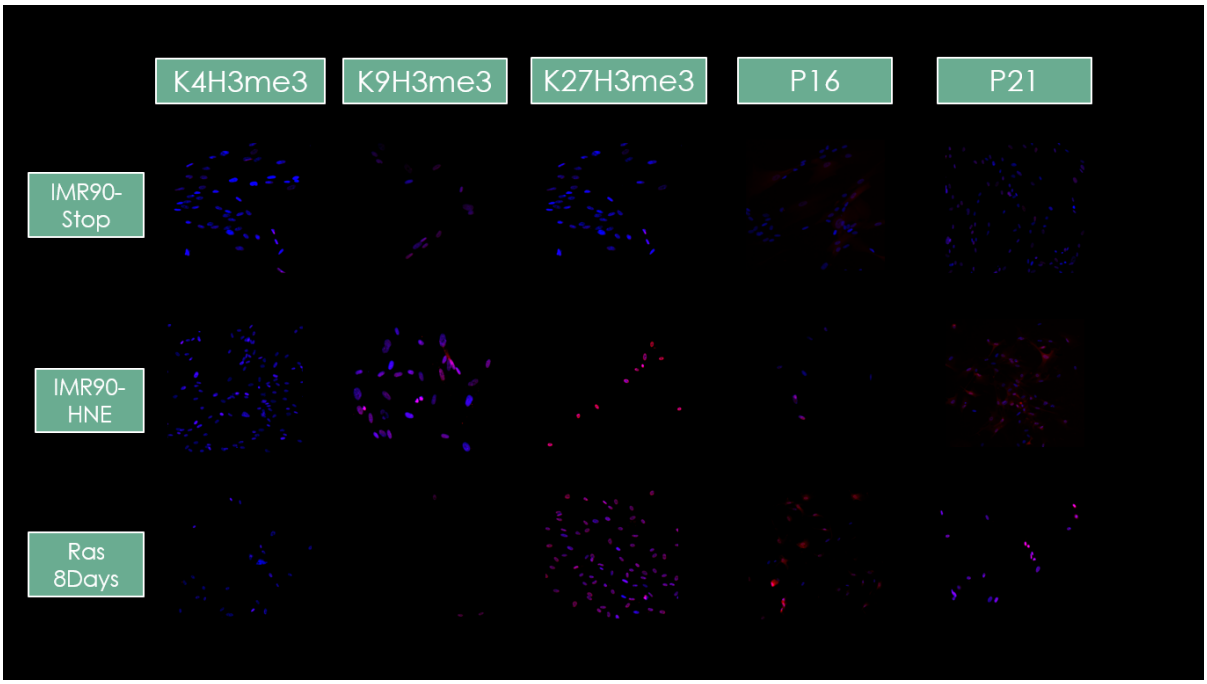
3.6 4-hydroxynonenal treated cells incorporated less BrdU and have an upregulation of p21.

In order to reinforce the study in the histone marks, it was realized an immunofluorescence combining antibodies for histone marks together with some senescent markers.

IMR90-Stop, IMR90-HNE and IRAS 8days were seeded at 2000 cells per well. The plate was fixed and stained for BrdU incorporation, 4',6-diamidino-2-phenylindole (DAPI), anti-H3K4me3, anti-H3K9me3, anti-H3K27me3, anti-p16 and anti-p21, all of them are represented in red except for DAPI which is represented in blue. The graph 2 summarizes the results acquired from the experiment, whilst the figure 8 displays some of the pictures took from the cells.

The result of 5-Bromo-2-Deoxyuridine (BrdU) incorporation was the expected result. There was a significant drop in BrdU incorporation between IMR90-Stop and the other two: IMR90-Stop has a mean absolute difference (MD) of 37.9 to IMR90-HNE and 67.3 to IRAS 8Days. Other senescent markers used were p16 and p21. In opposite to p21, the p16 did not have the results expected. The p16 level was identical between IMR90-Stop and IMR90-HNE (MD = 2.4), the level in IRAS 8days was higher and the MD increased to 34.9 as it was expected. The p21 was more alike with the expected. IMR90-Stop had a low level, whereas IMR90-HNE and IRAS 8days were higher. IMR90-HNE was even higher than IRAS 8days. IMR90-HNE and IRAS 8days had MDs of 90.7 and 75.5 respectively in comparison with IMR90-Stop.


150
100
50
0
% (positive cells)
BrdU
p16
p21
*
**
ns
*
****
****
IMR90-Stop
IMR90-HNE
IRAS 8days
IMR90-Stop
IMR90-HNE
IRAS 8days
IMR90-Stop
IMR90-HNE
IRAS 8days

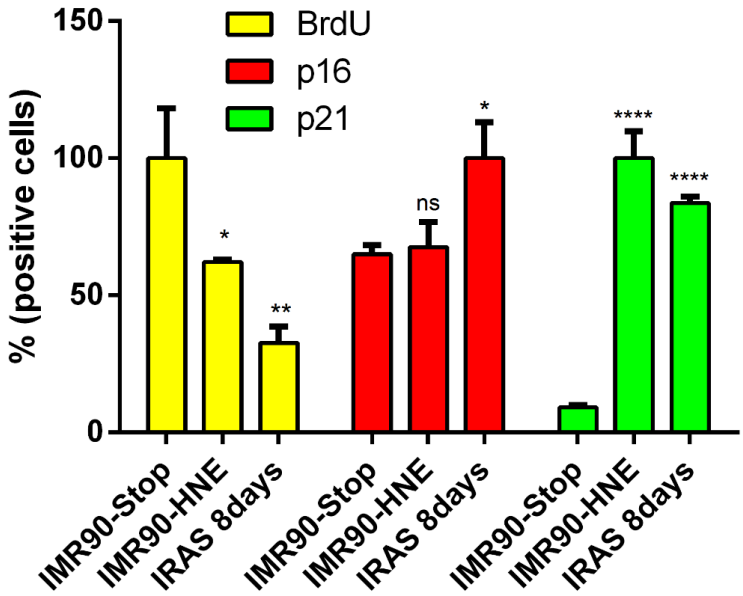

K4H3me3
K9H3me3
K27H3me3
P16
P21
IMR90-Stop
IMR90-HNE
Ras 8Days

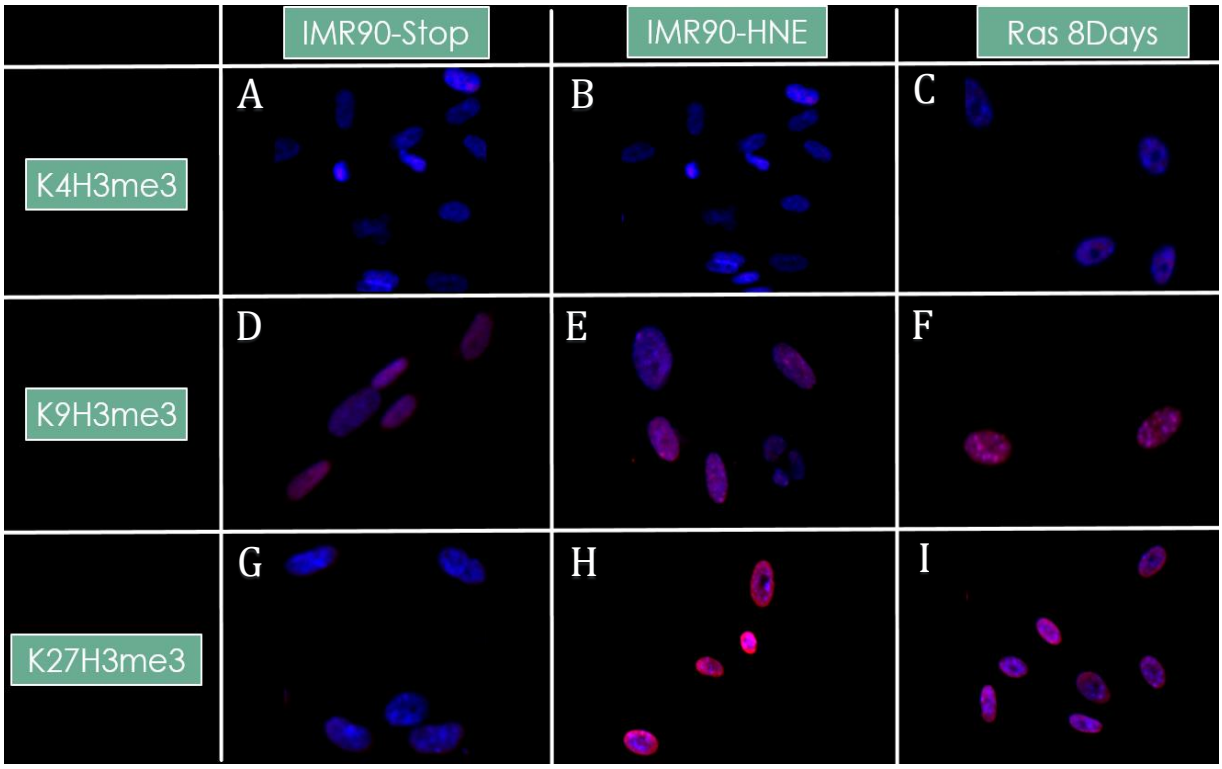
Graph 2 and Figure 8 (previous page): 4-hydroxynonenal treated cells have less proliferation and upregulation of p21. All cells were treated with tamoxifen during 8 Days. IMR90-HNE cells were treated with 10μM of HNE two hours before the staining. BrdU was added 16 hours before the exposure.
The graph represents the statistical information adopted from the immunofluorescence. The figure represents a single slide shoot from the same immunofluorescence.
All the significant values (*) are related with IMR90-Stop. The BrdU decreases significantly from IMR90-Stop to IMR90-HNE and IRAS 8Days. The p16 levels are close between IMR90-Stop and IMR90-HNE, but are elevated in IRAS 8Days. The level of p21 is maximum in IMR90-HNE, and follow closely by IRAS 8Days. In IMR90-Stop the value of p21 is really low.
For BrdU *p=0,0035 and **p=0,0002; for p16 *p=0,008 ; for p21 ****p<0,0001 for both IMR90-HNE and IRAS 8days The graph was done with the assist of graphpad®
In the figure, anti-H3K4me3, anti-H3K9me3, anti-H3K27me3, anti-p16 and anti-p21, are all represented in red and DAPI is stained in blue.

3.7 4-hydroxynonenal treated cells exhibit a senescence-associated heterochromatic foci.

The results for histone marks were correlated with the ones obtained in the previous western blots. The lysine 4 methylation levels decreased from IMR90-Stop to IMR90-HNE with a mean absolute difference of 22.0, but it was not considered significant by graphpad®. The difference was even more to IRAS 8days (MD = 52.9). The opposite happen with methylation of lysine 9: the MD between IMR90-Stop and IMR90-HNE is 29.8 which is considered a significant increase. The increase was even bigger with IRAS 8days 52.9. In lysine 27 trimethylated the results were even more expressive for IRAS 8days, with MD of 86.1, against 30.2 of IMR90-HNE. These results can be seen in graph 3.

After realizing that histones change their patterns in the presence of 4-HNE, it was decided to observe if 4-HNE also has a role in senescence-associated heterochromatin foci formation. So some of the images of the histone marks were amplified, stained and analysed the inner part of the nucleus. The results can be seen in figure 9. Again, anti-H3K4me3, anti-H3K9me3 and anti-H3K27me3 are stained in red, whereas DAPI is stained in blue.





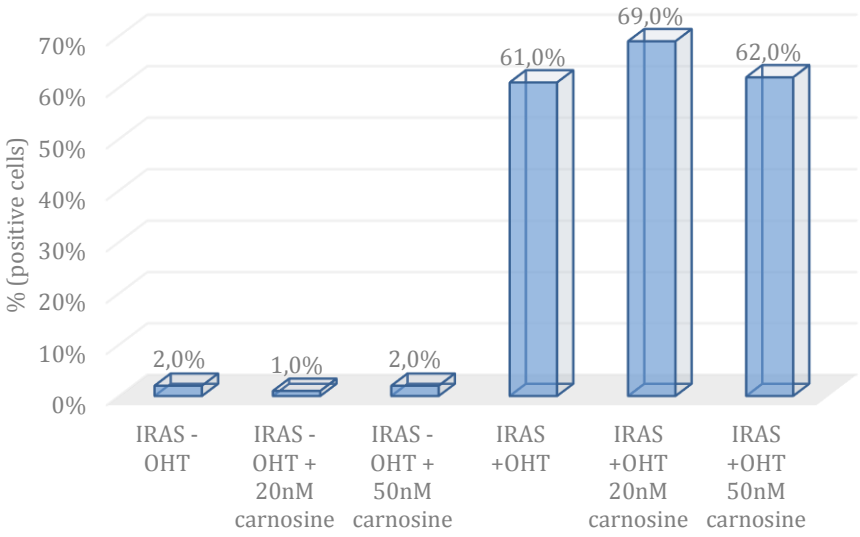
Graph 3 (previous page) and Figure 9: 4-hydroxynonenal plays a role in SAHFs formation. All cells were treated with tamoxifen during 8 Days. IMR90-HNE cells were treated with 10µM of HNE two hours before the staining. BrdU was added 16 hours before the exposure.
The graph represents the statistical information adopted from the immunofluorescence.
All the significant values (*) are related with IMR90-Stop. The K4H3me3 decreases from IMR90-Stop to IMR90-HNE and to IRAS 8Days. Still, the decrease from IMR90-Stop to IMR90-HNE was not significant. The H3K9me3 increased significantly from IMR90-Stop to IMR90-HNE, but the increase was even bigger for IRAS 8Days with three degrees of significance. The level of H3K27me3 also increased significantly from IMR90-Stop to IMR90-HNE, but is IRAS 8Days again with the maximum value.
For H3K4me3 *p=0,0036; for H3K9me3 *p=0,0022 ****p<0,0001; for H3K27me3 *p=0,009 ****p<0,0001 for both IMR90-HNE and IRAS 8days The graph was done with the assist of graphpad®
In the figure, anti-H3K4me3, anti-H3K9me3 and anti-H3K27me3 are stained in red, whereas DAPI is stained in blue.

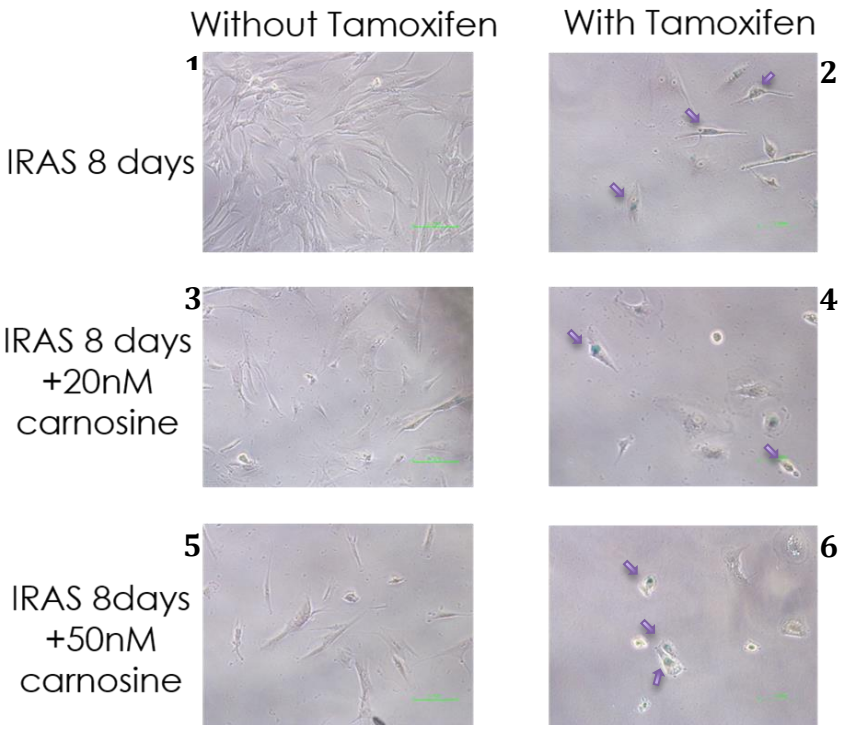
3.8 Carnosine does not interrupt the 4-hydroxynonenal-depedent senescence.

Lastly, it was desired to see if the effect of 4-HNE could be delayed or stopped with the use of a 4-HNE scavenger - carnosine. For this purpose, it was requested the β-galactosidase technique, which is a great marker for senescence. In this experiment, there are only IRAS 8days cells. Half of the IRAS cells (squares 2, 4 and 6) were treated with tamoxifen (senescent cells) whilst the other half (squares 1, 3 and 5) did not have tamoxifen (non-senescent cells). To one-third of the cells it was added 20nM of Carnosine (squares 3 and 4), to another third was added 50nM of Carnosine (squares 5 and 6), and to the rest of them there was no carnosine added (squares 1 and 2).

The percentage of positive cells for β-gal is represented in Graph 4 as a statistical analysis obtained from figure 10. It is considered a positive cell a viable cell with a blue coloration like the ones marked with a yellow arrow. As it was expected IRAS cells without tamoxifen did not have a lot of positive cells, in fact IRAS 8 days without 4-OHT (square 1) and IRAS 8 days without 4-OHT plus 50mM of carnosine (square 5) only have 2% of positive cells (4 blue cells in a total of 200). With even less percentage (1%) is IRAS 8 days without 4-OHT plus 20mM of carnosine (square 3).

The IRAS 8days with 4-OHT had some results unexpected. The 61% of positive cells in IRAS with 4-OHT (square 2) is around the normal values, but it was expected a drop in this percentage when adding carnosine to this cells. Instead, the values went up to 69% and 62% for IRAS 8 days with 4-OHT plus 20mM of carnosine (square 4) and IRAS 8 days with 4-OHT plus 50mM of carnosine (square 6), accordingly.





Graph 4 and Figure 10: Carnosine does not interrupt the 4-hydroxynonenal-depedent senescence. Carnosine has a toxic effect on cells. Cells described as positive for tamoxifen grow with it during 8 Days. All the cells were stained with β-gal. Cells were observe by optical microscopy.

The graph represents the statistical information adopted from the β-gal counting. IRAS 8 days without 4-OHT (square 1) only have 2% of positive cells the same ones as IRAS 8 days without 4-OHT plus 50mM of carnosine (square 5). IRAS 8 days without 4-OHT plus 20mM of carnosine had the lowest percentage, 1%. 61% of the cells were positive for β-gal in IRAS with 4-OHT. IRAS 8 days with 4-OHT plus 20mM and IRAS 8 days with 4-OHT plus 50mM have 69% and 62% respectively. The graph was done with the assist of excel™

3.9 Carnosine does not interrupt the 4-hydroxynonenal-depedent senescence.

In order to explain this results, the experiment was repeated but this time it was not stained for β-gal, in lieu cells were visualized through an optical microscope (figure 11). The cells without carnosine had the expected result. IRAS without OHT were confluent and viable, whereas IRAS with OHT (square 2) had fewer cells and the cells appear to have a long and flat morphology, some of them are indicated with a green arrow.

All the wells to which carnosine has been added had a lot of dead cells, their appearance was round and pale and are indicated with a black arrow. In top of that, there were a lot of rounded and bright membranes with a characteristic morphology of vesicles (marked with a yellow star), a signal of a highly noxious effect.

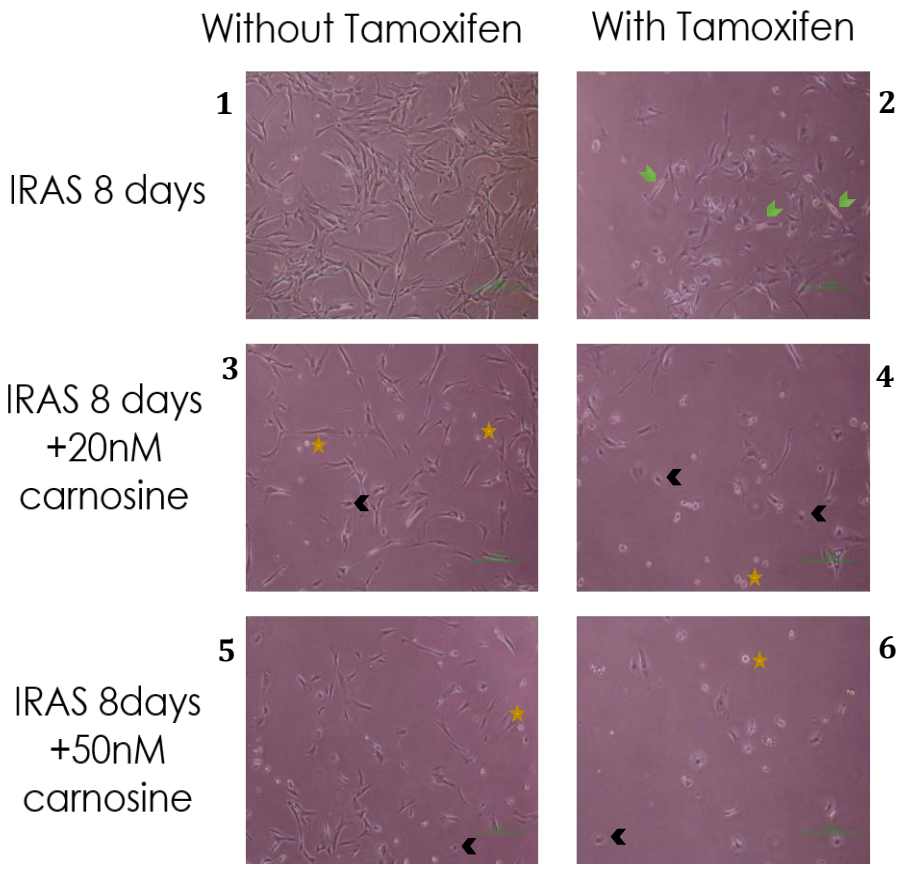


Figure 11: Carnosine has a toxic effect on cells. Cells described as positive for tamoxifen grow with it during 8 Days. Cells were observe by optical microscopy.
The green arrows represented in the figure, point to senescent cells. The black arrows indicate the dead cells, whereas the yellow stars inform the position of vesicles.

4: Discussion

4.1. Levels of lipid peroxidation and its product, 4-hydroxy-2-nonenal, increase during senescence

One of the main focus of this study is to discuss the importance of 4-hydroxy-2-nonenal in senescent phenotype. Some studies have demonstrated a increased 4-hydroxy-2-nonenal levels in cancer tissues (Karihtala et al. 2011) but few information is found about the same levels in senescent cells. Taking that into consideration, the first important step was to demonstrate a correlation between lipid peroxidation, 4-HNE and senescence.

Experiments previously done in the laboratory can not be shown because they are going to be published. However they are important for the story, reason why it is important to recreate them.

4-HNE is one of the main products of lipid peroxidation (Schneider et al. 2008), therefore when lipid peroxidation increases 4-HNE also increases. As mention in the introduction lipid peroxidation increases during senescence, although there is always a basal level of it, especially in culture cells which are under of a lot of stress. This stress is correlated with an increase in reactive oxygen species (Halliwell et al. 2014) and the consequent generation of lipid peroxidation products. In this experiment there was three types of cells: human fibroblasts (non-senescent cells), cells expressing RAS oncogene (senescent cells), and cells expressing RAS oncogene but with inhibitors of p53 and RB (cells bypassing senescence). The main conclusion acquire from this study was: senescent cells increase the percentage of lipid peroxidation and 4-HNE accordingly, non-senescent cells and cells bypassing senescence just maintained the basal level.

This not only evidences that 4-HNE increases during senescence but it also pronounces that this increase is p53/Rb dependent.

4.2. 4-hydroxy-2-nonenal bounds to recombinant histone H3

Ultimately, the main aim is to find if 4-hydroxy-2-nonenal modifies histones and if it plays an important role on post-translational modifications in histones. The experiment resume in figure 4 was the first step to fulfil that goal.

The experiment expressed in figure 4 A tries to demonstrate that both 4-hydroxy-2-nonenal and the antibody anti-4-hydroxy-2-nonenal are working properly. The fact that all the lanes incubated with 4-HNE have a band against 4-HNE and the lane that does not have a band is the one without 4-HNE incubation is a signal that the antibody is specific. In fact, it looks also accurate, as the intensity of the bands increase with the concentration of BSA. Important fact to mention is that this antibody does not detect 4-HNE free, it only detects Michael adducts formed specifically by 4-HNE with an unspecific protein. This interaction is unable for detection if 4-HNE is lower than 1µM. The reason why we used bovine serum albumin in this experiment is because it is rich in lysine and is a protein well described in the literature.

Although it was with recombinant histones, the figure 4 B is our first prove of a link between histones and 4-HNE. The lane with recombinant histone H3 incubated with 4-HNE presented an intense band, in contrast, recombinant histone H3 without 4-HNE does not have a band. The band is at a molecular weight of 16kDa, normally histones have a molecular weight of 15KDa. The anti-4-HNE band appears a kilo Dalton above the molecular weight of the protein with which it is linked.

At this point, we were having a problem. As seen previously we were able to see 4-HNE in membranes when it was added to recombinant histone H3 but we could not see it in membranes when we were using histones purified from our cells. Due to that fact we decided to do the same incubation but pass the cells through our purification protocol, described in methods. The result was clear, the recombinant histone H3 incubated with 4-HNE that proceed through the histone purification lose the bound

with 4-HNE or, as a second hypothesis, the bound was transformed (figure 4 C). Without knowing the strength and nature of the ligation between histone H3 and 4-HNE is hard to know if there is a possibility to its disruption. As a comparison, the methyl groups covalently bound to histones were not lost during the protocol. However, the fact is anti-4-HNE band seen after histone H3 incubation with 4-HNE (lane 3) is lost after the purification (lane 1).

4.3. The histone purification protocol promotes loss of 4-hydroxy-2-nonenal

Considering the last results, we decided to figure out where we were losing 4-HNE in the histone purification protocol. This would allow us to think in a possible change of step without compromising the purification.

In figure 5, lane 2 and 3 do not show a loss of signal, concluding that 4-HNE is not lost in this step. The lanes 4 and 5 have a fainted signal this implies that we have 4-HNE in the pellet, which is not good has the pellet is usually discarded. For 4-HNE be seen in the pellet, it might have happened a small precipitation of some of the histones, or other protein that has a molecular weight close to 16KDa and has an affinity for 4-HNE. After those lanes, none of the other steps has a band until the last one. The last one is the input passing through the column, the band looks fainted so probably some of the 4-HNE is also lost in the column step. At the end of this experiment, we might have more questions than the ones that we started. The main one is the still uncertainty about the crucial step where we are losing 4-HNE. Because of that we decided to go in another direction.

4.4. The histone purification protocol promotes loss of 4-hydroxy-2-nonenal

We could not advance without having a clear evidence that 4-HNE is in fact in our cells and reacts with histone H3. There were a vast number of techniques that we could use, but the chromatin immunoprecipitation look like the most appropriate one. By targeting the histone H3, the chromatin immunoprecipitation allows to pull down every protein attach to it. Is important to refer that this was a one shoot experiment.

Together with the anti-histone H3 antibody it was also used an anti-IgG negative for control purposes. To be a trustful band, it needs to appear only in the lanes where we have our antibodies of interest, in this case anti-histone H3, and should be absent from anti-IgG negative lane.

Figure 6 is divided in images of two membranes, the first represents an anti-histone H3 membrane. There are no bands at 16KDa in IgG lanes, which is a sign that there was no contamination and histone 3 was not present in those samples. The lanes that comprise the histone H3 pull down all have bands at 16KDa which demonstrates a successful chromatin immunoprecipitation. The only downfall of this membrane is the fact that IMR90-HNE with histone 3 pull down (lane 4) has more histone than the other two.

The anti-4-HNE membrane was more difficult to work with. The anti-4-HNE is produced in mouse, therefore it is needed an anti-mouse to recognize it. The problem is the anti-mouse not only recognizes the 4-HNE but also a lot of unspecific proteins from the beads. This generated a lot of background noise and as a consequence a lot of difficulties when detecting the 4-HNE band. Nonetheless, in the end we were able to detect a good size band at 16KDa in both IMR90-HNE and IMR90-RAS 8 days, the only downfall is the fainted band in IMR90-HNE IgG pull down (lane 3).

Putting this together with a lack of a band in IMR90-Stop, was the evidence needed. This results demonstrate that we were able to detect a bound between 4-HNE and histone H3 in our 4-HNE treated cells and senescent cells. This is a great achievement that allow us to proceed and validate the next results.

4.5. 4-hydroxynonenal is a histone H3 pattern modifier. Histone H3 lysine 4, 9 and 27 change their methylation pattern due to 4-hydroxynonenal

There were already reports of 4-hydroxy-2-nonenal as a carcinogenic promoter (Pizzimenti et al. 2010), as an inhibitor of proliferation (Sunjic et al. 2005) and a promoter of histone oxidation (Drake et al. 2004), although, to our knowledge, there is not yet paper reporting 4-hydroxy-2-nonenal has a histone pattern modifier. In figure 8 is represented one of several repeated blots with analogous results, and graphs 1, 2 and 3 represent their outcomes.

As explained before in the introduction (Chromatin modifications implicated in senescence segment) there is a change on the global epigenetic structure, and it is mostly characterize by: increase of trimethylation of histone 3 on both lysine 9 and lysine 27 (H3K9me3 and H3K27me3), and loss of methylation in histone 3 lysine 4. This is a generally accepted feature reported in a great number of papers. Cases in point: (Chandra et al. 2012), (Esteller 2007) and (Decottignies & d'Adda di Fagagna 2011).

By analysing the blots we can say the histone H3 is even between all of the conditions. The membrane anti-Histone H3 (figure 7D) works as a control, to see if the same amount of histones has been added, whilst the coomassie staining (figure 7E) works as a control for anti-histone H3 membrane, it makes sure that the signal is not saturated. After we made sure that the controls are inside of the conditions, we can analyse the results of interest.

The membrane against histone 3 lysine 4 trimethylated has a high-intensity band in IMR90-Stop (non-senescent cells) (lane 1), the IRAS 4 days and 8 days have a huge decrease of intensity which is according with literature. IMR90-HNE (lane 2) did not have a drop as big as the IRAS cells, but it still is a significant difference. The graph for lysine shows the same results, IMR90-HNE has a significant loss of H3K4me3 but is not as significant as the IRAS cells. In IMR90-HNE, the drop of lysine 4 methylation levels represents a cut down on an activation mark and an approximation to a senescence-like epigenetic phenotype.

The anti-histone 3 lysine 9 trimethylated membrane shows what was expected in non-senescent and senescent cells. The level of H3K9me3 in IMR90-Stop is low, whilst in IRAS cells, 4 and 8 days, is higher. Elevated levels of H3K9me3 is characteristic of a heterochromatin state (Stewart et al. 2005), always present in senescent cells. 4-HNE mimics the results of IRAS cells, having a band with similar intensity to this ones. Again, the graph is comparable with what we see in the image.

To finish, we have histone 3 lysine 27 trimethylated another histone pattern characteristic of heterochromatin (Kouzarides 2007). For what we can observe from the image and the graph, it happens a similar change to what we see in H3K9me3, but H3K27me3 has a small change. IMR90-Stop is still low in this histone pattern and IRAS 8 days still has an intense band. However, this time IRAS 4days does not have a significant difference for IMR90-Stop. The cells with higher levels of H3K9me3 are IMR90-HNE. This goes against what (Drake et al. 2004) reported with histones H2A. They said that 4-HNE has a bigger affinity to histones previously acetylated. But usually, in non-senescent cells, H3K9 is the one acetylated and needs to lose this acetylation in order to become methylated. The most likely hypothesis for this result is that H3K27me3 only increases during late senescence (after the fourth day). Most likely does not have an important work during early senescence delaying its activation for when it is needed.

4.6. 4-hydroxynonenal treated cells incorporated less BrdU and have an upregulation of p21.

As described before, the lack of proliferative markers is one of the features for a senescence phenotype, but we cannot declare a senescence state only in base of that, because it does not distinguish between the different types of proliferation arrest (senescent, quiescent or terminally differentiated cells), therefore there is a need for supplementary information. The p53 and $p16^{INK4a}$-Rb signal transduction cascades are usually activated in senescence (Serrano et al. 1997). The p21 is a downstream protein of p53 and it has been used as biomarkers to classify senescent cells. In this immunofluorescence, we used both p21 and p16 as complementary information to the proliferative marker, BrdU.

The BrdU incorporation gives us information about the strength of 4-HNE as a cell cycle inhibitor and how the proliferation was affected on each cell types. As it is possible to observe in graph 2, Brdu has a significant reduce when comparing IMR90-STOP with IMR90-HNE and IRAS 8Days, although IMR90-HNE it is still far from the reduced observed in IRAS 8Days. The IRAS 8Days incorporated two-thirds less of BrdU than IMR90-STOP.

The results for p16 were not the expected. There is no significant difference between IMR90-Stop and IMR90-HNE. P16 is an important protein in human senescence. A senescence with high levels of p16 becomes almost irreversible, even with the inhibition of p53 (Beauséjour et al. 2003), thus p16 provides a second barrier against proliferation. This barrier is the formation of SAHFs which is p16-Rb dependent, as discussed in the introduction (The senescence-associated heterochromatic foci section). In order to have SAHFs we need to have p16 and as we are going to see in the next segment 4-HNE induces a SAHFs phenotype, so it is only logical that it also increases p16 levels. The justification for this result might be the few number of cells and they conditions. As it is possible to observe, the IMR90-HNE cells for p16 are in

low number and in bad condition (figure 8). This can happen when the wells get dry, especially the ones on the end of the plate as it was the case with p16.

The p21 is close to what is expected. In fact, IMR90-HNE has high levels of p21, bigger than IRAS 8days. The increase in p21 levels needs to be associated with the increase of its upstream factor – p53.

Considering the results of p21 and BrdU we could affirm that 10µg 4-HNE, is enough to promote a senescence phenotype.

4.7. 4-hydroxynonenal treated cells exhibit a senescence-associated heterochromatic foci.

As explained before, senescence-associated heterochromatic foci is enriched with repressive epigenetic marks, such as histone 3 Lysine 9 trimethylated (H3K9me3) and histone 3 Lysine 27 trimethylated (H3K27me3)(Chandra et al. 2012), and lack of histone 3 lysine 9 acetylated (H3K9ac)(Narita et al. 2003). This are the same histone patterns that we observed in previous western blots, and we observe now as well in the immunofluorescence. Is important to refer that with two different techniques we acquired the matching results.

SAHFs is an important mechanism because defines the point of “not going back” in terms of growth arrest, and indeed 4-HNE promotes these chromatin structure. If we look to figure 9 we can see the nuclear DNA domains stained densely by DAPI (blue). In IMR90-Stop we can see the blue coloration dispersed through all the cell, whilst in IMR90-HNE and IRAS 8days we can see “nodes” where the DAPI (the DNA) concentrates. This is one of the chromatin rearrangement features promoted by SAHF.

The other is the already described multi-layered structure with an H3K9me3 core bordered by a ring enriched with H3K27me3 (figure 2). In figure 9 we can see high levels of H3K9me3 in squares E and F (IMR90-HNE and IRAS 8 days respectively) and H3K27me3 in squares H and I (IMR90-HNE and IRAS 8 days respectively). In normal cells, these repressive marks are close to the nucleus membrane, but because in SAHF the nucleus architecture gets disrupted, the repressive marks do not obey to these rule and disperse through the cells.

4.8. Carnosine does not interrupt the 4-hydroxynonenal-depedent senescence.

Tumor cells can exhibit higher expression of detoxification enzymes and antioxidant proteins that permit a better HNE extrusion (DeNicola et al. 2011). And it is also been proven that antioxidant proteins suspend senescence (Macip et al. 2002). This has been discussed more deeply in the introduction (reactive oxygen species segment). A 4-HNE-specific quencher, L-carnosine, immobilizes and expels 4-HNE from the cell (Carini et al.) (DeNicola et al. 2011) (Liu et al. 2003), and thus we tested it to see if had some effects in our senescent cells.

By adding carnosine to cells IRAS 8 days, we were expecting an improvement in senescence. To test our hypothesis we did a β-gal test described in the results section figure 10 and graph 3. As expected, the non-senescent cells (IRAS 8 days without 4-OHT, IRAS 8 days without 4-OHT plus 20mM and 50mM of carnosine) had a low percentage of cells positive for β-gal. Senescent cells (IRAS 8 days with 4-OHT, IRAS 8 days with 4-OHT plus 20mM and 50mM of carnosine) exhibit a high percentage of positive cells, and in opposite to what we were expecting there was no decline from the cells without carnosine to the cells with carnosine. This was an odd result that we could not explain, therefore, to have a clarification for this we decided to go deeper into the effects of carnosine.

4.9. Carnosine exhibits a toxic effect on cells.

In order to explain why the cells treated with carnosine were not reduced in the percentage of positive cells for β-gal staining, we decided to see the morphology of these cells. The first big difference was seen between non-senescent and senescent cells is the confluence and viability of the cells. Non-senescent cells are way more confluent and look more viable, whilst senescent cells had a flat and long morphology. This flat and long characteristic is common in senescent cells (Cho et al. 2004). The second thing that caught our attention was the large number of dead cells when this were treated with carnosine (senescent and non-senescent). Pointed with a black arrow, a lot of dead cells appeared during the treatment with carnosine, which made us think that carnosine has some toxic effect on cells. This theory won more strength when we saw a lot of round and bright membranes (marked with yellow stars) that we associated with vesicles. Although senescence is associated with some production of vesicles (as reported in the introduction) this were too many to just being an effect of the senescence phenotype. Plus, non-senescent cells treated with carnosine shown the same vesicles. Probably these vesicles may be expelling carnosine due to its toxic effects or even proteins that associate with it. Nonetheless, the hasty increase of vesicles will promote an increase of lysosomes, which by its turn, will increase the lysosomal content. As discussed in the introduction (β-galactosidase segment), β-galactosidase activity is based on the increased lysosomal content, therefore the vesicles are disturbing the result of β-gal. In the end, vesicles and cell death made this result unreliable.

CHAPTER 6: Conclusions & Future Directions

6.1. Summary

As discussed in introduction, the epigenetic phenotype is triggered by changes in the environment of the cell, and although the importance of lipid peroxidation in senescence has been increasing in the lasts years (Barrera 2012) (Mladenov et al. 2015), a few studies have considered this major change in senescent cell environment as possible trigger for an epigenetic change.

The main aim of this report was to study if 4-hydroxy-2-nonenal, a product of lipid peroxidation during senescence, could change the conformation of histones. In general the results point in that direction. In fact, the changes promoted by 4-HNE in lysine methylation are similar, or at least an approximation, to what happens in senescent cells, as you can see in a resume table (table 3) Furthermore, these histone post-translational modifications are followed by what looks like a senescent phenotype (absence of proliferation, upregulation of p21 and formation of SAHFs).

Table 3. Schematic resume of histone 3 post-translational modifications. The signals represent: (-) very low levels; (+) low levels, (++) high levels (+++) strong levels of methylation.

	Non senescent cells	Cells treated with HNE	Senescence (short time) 4 Days	Senescence (long time) 8 Days
H3K4me3	+++	++	+	-
H3K9me3	-	++	++	+++
H3K27me3	-	+++	+	+++

Additionally, we reach as well a link between 4-HNE and senescence-associated heterochromatin foci. This is an important feature because the formation of SAHFs is the point where the possibility for replication ends. This is an information worth to be explored, and to reach new and better information in the relation between SAHF and 4-HNE it would be better to do the same experiment but in a confocal

microscope. Confocal can give a bigger resolution and healthier pictures for the experiment. It would be better as well to have an anti-histone 3 lysine 9 trimethylated antibody and an anti-histone 3 lysine 27 trimethylated antibody produced in different animals. This would allow to use two different secondary antibodies which would permit to see if there are an H3K9me3 core and an H3K27me3 ring in cells treated with 4-HNE.

Taking this results into consideration, 4-HNE might work as an instrument of senescence reinforcement. If 4-HNE is a product of senescence and at the same time it reinforces it, this might indicate that it works as a loop. With activation senescence, by an oncogenic or telomere dysfunction pathway, ROS and phospholipidosis levels rise (figure 2), consequently, the combination of both leads to lipid peroxidation intensification. The lipid peroxidation progresses to the production of 4-HNE. And so as senescent increases, 4-HNE also increases which ultimately promotes an epigenetic endorsement of the senescence phenotype. Concluding, senescence increases 4-HNE and 4-HNE endorses senescence.

As it usually happens, this new discovery open doors to new questions. This questions can be seen as future perspectives and new trails to work on. The most obvious question is: How is 4-HNE modifying the lysine methylation? Is 4-HNE an epigenator signal or an epigenetic Initiator?

There are 3 big hypothesis for this question. The most likelihood is that 4-HNE alters some of the enzymes responsible for changing the histone patterns (methyltransferases). 4-HNE does not react only with histones, in fact, in data not shown, we demonstrate that 4-HNE reacts with a vast number of proteins in IRAS cells. By reacting with methyltransferases, directly or indirectly from upstreams proteins, it changes their conformation, making them more capable or incapable to apply their activity. In this case, we can see 4-HNE as an epigenator signal.

The second hypothesis involves the affinity of histones with methyltransferases. Considering that 4-HNE bounds histones, as proven in this report, and the target is most likely lysines, this interaction would create a change in lysine structure that might change the affinity on some of the methyltransferases. Consequently, this would end in a change of histone patterns.

The third hypothesis involves mitochondrial dysfunction. 4-HNE is a good autocrine molecule, but due to its reactivity it tends to react in its local of production – mitochondria. A high reactive molecule such as 4-HNE can easily cause a lot of mitochondrial stress and lead it to dysfunction. Mitochondrial dysfunction can activate a pathway already discussed in the introduction- retrograde response pathway. This pathway can cause changes in gene expression (Butow & Avadhani 2004) and one of its hallmarks is the activation of senescence.

One of this theories, or more than one, or even none of them, might be true, nonetheless this is an important question that might take some time to be answered.

In conclusion, the experiments done in this thesis have identified a new link between reactive oxygen species, lipid peroxidation, epigenetics and senescence, through a single molecule - 4-hydroxy-2-nonenal. This can be the way that takes lipid peroxidation to be considered as a key feature in senescence phenotype.

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