Response of nuclear lamins upon induction of therapy-induced senescence in cancer cells

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by

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Certificate

This is to certify that this dissertation entitled Response Of Nuclear Lamins Upon Induction Of Therapy-induced Senescence In Cancer Cells towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by K Sai Vignaish at Indian Institute of Science Education and Research under the supervision of Kundan Sengupta, Professor, Department of Biology, during the academic year 2021-2022.

Kundan Sengupta
13.05.2022

Committee:

Kundan Sengupta

Krishanpal Karmodiya

This thesis is dedicated to Mom, Dad and Arun

Declaration

I hereby declare that the matter embodied in the report entitled Response Of Nuclear Lamins Upon Induction Of Therapy-induced Senescence In Cancer Cells are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr Kundan Sengupta and the same has not been submitted elsewhere for any other degree

K Sai Vignaish

Date: 31 March 2022

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Abstract

The cellular senescence pathway is a stress response pathway involved in limiting the proliferation of cells that are at risk of becoming cancerous. Tumour suppressor genes of the cells get activated during senescence induction to deactivate cell cycle proteins and enter a state of growth arrest, and at the same time, cells show hyperfunction, which results in the cell-type dependent secretion of pro-inflammatory cytokines and altered tissue microenvironments. Cellular senescence can be induced by a number of methods such as gamma irradiation, treatment with chemotherapeutic or DNA damage agents and telomere shortening due to repeated replication. The nuclear architecture of senescent cells has common features and deformations compared to both normal aging cells and premature aging models such as Hutchinson-Gilford progeria syndrome (HGPS) [Yoon et al., 2019]. Our experiments involve inducing and characterising senescence in cell lines using etoposide, a well-known chemotherapeutic agent used to treat cancers. The gene expression status of lamins and LINC complex proteins is analyzed after etoposide treatment and with serum starvation. While cellular senescence is classically thought to be a permanent state of cell cycle arrest, tumour cell escape from senescence has also been observed [Saleh et al., 2019]. This is a cause for concern since senescence reversal can promote cancer relapse, and shows the complex ways in which senescence may affect in vivo cancer progression and tissue remodelling. Our studies aim to elucidate the role of lamins and the LINC complex in the senescence mechanism in order to find specific biomarkers of senescence and better avenues in cancer therapy and senolytics.

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Chapter 1 Introduction

Cellular senescence is a well-studied pathway involved in the cellular regulation of the risk of neoplastic transformation. When Leonard Hayflick and others studied cellular replication in the 1960s, they found that normal fibroblast cells in culture stopped dividing after a certain number of population doublings [Hayflick, 1965]. This was the first time that the limited division of "normal" cells was shown as compared to "immortalized" cells (malignant or stem cells). These cells also showed apoptotic resistance along with a distinct morphology. Cellular senescence is correlated to in vivo aging. Cells isolated from longer living organisms in a species showed longer replicative life spans (Röhme, 1981). Thus, cellular senescence became an intrinsic cellular model of organismal aging. Cellular senescence is included as one of the nine hallmarks of aging (López-Otín et al., 2013). Both apoptosis and senescence prohibit the expansion of stressed cells by cell death and proliferation inhibition respectively. Cellular senescence induction is accompanied by a variety of morphological and molecular signatures such as increased nuclear and cellular size, lamin B1 downregulation, upregulation of growth pathways such as mTOR, activation and stabilization of a host of tumour suppressor proteins such as p53, p16 and p21, pRB, subsequent downregulation of cell proliferation proteins, increased lysosomal activity as observed in the Senescence-Associated Beta-galactosidase assay, nuclear deformations and modified chromatin architecture. Senescent cells also modify the properties of the ECM by releasing proteinases and immune factors, which may induce problematic paracrine effects.

1.1 Routes leading to senescence:

Multiple modes of senescence induction have been documented that are classified into three broad categories: Replication-induced senescence (or replicative senescence), and two types of premature senescence- Stress or DNA Damage-induced senescence and oncogene-induced senescence.

Replicative senescence, as observed by Hayflick, is caused by reaching critical telomere length over multiple replicative cycles. Telomeres are short tandem repeats found at the end of chromosomes (TTAGGG in vertebrates), which protect core DNA from replicative erosion. Each time a cell undergoes division, it loses a part of the telomere end- instead of losing core

DNA which could be deleterious for the cell. After continuous replication and gradual shortening of telomeres, dividing cells go from what Hayflick described as Phase II division (rapid proliferation) to Phase III division, where proliferative capacity keeps decreasing with passages and then grinds to a halt. Induction of replicative senescence is cumbersome and time-consuming. It takes about a year of culturing in fibroblasts to attain replicative senescence and continuous serial passaging increases the chances of contamination. Certain mammals, such as mice, show large telomere lengths which require very low telomerase activity for maintenance as compared to human beings and prevent senescence occurrence, yet their lifespan is 30 times shorter than ours [Calado and Dumitriu, 2013].

Oncogene-induced senescence is caused by the sustained activation of an oncogene or inactivation of tumour suppressor genes. An oncogene is a mutant form of a gene that aids in the malignant transformation of the cell. Ras and BRAF are two genes whose oncogenic forms may induce senescence. In the BRAF context, the oncogenic conversion of BRAF into BRAFV600E resulted in IGFBP7 gene activation, a secretory gene that acts on cell surface receptors and suppresses the BRAF-MEK-ERK pathway to induce senescence [Wajapeyee N et al. 2008]. It is widely known that oncogenic mutations rise every minute in the body of higher organisms and that most of these mutations do not give rise to lasting malignancies. Oncogene-induced senescence might play a role in the suppression of malignancies, alongside many other mechanisms. In the same example, IGFBP7 injection in BRAFV600E mice resulted in smaller tumours, but not in BRAF mice. Oncogene-induced senescence and replicative senescence are irreversible and robust forms of senescence.

DNA Damage-induced senescence (also known as stress or therapy-induced senescence) occurs when cells face external stress causing persistent DNA damage and disturbed cellular function. Oncogene-induced senescence and stress-induced senescence are collectively known as premature senescence since they show the senescent phenotype much earlier than telomere erosion would occur. A variety of agents inducing senescence via DNA damage include anti-cancer therapies such as chemotherapeutics (doxorubicin, etoposide), radiotherapy, CDK4/6 inhibitors (ribociclib, palbociclib) and epigenetic modifiers (5-Aza-2' deoxycytidine) [Wang et al. 2020]:

Agent	p53 status†	Mechanism	in vivo
Aphidicolin	+	DNA polymerase α and δ inhibitor	
Bleomycin	+	DNA strand break	
Camptothecin	+/−	Topoisomerase I inhibitor	
Cisplatin	+/−	DNA crosslinking	
Diaziquone/AZQ	+/−	DNA crosslinking	Prostate xenograft tumours
Doxorubicin	+/−	Topoisomerase II inhibitor	
Epigallocatechin gallate	+	Telomerase inhibition	
Etoposide	+/−	Topoisomerase II inhibitor	
Gamma irradiation	+/−	DNA damage	+
Hydroxyurea	+	Reactive oxygen species (ROS)	
K858	+/−	KIF11	Xenografts tumours
Lovastatin	−	HMG-CoA- reductase inhibitor	
Mitoxantrone	+/−	Topoisomerase II inhibitor	Human prostate tumours
MLN4924	−/+	Cul1 SCF subunit inhibitor	Prostate xenografts tumours

MLN8054	+	Aurora kinase A inhibitor	Colon xenograft tumours
Pyrithione	+/−	Zinc/calcium regulation, ROS	
Resveratrol	+	Reactive oxygen species (ROS)	
Retinols	+	Differentiation	
TPA, PEP005, PEP008	+	Protein kinase C activating	
VO-OHpic	+	PTEN regulator	Prostate xenograft tumours

Table 1. A table showing various drugs that are known to induce senescence in cancer cells. (†) p53 (+ or -) indicates the state of p53 in which these drugs activate senescence: '+' indicates that an active p53 is required for senescence, whereas '-' indicates p53 deletion or knockdown [Ewald et al. 2010].

1.2 Etoposide:

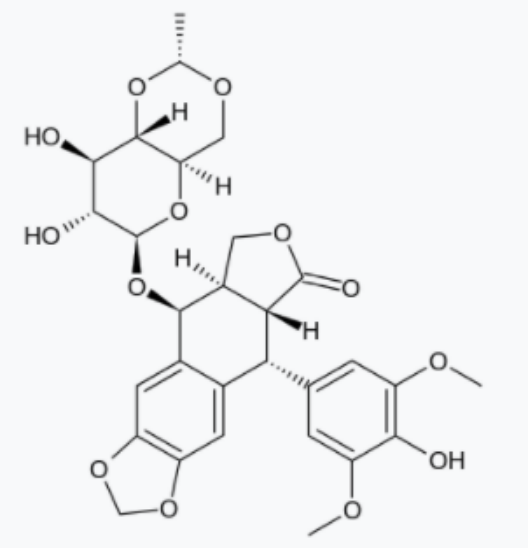


Figure 1. Molecular structure of Etoposide

In our experiments, etoposide has been used in low concentrations to induce senescence. Etoposide has been used as a chemotherapeutic agent in the treatment of various cancers since

the early 1980s. It was first isolated in 1966 by synthetic modification of a highly toxic glycoside, Podophyllotoxin, derived from the rhizome of *Podophyllum peltatum*. It acts as a topoisomerase II enzyme inhibitor, by binding to and arresting topoisomerase II in its catalytically active state along with the broken DNA strand. It does so by preventing the ligation activity of the enzyme after making a DNA cut and then forming a ternary complex with the broken DNA and enzyme. Thus, etoposide results in both persistent DNA damage and loss of topoisomerase II in the S-phase. This marks the beginning of the signal for senescence induction. Lower concentrations of etoposide result in a senescence response in cells, whereas higher concentrations result in an apoptotic response [Saleh et al. 2019]. In cancer treatments, low concentration exposure of etoposide has been seen to induce sustained apoptosis and treatment resistance. This could result in the formation of secondary cancers in vivo [Liu et al., 2002]. Strangely, this effect was seen more prominently in topoisomerase inhibitors, like doxorubicin and etoposide, but not in other agents of senescence induction, such as cisplatin. Study in this area could thus help elucidate mechanisms connecting senescence with aging and cancer.

1.3 Biomarkers of cellular senescence:

The senescent cell phenotype is well-documented across a plethora of studies in the past few decades. However, the study of cellular senescence in vivo and connecting it to in vitro studies is still difficult due to the absence of universal senescence biomarkers and heterogeneity of phenotypes in various kinds of senescent cells. Trying to connect processes like aging, cellular senescence and tumour suppression suffers from loss of context across various experimenters who often fall prey to inconsistencies. Senescence is a state of the cell, but it can be identified only by a multitude of features, none of which is exclusive to senescence [Ewald et al., 2010]. Induction of senescence features and the gene expression changes that underlie them take at least a few days to manifest, as compared to the apoptotic route of stress management, where the cellular program is established within 24 hours. Here we discuss a few of the biomarkers that have been used in standard senescence detection in vitro and in vivo.

Senescent cells in vitro and in tissue samples have a distinct morphology compared to normal cells. They appear stressed, and have an enlarged and flattened cellular and nuclear size when viewed in a 2D culture dish. This might be due to increased growth pathway activation such as MAPK and mTOR [Blagosklonny, 2011]. Senescent cells also show higher side scatter (SSC)

in flow cytometry as compared to normal cells. Higher SSC is generally correlated with increased granularity or 'complexity' of the cell, however this correlation with granularity is strong only for certain kinds of cells, such as blood lymphocytes. In senescent cells, this increase in granularity may be a result of larger vacuolic compartments in the cytosol [Ewald et al., 2010].

One of the most widely used biomarker for senescent cell detection is the senescence-associated beta-galactosidase assay (SA β-gal assay). Senescent cells show a characteristic increase in the concentration and enzymatic activity of GLB1 (lysosomal beta-galactosidase) at pH 6, which is generally not seen in normal cells where the enzyme works at pH 4 [Wagner et al., 2015]. This increase is seen as more of an effect or feature of senescence since removal of the enzymatic activity does not seem to influence senescence induction, and senescence induction has been observed without any increase in SA β-gal activity. SA β-gal assay being used as an in vivo marker for senescence is questionable- the staining is not specific and has been reported to give positive results by the action of Transforming Growth Factor-β pathway completely independent from senescence [Ewald et al., 2010]. Certain lower passage cell cultures derived from various skin and duct tissue samples also show β-gal staining at pH 6 without senescence induction. The other problem with the assay is that it requires functioning enzymatic activity to show results, which is an issue with frozen or fixed samples. This problem is effectively solved by immunohistochemical staining of GLB1 which was recently discovered as the protein responsible for the irregular lysosomal activity [Wagner et al., 2015].

A stable proliferation arrest is one of the defining features of senescence. Senescent cells are thought to have evolved to prevent proliferation upon facing persistent stress in order to minimize the expansion of transformed cells [Ewald et al., 2010]. Proliferation arrest can be observed by analyzing G1, G2 and S-phase markers via flow cytometry. The fraction of cells in S-phase to G1/G2 is seen to decrease. Metaphase analysis of senescent cultures would result in a significant decrease in the number of metaphases in senescent cultures. Standard proliferation assays, such as bromodeoxyuridine (BrdU) incorporation assay and Ki-67 staining are generally used to quantify proliferative decline. BrdU incorporates itself into the DNA and can be labelled and tracked across replicative cycles. Ki-67 is a protein that is expressed in

proliferating cells, but not in quiescent cells. Both these markers are non-specific to the senescence program.

Proliferation arrest in senescent cells is one of more universal and defining feature of senescence and mediators of cell cycle arrest qualify as important biomarkers of senescence. However it must be noted that cell cycle arrest is not exclusive to the senescence process, it is observed, for example, in quiescence. Another point to note is that from a functional point of view, cell cycle arrest mediators are not good markers of senescence since they get bypassed easily to let the cell enter normal or cancerous state. Cell cycle arrest is mediated by the action of tumour suppressor genes such as p53 and Rb and cyclin dependent kinase inhibitors (CDKi) such as p16 (aka INhibitor of CDK4- INK4), p21 (aka CDK interacting protein 1- Cip1), p27 (aka Kip1). Many types of cancer show mutations and downregulation in these proteins, suggesting the anti-cancer theory of senescence evolution.

Many types of senescent cells exhibit a secretory phenotype, known as the senescence associated secretory phenotype (SASP). These include growth factor pathway proteins, pro-inflammatory cytokines, matrix metalloproteinases and other extracellular matrix modifiers. These proteins are heterogeneous in the various forms of senescence induction and cellular subtypes, and sometimes even absent, but they have the advantage of being able to be easily detected in tissue and cell samples by immunohistochemistry (IHC). SASP also provides a functional angle to senescence by mediating autocrine and paracrine effects [Ewald et al. 2010].

Senescence and apoptosis are the two main responses of the cell in response to external stresses. The two processes are hence also very similar in terms of their certain phenotypes, such as those involved in the DDR pathway. Therefore markers of apoptosis such as cleaved caspase and lamin A, and flow cytometry techniques must be used to exclude apoptosising and senescing cells. Another biomarker of senescence, the senescence associated heterochromatin foci (SAHF) will be discussed in the following subsection.

1.3.1 Nuclear architecture of senescence

Senescent cells show widespread nuclear deformations, which is also consistent with non-senescent aging cells. Characteristic nuclear morphology defects such as multinucleated

cells and fragmented nuclei can be observed by 4,6-diamidino-2-phenylindole (DAPI) staining. A nuclear biomarker of senescence is the loss of heterochromatin integrity (both markers and structures) and formation of SAHF, which are condensed foci of unanchored heterochromatin colocalizing with epigenetic marks such as H3K9me3 and HP-1, and DNA damage marker γ-H2AX. SAHF are formed when there is widespread heterochromatin loss due to epigenetic remodeling. While SAHF is a marker more specific to senescence as compared to other forms of heterochromatin loss phenotypes such as in HGPS, SAHF is not a universal marker of senescence. [Wang and Dreesen, 2018]. Senescent cells show increased cytoplasmic chromatin fragments (CCFs) which indicate defects in nuclear architecture. Normal aging cells show a loss of lamin B1, which is also a biomarker of senescent cells. Studies have shown widespread mislocalization and misregulation of lamins and LINC complex proteins, especially Lamin B1. Lamins are intermediate filaments which are involved in gene expression and maintenance of chromatin and nuclear architecture. LINC complex proteins serve as a signal transducer between the nucleus and the cytoplasm by connecting the two phases via the nuclear membrane. Defects and dysregulation of lamins and LINC complex proteins result in modified gene expression due to chromatin remodeling. Loss of lamin and heterochromatin integrity has been well-documented in normal aging. Lamin B1 and lamin A/C are related to the aging process due to their roles in cellular senescence and disease states. Lamin B1 levels vary across cell types, therefore use of cell-type specific marks along with lamin B1 analysis proves as a robust marker of senescence. Our studies use Lamin B1 downregulation as a marker of senescence.

Biomarker of senescence	Marker description
Cellular phenotypes	
Morphology	Visually observed (Brightfield imaging)
SA-β-gal activity	Enzyme activity and staining
Glb1	RNA, Immunohistochemistry (IHC)
Side scatter (SSC)	Flow cytometry

Telomere staining (RS only)	Fluorescence in situ Hybridization (DNA FISH)
Arrested Proliferation	
BrdU Incorporation	Immunohistochemistry, flow cytometry
Decreased Ki-67	Immunohistochemistry
Apoptosis exclusion	
Propidium iodide/annexin V staining	Flow cytometry
Cleaved PARP	Immunohistochemistry
Cleaved caspase 2/3/9	Immunohistochemistry
TUNEL staining	Immunohistochemistry
CDK inhibitors	
p16(Ink4a)	Immunohistochemistry
p21(waf1/cip1)	Immunohistochemistry
p27(kip1)	Immunohistochemistry
Heterochromatin foci	
DAPI/Hoechst 33342	DNA Staining
HIRA	Immunohistochemistry
H3K9me3	Immunohistochemistry
HP1-γ	Immunohistochemistry

DNA Damage	γ-H2AX staining
Secretory proteins	
IGF2	RNA, Immunohistochemistry
IGFBP3, IGFBP5, IGFBP7	RNA, Immunohistochemistry
IL-6, IL-8, CXCR2, and others	RNA, Immunohistochemistry
Miscellaneous	
Versican*	RNA
CXCL14*	RNA
Mitochondrial fusion/hFis1/OPA1 §	Immunohistochemistry
Dec1‖	Immunohistochemistry
DcR2‖	Immunohistochemistry

Table 2. Biomarkers of senescence. (*) Marker observed only in prostate samples so far. (§) Marker observed in HeLa cells. (‖) Marker observed in non-transformed cells. [Ewald et al. 2010]

1.4 Fasting, autophagy and senescence

Serum starvation of cells promotes quiescence and autophagy. Autophagy is the cellular mechanism by which unwanted molecules, such as misfolded proteins and dysfunctional organelles are targeted for lysosomal degradation. Studies have shown an intricate connection between autophagy and growth pathways and senescence pathways. Induction of autophagy has been studied as a way of clearing senescent cells and restoring youthful markers in age-related diseases and promoting longevity, especially when synchronized with the circadian rhythm, i.e. Night-time or Circadian-dependent autophagy [Helfand and de Cabo, 2021]. Common stimuli are known to induce autophagy and senescence, such as DNA damage,

reactive oxygen species (ROS) production and oncogenic stress. At the cellular level, autophagy has been seen to both promote and repress markers of senescence. Removal of dysfunctional organelles reduces cellular waste load and stops senescence, whereas autophagy also promotes the secretion of SASP. Autophagy itself is generally seen to increase upon senescence induction, however mechanisms by which autophagy regulates senescence are unknown. The issue of the relationship between autophagy and senescence is highly dependent on the type of autophagy established, cell-type and stemness and even the intracellular compartment where autophagy occurs [Kwon et al., 2017].

Chapter 2 Materials and Methods

Cell culture techniques

- HCT116 and SW480 (<15 passages) cells were cultured in complete DMEM (cDMEM= DMEM + 10%FBS + 1% penicillin-streptomycin) in 60mm (5mL media) and 100mm dishes (8mL media). Cell plating densities were around 0.8 million/plate and 2.2 million/plate respectively for the two dishes. Cells are cultured in a 5% CO2 incubator at 37°C.
- Media conditions and cells were checked regularly for signs of stressed growth or contamination- media colour changes, black spotting on the dish, high cell death. Media was changed every couple of days and cells were split upon reaching 80-90% confluency.
- Cells were checked for mycoplasma contamination after every split and before seeding for experiments:

A. B.

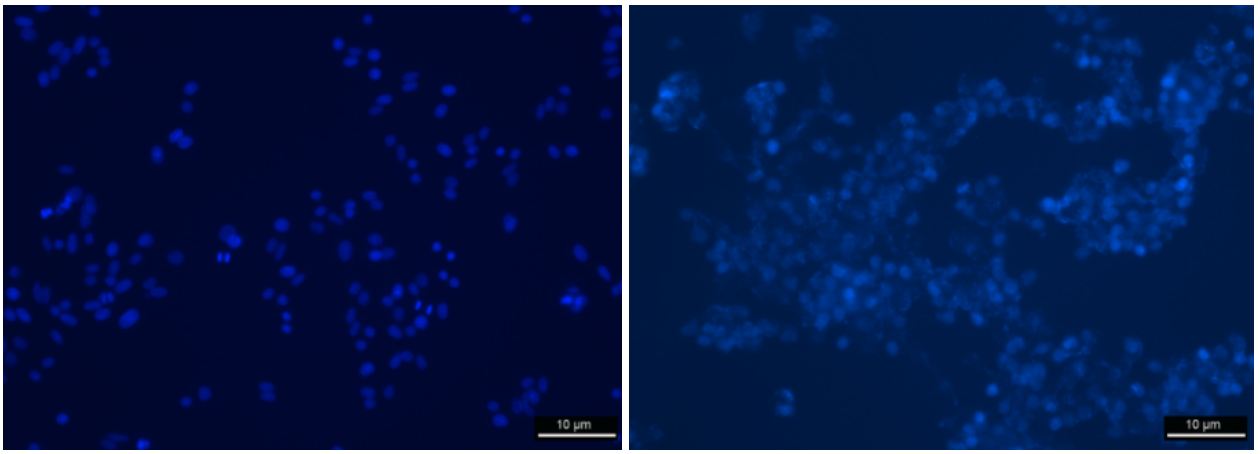


Figure 2. DAPI staining of cells after fixation with 4% PFA shows the presence of mycoplasma in the cytosolic compartment in panel B, whereas panel A shows mycoplasma negative cells, with DAPI staining cell nuclei exclusively.

Cryopreservation and thawing of cells:

- 1 million cells are taken in 1 mL of freezing media (9:1:: complete DMEM: DMSO). Cells are gradually frozen in an isopropanol chamber and stored at -80°C until revival.

- For cell revival, a small water bath and media is first pre-heated to 37°C.
- The cryovial is removed from -80°C and quickly thawed in under a minute in the water bath. The vial is sterilized with ethanol before adding pre-warmed media, after which the cells are centrifuged.
- Cells are then resuspended in fresh media and added to a T25 cell culture flask or a 100mm cell culture dish.

Serum starvation cell culture experiments:

- 24h post-seeding, HCT116 cells were cultured in either FBS-starved, i.e. "plain" DMEM, or cDMEM for 24h after which Brightfield images were taken, cell count was performed and RNA isolation was performed for further analysis. For serum rescue experiments after FBS starvation, at the end of 24h FBS starvation, the media is replaced with cDMEM and cells are harvested after 24h.

Senescence induction using Etoposide (E1383)

- Upon reviewing literature, 1-10μM etoposide treatment was found to be the ideal concentration for inducing senescence [Jochems et al. 2021; Saleh et al. 2019].
- Cells are seeded for experiment after performing cell count. 24h after seeding cells, 'Test' cells were treated with 1μM etoposide, whereas 'Control' cells were treated with DMSO.
- 48-72h after treatment, cells were imaged for morphological changes and then harvested for further processing.
- For senescence induction in FBS-starved cells, 0.4-0.6 million HCT116 cells were seeded in 60mm cell culture dish in 4-5 mL media. After 24h, ensuring healthy cell attachment, the media in the test dish is changed to plain DMEM for 12h, and then replaced with 1μM etoposide in cDMEM for 72h before harvesting for RNA.

Analysis of Metaphases:

- Actively growing cells were treated with 1% Colcemid (1μL/mL of media) in fresh medium, followed by incubation for 90 minutes in 5% CO2 at 37°C
- Post incubation, media is removed and cells are washed with 10mL 1xPBS.
- Cells are then trypsinized and centrifuged. Cell pellet is resuspended in 0.075M pre-warmed KCl and incubated for 30 min in 37°C water bath.

- Fixative treatment (Methanol:Acetic Acid= 3:1 solution) and centrifugation performed twice before resuspending cells in 5mL fixative. Suspension stored in 4°C
- Slides are prepared for metaphase drops by washing serially with 70%, 90% and 100% ethanol. Cells are resuspended in fresh, cold 1mL fixative.
- Slides are humidified before dropping metaphases and labelled. DAPI staining and karyotyping performed.

BCA Protein Estimation:

- Standard solutions A to I are prepared by mixing the stock BSA and distilled water as described in the serial dilution protocol described in the Pierce™ BCA Protein Assay guide (cat. 23225).
- 25µL of standards A to I are added in double replicates in a labelled 96-well plate. The next two wells contain 25µL of RIPA, followed by sample protein replicate wells.
- 200µL of freshly prepared working reagent (solution A: solution B= 50:1) in each well followed by incubation at 37°C for 30-35 minutes. Colorimetry is done as soon as incubation is complete. Standard graphs are plotted and used to calculate the concentration of sample protein.

RNA isolation:

- Adherent cells are given a cold PBS wash. 1-2mL TRIzol reagent is added and pippeted repeatedly to ensure maximal cell lysis.
- Cells are kept in TRIzol in a 1.5mL vial for a few minutes before 200µL chloroform is added and mixed well by inverting gently multiple times.
- This mixture is kept for a few minutes before cold centrifugation (4°C) for 15 minutes at high speed (~15000 rcf).
- Post centrifugation, the aqueous top layer is carefully transferred to another vial without disturbing the interphase precipitate. An equal volume of isopropanol is added to the new vial and is invert mixed gently. Cold centrifugation was performed again after 15min at room temperature.
- Isopropanol is removed without disturbing the pellet and 1mL 75% RNase-free ethanol is added and gently resuspended. Cold centrifugation is performed. Ethanol wash is repeated a couple of times before removing ethanol completely and air-dried.

- Resuspend dried pellet in 10-15µL nuclease-free water. The RNA solution is stored in -80°C. RNA purity and concentration is checked with a Nanodrop spectrophotometer and checked for degradation with Agarose Gel Electrophoresis.

cDNA synthesis and RT-PCR:

- Isolated RNA is reverse transcribed to form cDNA using Takara cDNA synthesis kit as per instructions provided, using a thermal cycler. The reaction mixture os then subjected to 5 min at 25°C, followed by 30 min at 37°C and then 5 min at 85°C which deactivates the reverse transcriptase enzyme. The mixture is then cooled down to 4°C. Post cDNA synthesis, the solution is diluted and stored at -20°C.
- cDNA, SYBR Green master mix and paired forward and reverse primers of chosen genes are taken along with Nuclease Free Water (NFW) in 96-well plates. Polymerase chain reaction occurs in the thermal cycler. The sample is heated up to 95°C for 3 minutes followed by cycling 39 times between 95°C for 20 seconds and 65°C for 30 seconds. The fluorescent signal is captured at the end of each cycle by the thermal cycler which also records the cycle threshold (Ct) value for each well. After completion of 39 cycles, the sample is kept at 65°C for 5 seconds, followed by 95°C for 50 seconds and cooldown at 4°C for 4 minutes.

Chapter 3 Results

3.1 In silico analyses-

1. Human Ageing Genomic Resources (https://genomics.senescence.info/) was used to explore genes known to affect the aging process and compile a large list of genes based on the nature and quality of evidence. From the analysis, we identified Lamin A/C as an important modulator in aging and cancer. Lamin A/C (LMNA) is an important protein implicated in the aging and senescence process. The LMNA gene exists as various splice variants in the cell- the prominent Lamin A, lamin C, lamin A delta 10 and lamin C2. Mutations or modulation in the efficacy of the lamin genes loci result in various progeroid syndromes, such as HGPS, atypical-Werner syndrome and atypical Progeria- these are known as progeroid laminopathies. In HGPS, the accumulation of prelamin A or progerin due to improper post-translational modification results in altered signaling of mTOR pathway, altered epigenetics, modification of LINC complex and stress response [Cenni et al., 2020]. Transient prelamin A presence in cells might also act as a cellular stress signal.

GenAgeID	symbol	name	Entrez id	Organism(s)
12	ERCC8	excision repair cross-complem -entation group 8	1161	human
14	LMNA	lamin A/C	4000	human
13	WRN	Werner syndrome, RecQ helicase-like	7486	human, mammal, cell
230	ELN	elastin	2006	human
309	GDF11	growth differentiation factor 11	10220	mammal, human
123	FOXO3	forkhead box O3	2309	invertebrate, human

Table 3. A sample of aging-associated genes taken from the Human Ageing Genomic Resources

2. Signaling pathways: KEGG pathway database (https://www.kegg.jp/) was used to gain an understanding of the various interactions involved in modulating cell senescence responses via interactive pathway maps. The senescence program is initiated when the cell faces various kinds of stressors and developmental cues. When the cell faces lower stress loads, it chooses the senescence pathway over apoptosis, however our analysis shows that many of the pathways and molecular interactors are common in the two processes. Inhibition of caspases and B-cell CLL/lymphoma 2 (Bcl-2) overexpression result in apoptosis inhibition and senescence induction and present a point before which the two pathways diverge [Ewald et al., 2010]. DNA damage repair (DDR) pathways have been extensively implicated in the senescence process, partly due to many experiments being conducted by inducing senescence using DNA damage agents. However, the role of genomic instability and genotoxic stress is central to senescent mechanisms, since persistent low-grade DNA damage invariably results in a senescent phenotype in most cells. Proteins involved in the DDR pathway such as ataxia telangiectasia–mutated (ATM), checkpoint homologs 1 and 2 (Chk1 and Chk2) are implicated in the senescence process. Several tumour suppressor proteins and cyclin dependent kinase inhibitors are implicated in cell cycle arrest in the senescence process. These include, but are not limited to, p53, Rb, p16, p21 and p27. The functions of most of these proteins are taken over by other proteins and pathways to induce senescence in their absence, and their own activity is easily overpowered by strong apoptotic or oncogenic signaling. There is evidence suggesting that while p21 is involved in early stage senescence induction, p16 might help in maintaining the senescent phenotype. Growth factors such as insulin like growth factor binding protein 7 (IGFBP7) and IGFBP-rP1 are closely involved in the senescence process. Their expression increases upon senescence induction and they also act as senescence inducers [Wajapeyee et al., 2008]. Senescent cells also express a hyperfunction phenotype, as characterized by their sustained survival and enlarged state, due to the activation of mTOR and other growth signaling pathways. mTOR activation in cells that are proliferative locked results in senescence, which provides an important clue differentiating senescence and

quiescence [Blagosklonny, 2011]. Thus growth promoting pathways might form an integral part of senescence induction and maintenance. In low p53 conditions, cells seem to favour the senescence program due to their incapability to inhibit mTOR. Senescent cells also show widespread chromatin remodeling and nuclear deformation which results in heterochromatin loss and subsequent loss of heterochromatic marks such as H3K9me3. The mechanisms by which these effects are mediated are still being studied.

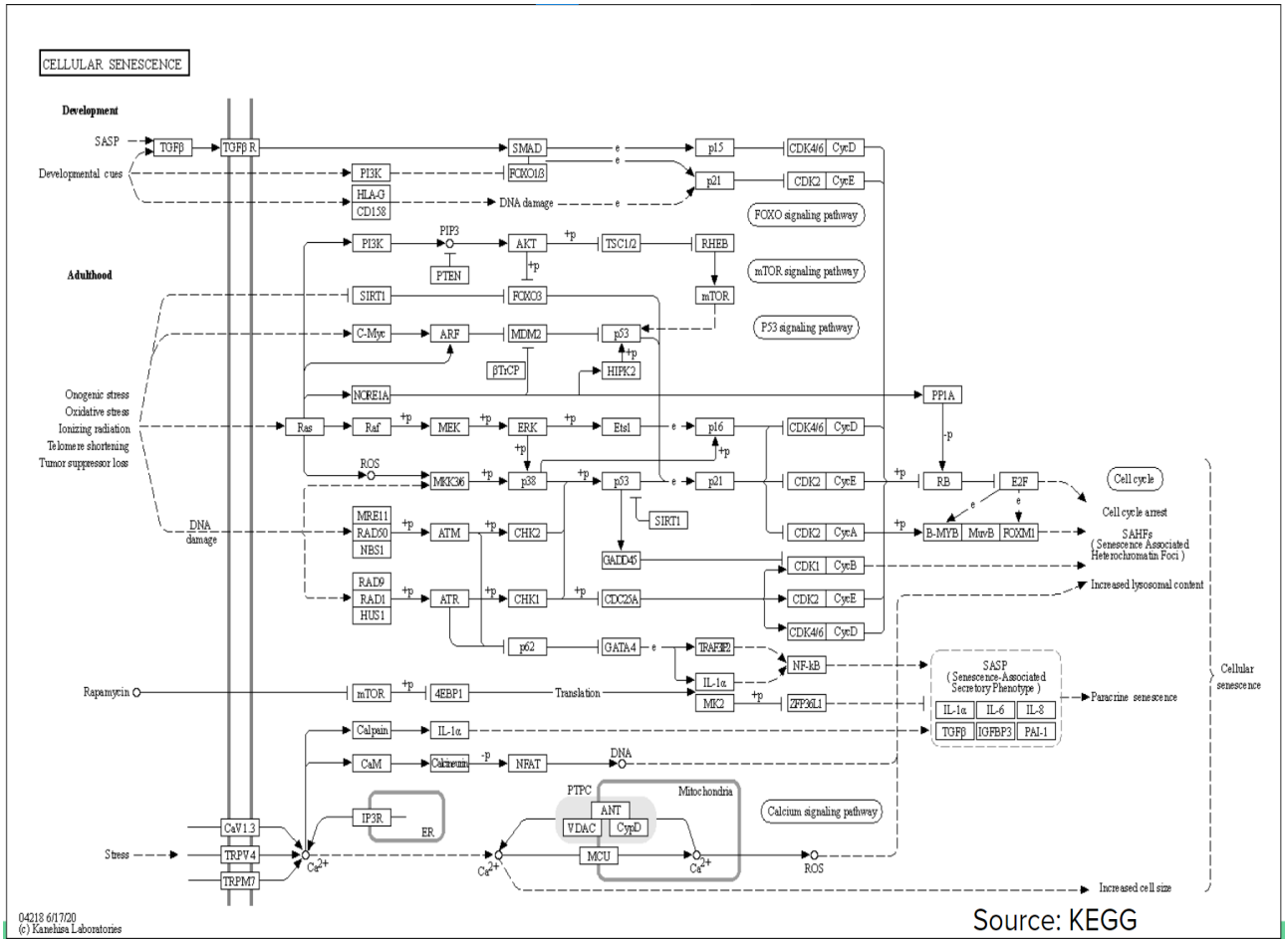


Figure 3. Various molecular interactions interplay in the senescence pathway (KEGG)

3. Protein-protein interactions: STRING database (https://string-db.org/) used to explore protein-protein interactions between candidate genes gated on evidence-based confidence levels. Interactions between retinoblastoma (Rb) protein pathway and p53 pathway influence the senescent phenotype across multiple forms of senescence by forming feedback loops with transcription factors, inhibitors and direct interactions. Stress signals induce p14, which increases p53 levels by inactivating MDM2, which acts is the inhibitor of p53. p53 has a pleiotropic effect on various cell cycle

modulators, such as p21 and apoptosis inducer BAX. The action of CDKIs such as p16 and p21 results in the maintenance of the active phosphorylated state of Rb, which results in cell cycle arrest, mediated by the transcriptional regulation of E2F. The apoptotic functions of p53 are also influenced by Rb levels due to the formation of p53-MDM2-Rb complexes. p53 has also been shown to interact with lamin A/C to increase p16 transcription and aid senescence induction [Yoon et al., 2019].

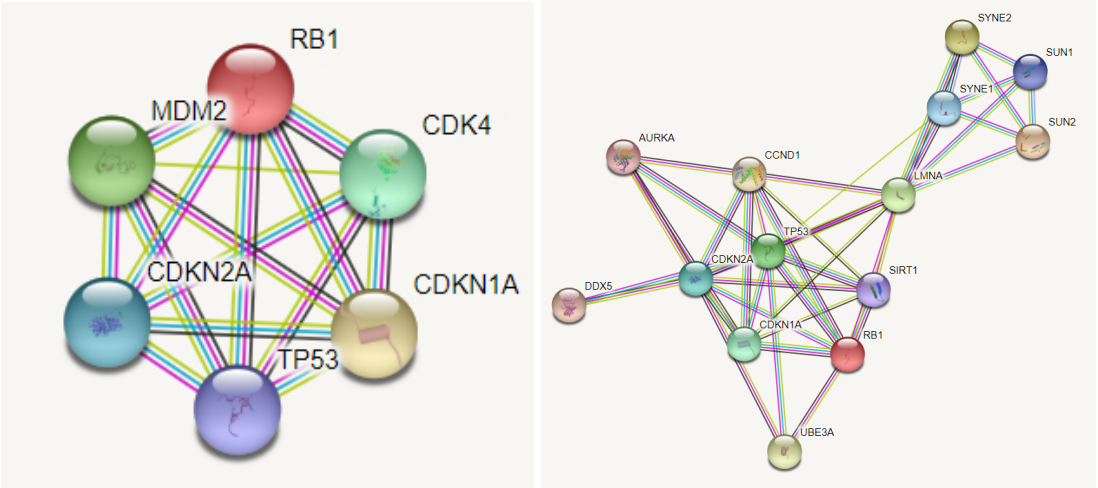


Figure 4. STRING analysis. The first image is an interaction map between proteins of two important pathways involved in cell apoptosis and senescence- the p53 and pRB pathways, while the second image connects important tumor suppressor proteins involved in senescence with nuclear architecture and envelope proteins such as lamin A, nesprin-1 and SUN1. Colour-coded interactions between molecules: Light blue and purple lines indicate known interactions, whereas green, red and dark blue lines indicate predicted interactions.

4. The Cancer SENESCopedia is an interactive explorer of the gene expression data published by Jochems et al., 2021. 13 cancer cell lines were subjected to treatment with either Etoposide or Alisertib and their gene expression data was obtained and published for set timepoints post-senescence induction- for etoposide treatment, data is available for 7 days post-treatment, whereas for alisertib, data for 7 and 14 days post-treatment is

available. Across cell lines, we can see downregulation of LMNB1, in the below figure:

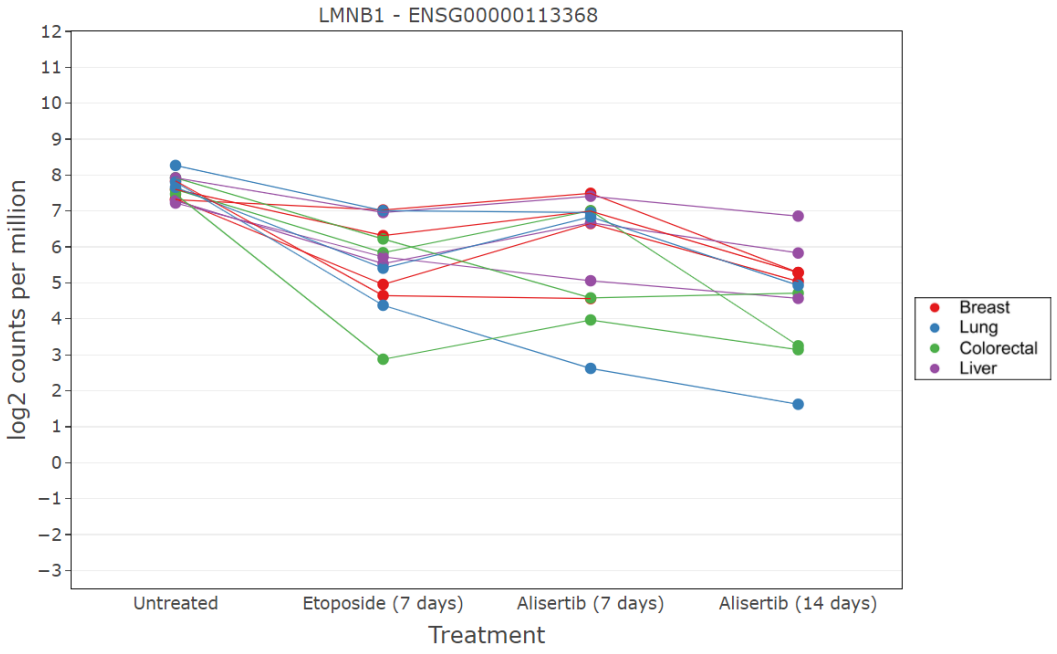


Figure 5. Schematic taken from the Cancer SENESCOpedia Gene expression viewer. RNA-Seq data from Jochems et al., 2021 has been plotted for three experimental conditions and timepoints- 7 days post-etoposide treatment, 7 days post-alisertib treatment and 14 days post-alisertib treatment.

3.2 BCA Protein Estimation:

1. 2.

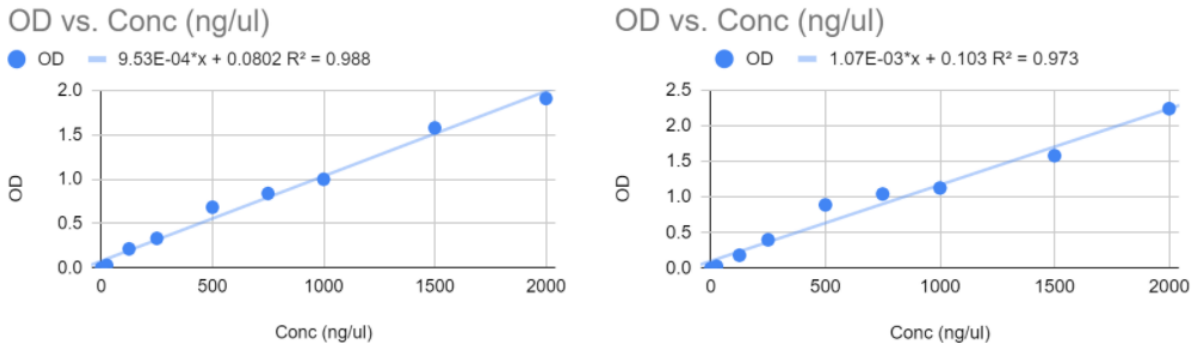


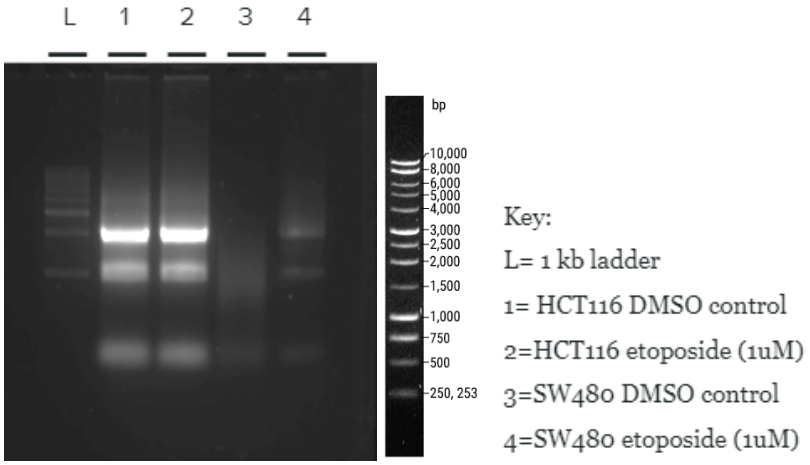
Figure 6. Graph showing the standard curve plotting used to quantify unknown protein concentration.

BCA protein assay is a simple technique used to measure the concentration of protein lysates. Increasing concentrations of proteins give rise to formation of higher amounts of BCA-cuprous complexes, which have maximum absorbance at 562 nm. In accordance with Beer-Lambert's

law, absorbance, reflected in optical density value (OD value), shows linear increase with concentration. The first biological replicate yielded more consistent results and a better R-sq value of 0.988, with a calculated concentration of 511.84 µg/mL. Absorbance data for sample 2 suggests pipetting errors due to differences in some of the double replicate wells and a lower R-sq value of 0.973. The concentration was calculated at 2305.2 µg/mL. Protein quantification using the standard curve equation also revealed that sample 2 was more concentrated than the working range of BCA protein assay and required to be diluted further before the assay.

3.3 RNA isolation:

(i)



(ii)

Source	Conc. in ug/ul	260/280 ratio	260/230 ratio
HCT116 +DMSO	7.97	2.03	2.17
HCT116 +Etoposide	7.1	2.05	2.21
SW480 +DMSO	0.42	2.08	1.79
SW480 +Etoposide	0.5	1.98	0.55

Figure 7. (i) Top panel shows the 1.2% agarose gel image of isolated RNA visualized by SYBR™ Safe nucleic acid stain. A reference 1kb ladder has been placed adjacent to the gel image for size comparison of RNA bands (ii) Bottom table shows the quantification of concentration and absorbance ratios of isolated RNA using Nanodrop spectrophotometer.

RNA sample isolated from HCT116 and SW480 control and etoposide-treated cells using TRIzol reagent (see Materials and methods). HCT116 samples show high concentration yield of RNA between 7-8 μg/μL, whereas SW480 show low concentration yield of 0.4-0.5 μg/μL. This might be due to the much lower confluency of SW480 dishes while harvesting (~40%) as compared to HCT116 dishes (~90%). Nanodrop spectrophotometer validated the purity of RNA with healthy values of 260/280 nm absorbance ratio (~2) for all 4 samples. SW480 etoposide-treated sample had an aberrant 260/230 value of 0.55. This suggests the presence of some impurities in that sample. RNA Agarose Gel Electrophoresis validated the quality of RNA with the sharp bands at 28S rRNA and 18S rRNA for both HCT116 lanes and SW480 etoposide-treated lane. SW480 control lane shows smeared bands, suggesting degradation of RNA. Since only one of the lanes was smeared in the gel, we may conclude that the degradation did not occur due to errors in electrophoresis, but during sample preparation or quantification after RNA extraction. Thus, HCT116 RNA was used for cDNA synthesis, whereas SW480 RNA was not used for further analysis.

3.4 RT-PCR of housekeeping genes:

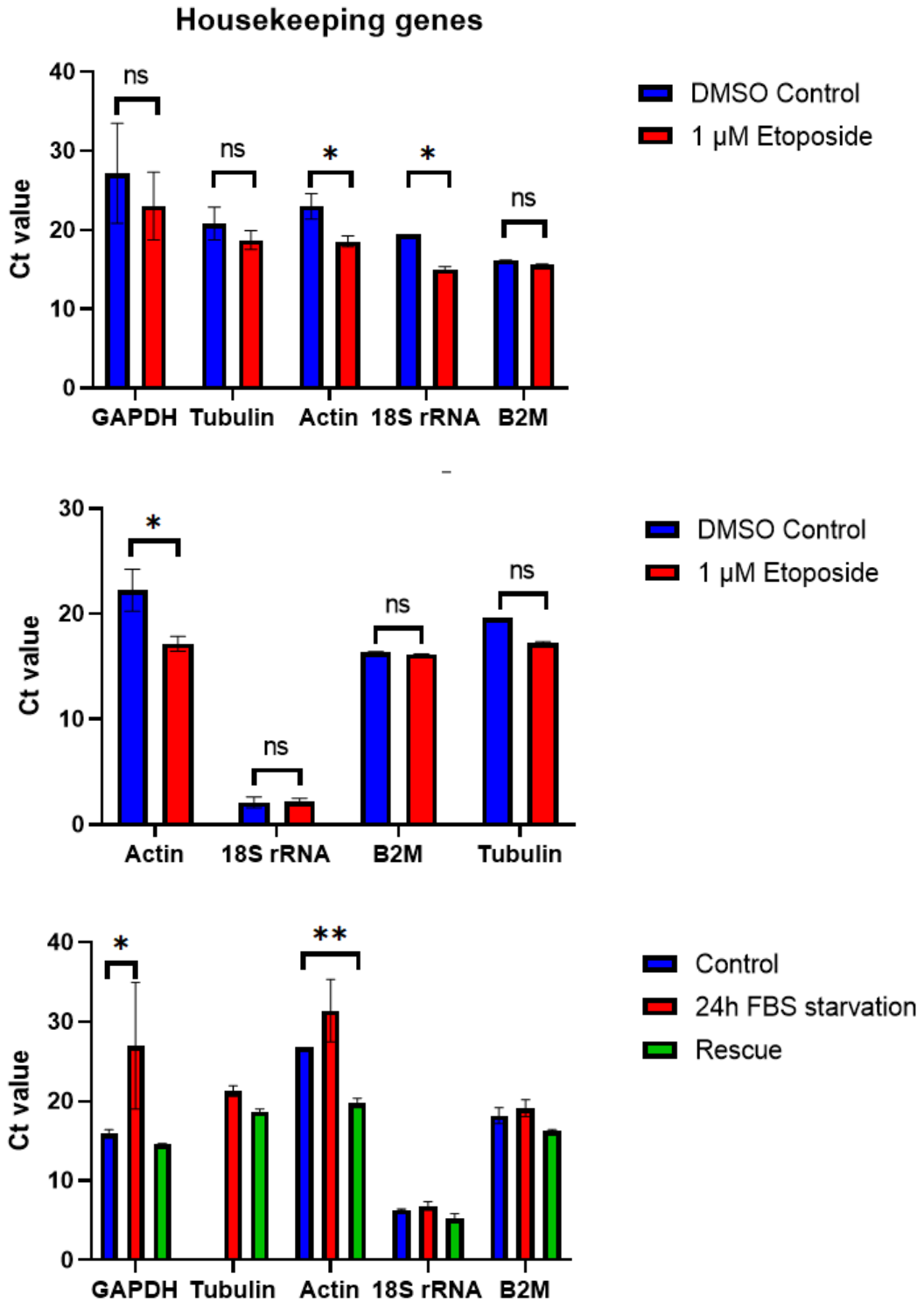


Figure 8. RT-PCR of housekeeping genes for three different experiments. No. of technical replicates for each experiment= 3. (i) Top panel shows housekeeping gene analysis for etoposide treatment of HCT116 cells. (ii) Middle panel shows the analysis for etoposide

treatment of SW480 cells. (iii) Lower panel shows the analysis of housekeeping genes for FBS-starvation and rescue experiments in HCT116. Error bars represent SEM.

Housekeeping gene analysis is done prior to transcriptional quantification of lamins and nuclear architecture genes. The housekeeping gene is chosen such that its expression is invariant across the experimental conditions, by choosing for the least difference in Ct value between Control and Experiment samples. Housekeeping gene analysis also acts as a pilot experiment to optimize for cDNA quantity required in the experiment. Then the chosen gene acts as the base normalising factor for the control and experiment groups allowing us to gather relative transcriptional data that is comparable across biological replicates. In all the three experiments, from amongst GAPDH, actin, tubulin, 18S rRNA and Beta-2 microglobulin (B2M), B2M was chosen as the housekeeping gene. B2M showed the least differential transcription across control and experiment groups, and also showed least variance in Ct values suggesting it a reliable gene for normalisation. Unpaired t-test was used in all experiments to measure the significance of the data.

3.5 RT-PCR pilot experiment: Transcriptional analysis of lamins upon etoposide treatment of HCT116 cells

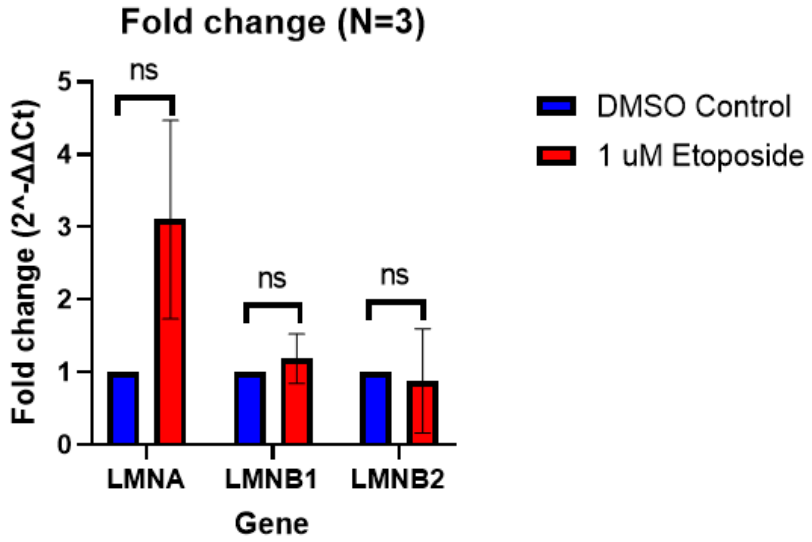


Figure 9. RT-PCR data for 3 biological replicates of the etoposide treatment experiment of HCT116 cells. Error bars denote SEM. Data includes 6 technical replicates (n) for Lamin A/C, n=5 for Lamin B1 and n=4 for Lamin B2.

RT-PCR data for etoposide-treated HCT116 cells show differential transcription of lamin A/C, lamin B1 and lamin B2. A consistent increase in lamin A/C transcription is observed, in accordance with data from Jochems et al., 2021. However, errors in conducting RT-PCR, such as improper sealing of 96-well plate (which resulted in empty wells and lesser technical replicates) and pipetting errors resulted in high variance and discarding outlier data. The first RT-PCR had 6 replicates for Lamin A/C, 5 for Lamin B1 and 4 for Lamin B2 across three biological replicates.

3.6 Transcriptional modification of lamins upon etoposide treatment in SW480 cells:

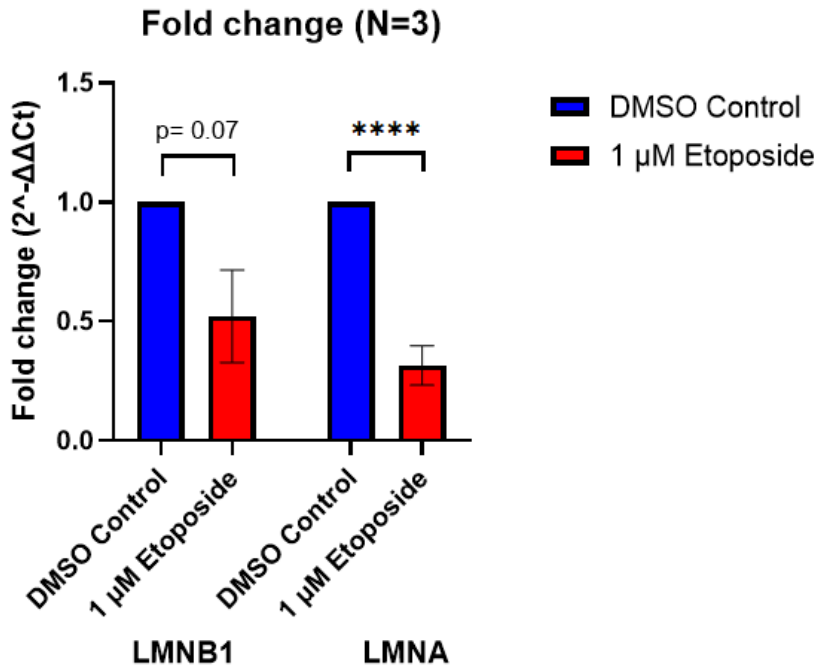


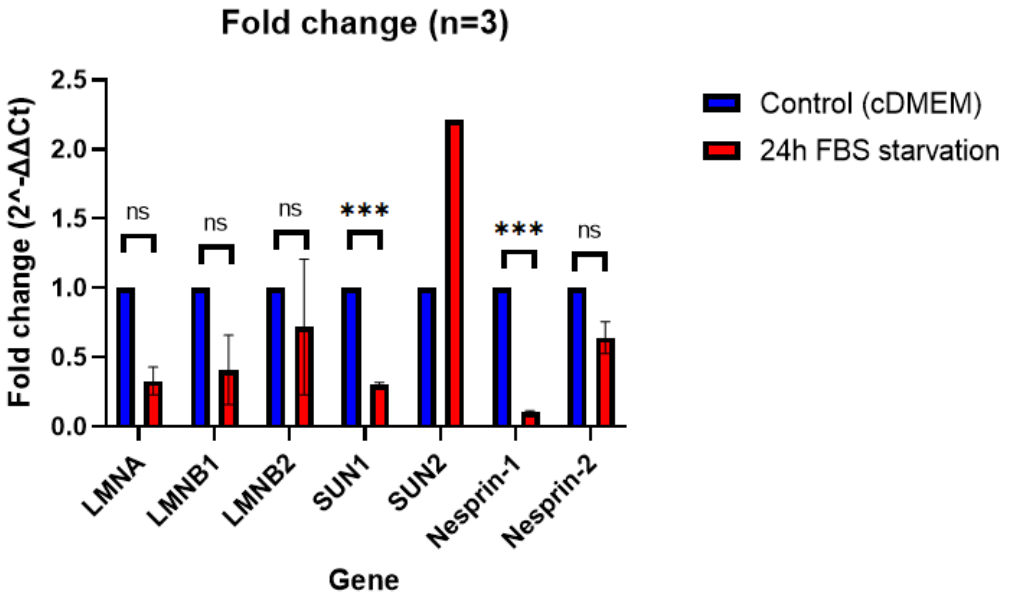
Figure 10. RT-PCR data showing downregulation of lamins upon low dose etoposide treatment of SW480 cells. Error bars represent SEM. Data spread across three biological replicates, which includes n=9 for Lamin A/C and n=5 for Lamin B1.

In our next set of experiments with SW480 cells, we analyze the transcriptional status of Lamin A/C and Lamin B1 upon low dose etoposide treatment for 72h. Data for 5 replicates for Lamin B1, and 9 replicates for Lamin A/C across three biological replicates has been presented. Low dose etoposide treatment for 72h in SW480 cells show an aberrant decrease in the transcriptional levels of Lamin B1 and Lamin A/C. Lamin B1 downregulation has been observed as a biomarker of senescence across various cell lines [Freund et al., 2012]. Senescence induction is accompanied by widespread nuclear deformations and enlargement of

nuclei, which might be related to the lamin dysregulation, since lamins function as the nucleoskeletal scaffolding in cells. Lamin B1 and lamin A/C downregulation has also been shown to occur with aging in normal cells in various cell types, which suggests that lamin downregulation might be a general marker in age-related diseases risk [Matias et al., 2022].

3.7 Lamin and nuclear architecture genes show differential gene expression with FBS-starvation:

(i)



(ii)

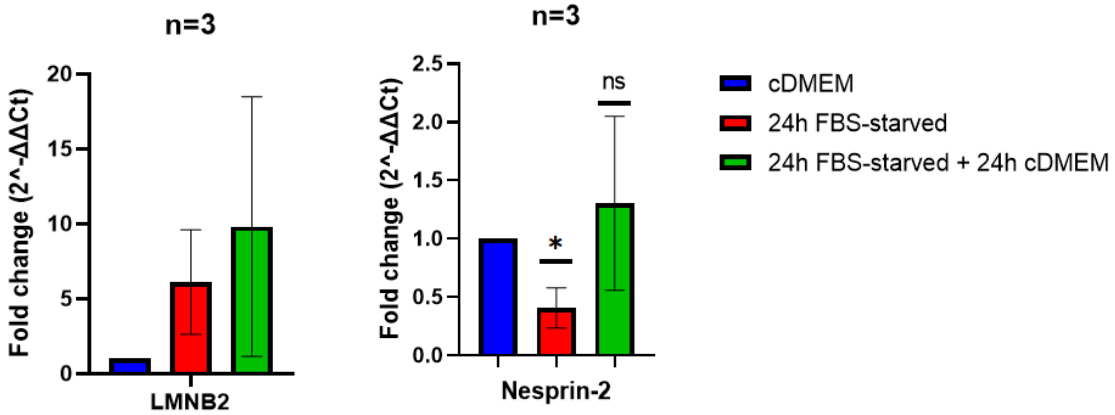


Figure 11. (i) Top panel shows RT-PCR data for lamins and LINC complex genes upon 24h FBS starvation. (ii) Bottom panel shows RT-PCR data for Lamin B2 and Nesprin-2 in the context of serum rescue after starvation. All experiments are in three technical replicates. Red

bars represent 24h FBS-starvation alone, whereas green bars represent 24h serum rescue after FBS-starvation Error bars denote SEM.

Next, we question whether lamin and LINC complex genes are modulated by serum starvation. Serum starvation results in a sharp decrease in proliferative capacity in culture by dampening growth pathway signaling, and decreased activity and biomolecules synthesis, and the effects are reversed when serum is added back to culture. This kind of reversible cell cycle arrest is termed as quiescence. Extended periods of serum starvation beyond 24h trigger the apoptotic pathway in cells. Data has been shown for 1 replicate for SUN2, 2 replicates for Lamin A/C, Lamin B1 and Lamin B2, and 3 replicates for SUN1, nesprin-1 and nesprin-2 in one biological replicate. Lamins A/C, B1 and B2 show a reduction in transcript levels on 24h FBS-starvation, along with SUN1, nesprin-1 and nesprin-2. This indicates that the stress placed on the cells by removal of serum transduces to the nuclear architecture proteins and alters the nuclear landscape. SUN2 has been shown to increase upon FBS starvation, however, the data is not reliable due to a lack of replicates. In the FBS rescue experiment, most genes failed to provide reliable results, as is seen in the case of Lamin B2, however, we can see nesprin-2 showing signs of recapture of transcriptional levels before FBS-starvation when serum is added back for 24h. Rescue experiment data for Lamin B2 and nesprin-2 are shown for 3 replicates in one biological replicate.

3.8 Lamin transcriptional downregulation upon etoposide treatment of FBS-starved cells:

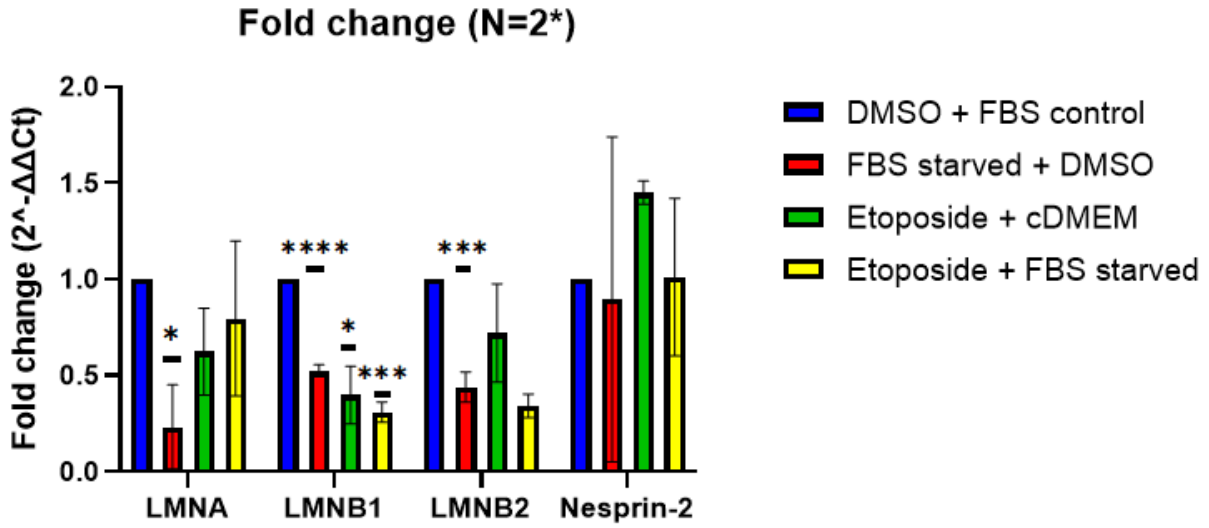


Figure 12. RT-PCR data showing downregulation of lamins A/C, lamin B1 and lamin B2

mRNA across three conditions: (i) 12h FBS starved+DMSO (Red) (ii) 72h Etoposide+cDMEM (Green) and (iii) 12h FBS starved + 72h Etoposide treatment (Yellow). (*)= Nesprin-2 is in 1 biological replicate, whereas Lamin A/C, Lamin B1 and Lamin B2 are in 2 biological replicates.

Here we present the RT-PCR data for etoposide treatment of HCT116 cells after FBS-starvation treatment. The rationale to do this experiment came from studies that show a close connection between the growth pathways involved in serum starvation-induced quiescence and senescence. Experiments that modulated the levels of important senescence markers like p53 in proliferation-arrested cells show that mTOR signaling is essential for senescence induction and senescence was established when p53 failed to inhibit the mTOR pathways due to low levels of induction [Leontieva and Blagosklonny, 2010]. Thus, it is hypothesized that FBS-starvation induced inhibition of growth pathways might have a differential effect in the senescent phenotype. For the combined FBS starvation and etoposide treatment experiment, data has been presented for 4 replicates of Lamin B1 and Lamin A/C in two biological replicates and 2 replicates for Lamin B2 in one biological replicate for the relative transcript level analysis of lamins. The two other experimental conditions which are FBS-starvation alone and etoposide treatment alone, are represented by 4-6 replicates in two biological replicates. Nesprin-2 data is in one biological replicate and shows high deviation. Lamins show consistent downregulation across the three experimental conditions. Lamin B1 shows higher downregulation upon senescence than in FBS starvation alone. This suggests that higher Lamin B1 downregulation functions as a better senescence specific marker than as a general stress marker. In the case of Lamin B2, transcript levels show more consistent downregulation in FBS-starved conditions. For Lamin A/C, transcript levels dropped drastically for the FBS-starved group rather than for the etoposide treated group. As discussed in earlier results, Lamin A/C transcription and accumulation of prelamin A might function as a cellular signal for acute stresses, showing increased transcription for combined stress group. However this result needs more replicates and protein level analysis to be validated. Cell line studies have shown that p53 acts as Lamin A/C stabilizer which results in the transcription of p16 while inducing senescence [Yoon et al., 2019].

Chapter 4 Discussion

In this project, we analyzed the transcriptional status of lamins in the context of low dose etoposide and in FBS-starved conditions. We observe downregulation of lamins across two types of stress situations, and observe that lamin B1 downregulation is not only a marker of senescence, but also for FBS-starved conditions. Please note that this statement requires more data for validation and for this conclusion the etoposide treatment of HCT116 shown in Figure 9 has not been included, since it was a pilot experiment with error-prone data. Lamin downregulation upon etoposide treatment can be observed. Therefore, lamin B1 downregulation as a biomarker of senescence must be used along with other markers, such as GLB1 or SA β-gal assay, proliferation arrest or SASP profiling. Various LINC complex proteins also show downregulation of transcription with FBS-starvation and etoposide treatment, suggesting chromatin remodeling due to lamin misregulation. Lamin modulation might be central to the reprogramming of the nuclear structures by their scaffolding action and regulation of lamina-associated domains (LADs).

During senescent induction, along with the downregulation of lamin B1, lamin B receptor (LBR) and lamina-associated polypeptides (LAPs) are also depleted. There is also an increase in damaged chromatin particles found in cytosolic compartments, suggesting chaotic nuclear architecture. While the role of lamin B1 downregulation in senescence induction and mediating senescence phenotypes is still unclear, we know that lamin B1 downregulation is caused by destabilization of lamin B1 mRNA and targeted degradation of mRNA by microRNAs [Wang and Dreesen, 2018]. Lamin B1 is also transported to the cytosolic compartment, where it is targeted by autophagy.

RNA quantification shows sensitive changes to the system, however, we must note that this is a quantification of global RNA. Therefore, it includes both kinds of cells- cells that show the least and the most differential expression due to the senescence induction. In *in vivo* conditions, this can mean a thousand-fold change in the expression of senescence markers and

SASP, which may have local and intense paracrine and autocrine effects [Wajapeyee et al., 2008]. This can cause tissue remodeling over long periods, suggesting a senescence theory of aging. However, we must note that aging as seen by people is caused due to the self-accelerating accumulation of damage and overdriven and inefficient systems at multiple levels. It is still a debate whether senescent cells and pathways are at the core of organismal aging or just a small clue on our way to understanding and tracking aging.

Studies on the effects of senescence may not need universal biomarkers. We need to employ different features and biomarkers of senescence and relevant senescence pathways in order to elucidate mechanisms by which senescence processes affect cancer or age-related disease progression. In that sense, the state of the senescent cell can be thought of as a quasi-stable equilibrium of various pathways at a higher cellular stress situation, but not high enough to induce apoptosis or transformation. The field of developmental senescence is burgeoning in the in vivo application of senescent markers. In certain developmental niches, markers of senescence such as proliferation arrest and SA β-gal are expressed whereas, widely accepted senescence pathways such as the DDR pathway and SASP, are not involved in the process. Thus, senescence biomarkers can be thought of as independent programs depending on the paradigm [Sharpless and Sherr, 2015].

Only recently has the role of senescent cells in multicellular organismal aging and disease conditions been observed, even though cellular senescence has been studied for decades. A lot is unknown with regards to the exact proteins and genes involved in senescence induction as opposed to processes that use a similar pathway but achieve entirely different outcomes, such as apoptosis and autophagy. The effects of senescent cells on ECM, cell-tissue interaction and subsequent formation of disease states need to be further explored. All of this requires a basic understanding of chromatin remodeling and nuclear architecture in senescence, which may be central to the way senescent cells express their variety of intracellular and intercellular effects.

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