

HETEROGENEITY EXHIBITED IN CELLS THAT ESCAPE FROM DRUG-INDUCED
SENESCENCE

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Abstract

Maintaining genomic integrity is key in the prevention of cancer. To limit the accumulation of mutations over time and stop damaged cells from continuing to proliferate and further gain subsequent mutations, intrinsic terminal cellular programs are activated. Senescence, an evolutionarily conserved stress state that traditionally has been viewed as an irreversible state of cell cycle arrest, acts as a tumour suppressive barrier. However, senescence acts as a double-edged sword, as senescent cells accumulate and contribute to age-associated diseases by altering tissue microenvironments and disrupting tissue homeostasis. Atrophied telomeres, mitotic errors, diminished DNA damage response and loss of nuclear envelope integrity all contribute to the increased rate of the formation of senescent cells during aging. In this study, I observed an age-associated accumulation of cytoplasmic DNA, in the form of micronuclei and chromatin bridges. I extended these finding to rare segmental accelerated aging diseases such as classical and non-classical Hutchinson-Gilford progeria syndrome, and Dyskeratosis Congentia. I further reveal that age-associated micronuclei and chromatin bridges undergo DNA damage and are recognized by cGAS that likely initiates an innate immune inflammatory response. My findings, along with recent reports showing chromosomal instability in senescent cells, led me to hypothesize that senescence may be reversible. To address this question, I utilized a drug-induced senescence model in BJ fibroblast and leukemia cells. Performing cloning experiments and monitoring single cells, I show that escape from a drug-induced senescent-like state occurs in a rare sub-population of cells for both transformed and normal cells. The escaped population displayed phenotypic heterogeneity, with respect to drug-response, gene expression, rate of proliferation and duration of arrest. RNA-sequencing highlighted the extensive transcriptional and metabolic reprogramming senescent cells undergo. Inhibition of lysosome function with FDA approved

drug, chloroquine, specifically kills some senescent leukemia cells. By stratifying acute myeloid leukemia patients based on expression of senescent-associated gene signatures, I show that senescent gene signatures are associated with poorer overall survival. My findings provide evidence challenging the previous notion that senescence is a permanent state of cell cycle arrest and may be a mechanism which cancer cells exploit to survive therapy.

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List of Abbreviations:

Abbreviation	Full form
AML	Acute myeloid leukemia
bp	base-pair
C12-FDG	5-Dodecanoylamino fluorescein Di- β -D-Galactopyranoside
C3	Complement 3
CB	Chromatin bridge
cGAMP	2'3'-cyclic guanosine monophosphate-adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase
CQ	Chloroquine
CRISPRi	CRISPR-mediated gene inactivation
D-loop	Displacement loop
D+Q	Dasatinib and quercetin
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DDR	DNA damage response
Diapause	Embryonic-diapause
DKC	Dyskeratosis Congentia
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
Dox	Doxorubicin
DS	Down Syndrome
DSBs	Double-stranded breaks
dsDNA	Double stranded DNA
DTP	Drug-tolerant persister
EPO	Erythropoietin
EPOR	Erythropoietin- receptor
ESAs	Erythropoietin stimulating agents
FKBP-Casp8	FK506-binding-protein-caspase 8
GSEA	Gene Set Enrichment Analysis
HGPS	Hutchinson-Gilford progeria syndrome
HR	Homologous recombination
IFN	Type-I interferon
IKK	I kappa B Kinase
IRF3	Interferon Regulatory Transcription factor 3
LMNA	Lamin A/C
LSC	Leukemia stem cells
MEF	Mouse embryonic fibroblasts

MN	Micronuclei
MOI	Multiplicity of infection
NC	Negative control
NE	Nuclear envelope
NH ₄ Cl	Ammonium chloride
NHEJ	Non-homologous end joining
NT	Not treated
OIS	Oncogene-induced senescence
p53DD	Dominant negative p53
PBS	Phosphate-buffered saline
Pol	Polymerase
PRR	Pattern recognition receptors
qRT-PCR	Quantitative real-time PCR
Rapa	Rapamycin
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
SA- β -gal	Senescence-associated β galactosidase
SAS	Senescence-associated stemness
SASP	Senescence-associated secretory phenotype
SAT	Senescence-associated tagged
ssDNA	Single-stranded DNA
STING	Stimulator of Interferon Genes
TBK1	TANK-binding kinase 1
TCGA	The Cancer Genome Atlas
TIS	Therapy-induced senescence
UV	Ultraviolet
XBJ	Drug-recovered BJ fibroblasts
YO	Year old
γ H2AX	Phosphorylation of H2AX serine 139

Chapter 1

General Introduction

1.1 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a clonal malignancy arising from myeloid progenitor cells that are incapable of fully differentiating. Subtypes of AML are defined by differences in morphology, genetic abnormalities and immunological markers. In spite of extensive research, the prognosis of AML remains poor and has not changed over the past 30 years. AML remains the most lethal form of blood cancer, with a mortality of nearly 70% in patients 65 years and older. Genotoxic chemotherapy involving daunorubicin and cytarabine, known as the “3+7 regimen” (3 days daunorubicin and 7 days cytarabine,) is commonly used to treat AML. For a majority of patients who respond well initially, the disease recurs within 3 years of diagnosis (Döhner et al., 2015). Despite extensive research, finding genetically altered resistant clones has proved difficult, suggesting that non-genetic means may be responsible for relapse and tolerance to therapy (Fennell et al., 2021; Kreso and Dick, 2014).

Leukemia stem cells (LSCs), also referred to as leukemia initiating cells, are similar to hematopoietic stem cells, as LSCs are self-renewing and give rise to leukemic blasts with varying degrees of aberrant differentiation. Incomplete differentiation or lineage infidelity is commonly seen in AML and contributes to both inter- intra-tumoral heterogeneity; driver gene mutations in subclones are correlated with different cell hierarchies (van Galen et al., 2019; Smith et al., 1983). The levels of LSCs signatures are a predictor of patient survival and response to therapy (van Galen et al., 2019; Ng et al., 2016). However, recent work tracing single cell dominance and

transcriptional states demonstrates that not all dominant AML clones that give rise to a tumour contain the LSC signature and display heterogeneous transcriptional profiles (Fennell et al., 2021).

The acquisition of driver gene mutations in immature progenitor cells fuels proliferation and survival of leukemia cells. A hallmark of cancer is the ability to sustain proliferation; some become independent of extrinsic growth factor signalling. Several mechanisms can be responsible for the independence from extrinsic growth signals: 1) activating mutation within extracellular receptors; one of the most common driver mutations in AML occurs in FLT3. FLT3, a split tyrosine receptor kinase promotes proliferation and survival by activating downstream targets such as AKT, STAT5 and Ras pathways. Under normal circumstances, FLT3 binds the extracellular ligand FL, which promotes FLT3 dimerization and activation that subsequently undergoes auto-inactivation. Internal-tandem duplication of the juxtamembrane domain within FLT3, the most common FLT3 mutation in AML, prevents auto-inhibition and leads to a constitutively active FLT3. AML patients harbouring an FLT3 mutation have a poor prognosis and increased frequency of relapse (Kiyoi et al., 2020). The ability of cytokine receptors to stimulate pro-survival pathways in AML and other cancers is a common occurrence but may not require mutational events to promote chronic activation; high levels of endogenous cytokines or cytokine-related therapies may promote chronic activation of cytokine receptors and downstream survival pathways. 2) autocrine production; amplification of ligand encoding genes, or removal of silencing epigenetic modifications resulting in the de novo expression or overexpression of pro-survival ligands can lead to aberrant autocrine signalling. 3) overexpression of receptors; amplification of receptor genes or epigenetic changes resulting in ectopic expression or overexpression of receptors can result in proliferative and pro-survival stimulation. Expression of ectopic receptors in tumorigenic

cells can facilitate novel ligand stimulation and pathway activation (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

Production of poorly differentiated, rapidly proliferating leukemic blasts promotes overcrowding of the bone marrow, bone marrow failure and prevents efficient erythropoiesis. As a result, AML patient commonly experience recurrent infection, bruising and anemia. Additionally, genotoxic therapy aimed to eliminate the malignancy adversely affects normal hematopoiesis and can further immunocompromise patients and contribute to anemia. Malignancy- and chemotherapy-induced anemia in cancer patients have been managed with erythropoietin (EPO) and erythropoiesis-stimulating agents (ESAs) (Aapro et al., 2019). EPO, a cytokine that canonically binds to the EPO-receptor (EPOR) triggers differentiation of immature erythroid progenitors into mature red cells. However, EPO stimulation additionally promotes the activation of pro-survival signals mediated through activation of the JAK2, STAT5, and Ras pathways. Several studies have reported that treatment of anemic cancer patients with ESAs increases tumour progression, tumour recurrence, thrombosis and death (Glaspy, 2009; FDA, 2017). These serious safety concerns have restricted the use of ESAs in patients receiving chemotherapy. EPO protects DA3/EPOR leukemia cells and DP.16 erythroleukemia cells from genotoxic drug-induced cell death (Lin and Benchimol, 1995; Pham et al., 2019).

1.2 Senescence

Cellular senescence is a stress response state triggered by various endogenous and exogenous sources. There are three main types of senescence: 1) replicative senescence; a telomere-dependent process, 2) oncogene-induced senescence; a telomere-independent process, 3) therapy-induced

senescence; a telomere-independent process. Senescent cells remain viable and resist death by upregulating pro-survival pathways, are resistant to mitogenic stimulation and are stably arrested in G1 or G2 of the cell cycle with an enlarged morphology (Hernandez-Segura et al., 2018). Due to the seemingly irreversible arrested nature of senescence, senescence is viewed as a tumour suppressive barrier for pre-cancerous lesions and cancer cells. Typically, senescent cells display persistent DNA damaging signaling and attenuated DNA repair mechanisms. During senescence cells undergo metabolic reprogramming and begin secreting cytokines, chemokines, metalloproteases, and extracellular matrix proteins, known as the senescence-associated secretory phenotype (SASP). Senescent cells display phenotypic heterogeneity with respect to morphology, DNA damage response, and SASP genes produced, which is partially dependent on the stress promoting senescence and cell type (Hernandez-Segura et al., 2018; Coppé et al., 2010).

In addition to senescence being a tumour suppressive barrier, more recent work has shown that senescence has pleiotropic effects. Sporadic, acute appearance of senescent cells in young individuals occur at low frequency and play beneficial roles. The transient existence of senescent cells in young individuals helps promote tissue homeostasis. Transient expression of SASP plays a critical role in development, wound healing, assisting in immunosurveillance, and tissue plasticity (Di Micco et al., 2021). However, the accumulation of senescent cells is associated with aging and has been shown to promote various aging-associated diseases and reduce the health-span and lifespan of mice. Chronic production of SASP, due to the accumulation of senescent cells, contributes to pathology in an autocrine and paracrine manner. SASP has been shown to reinforce the senescent phenotype, and initiate senescence in neighbouring cells. The persistence of SASP results in ‘inflammaging’, where senescent cells continuously secrete pro-inflammatory

products. Despite senescence being an intrinsic barrier to tumorigenesis, the presence of senescent cells neighbouring transformed cells can aid in tumorigenesis. SASP can recruit immature myeloid immune suppressive cells, support angiogenesis, and promote invasion of tumour cells into the surrounding tissue (Di Micco et al., 2021).

1.2.1 Replicative Senescence

Initially described in 1961, Hayflick and Moorhead demonstrated that normal fibroblasts had limited replicative potential in culture and entered an irreversible cell cycle arrest, termed Hayflick's limit, now referred to as replicative senescence (Hayflick and Moorhead, 1961). Hayflick and Moorhead proposed that senescent cells represent aging *in vitro* and may contribute to organismal aging. In 1971, Olovnikov theorized, based on the structure of DNA and the semiconservative method by which DNA is replicated, that linear eukaryotic chromosomes would shorten and the process of chromosomal shortening would lead to cellular aging; cells that are able to maintain chromosome lengths would be endowed with unlimited replicative capacity (Olovnikov, 1971). Despite skepticism, both theorized biological processes were recognized to be accurate in 2011 and 1998, respectively (Vaziri and Benchimol, 1998; Bodnar et al., 1998; Baker et al., 2011).

1.2.1.1 Telomere structure

Eukaryotic chromosomes are linear and end in an evolutionarily conserved DNA sequence, known as the telomere. In humans, telomeres consist of perfect repeats of 5'-TTAGGG-3' over a length of 5-10 kilobase pairs. Human telomeres have a long 3' G-rich single stranded overhang ranging

in size from 50-300 nucleotides (nt). To prevent the linear ends of the chromosomes being recognized as DNA double stranded breaks (DSBs) by the DNA damage response (DDR), the telomere forms a t-loop. The 3' G-rich overhang invades and displaces the upstream G-rich strand and anneals with the 5' C-rich strand, forming the t-loop. The upstream G-rich strand that is displaced by the invading 3' overhang forms a displacement (D-) loop. Processing and maintenance of the t-loop is orchestrated by the shelterin complex, a protein complex composed of TRF1, TRF2, POT1, TPP1, TIN2, and Rap1. Disruption of the shelterin complex, by the expression of dominant negative TRF2, results in rapid telomere shortening and aberrant DDR (Maciejowski and de Lange, 2017).

1.2.1.2 Telomere attrition and the DNA end replication problem

At the origin of replication, the helicase complex unwinds DNA bidirectionally generating the replication fork bubble, composed of two symmetric replication forks. The replication fork is comprised of a leading strand, where the template DNA is in a 3' to 5' direction, and a lagging strand, where the template DNA is in a 5' to 3' direction. As the replication fork moves away from the origin, single stranded DNA (ssDNA) begins to be exposed. Polymerase (Pol) α synthesizes a *de novo* RNA primer (10-20 nt) in a 5' to 3' direction. The RNA primer is essential for high fidelity DNA polymerases to initiate DNA synthesis. Once the RNA primer is laid down, PCNA is recruited to the RNA primer and facilitates a docking site for DNA pol δ and ϵ , which synthesize DNA of the lagging and leading strands, respectively. The leading strand can be synthesized in a 5' to 3' direction following the unwinding of the replication fork, from a single RNA primer. Due to the necessity of DNA polymerases to synthesize DNA in a 5' to 3' direction, the lagging strand

requires RNA primers to be generated congruently with the movement of the replication fork. DNA pol δ is then able to synthesize short fragments of DNA from each RNA primer in a 5' to 3' direction, known as Okazaki fragments. The upstream RNA primer is removed and replaced by the downstream Okazaki fragment. The Okazaki fragments are then ligated together. The mechanism of DNA replication poses an inherent issue for linear chromosomes. At the end of the telomere, the lagging strand's RNA primer is removed but not replaced. The loss of the RNA primer results in the telomere ends shortening each round of DNA replication. As normal somatic fibroblasts increase in population doubling, the telomere length decreases (Harley et al., 1990). Cells taken from elderly individuals contain shorter telomeres than younger individuals.

In addition to the DNA end replication problem, the telomere is shortened by a secondary mechanism. For the t-loop to protect the ends of the telomere, the 3' G-rich single stranded overhang is generated by resectioning mediated by the Apollo nuclease. This process is regulated by the shelterin complex. Cumulatively, each round of DNA replication leads to 50-100 bp shortening of the telomere.

1.2.1.3 Telomerase extends telomere length and lifespan

Telomerase is a reverse transcriptase composed of two main subunits: hTERT, a catalytic subunit, and hTERC, an RNA subunit. Additional components of the telomerase complex are DKC, NOP10, NHP2, GAR1, and TCAB1. hTERC contains a template region with a 5'-CCCUAA-3' sequence; complementary to the G-rich telomere strand. hTERT extends the 3' end of telomeres by synthesizing DNA *de novo*, using the hTERC subunit as a template. Normal

somatic cells do not express telomerase, and therefore cannot maintain telomere lengths leading to replicative senescence. Mutations in telomerase subunits, are associated with Dyskeratosis Congentia, a rare disease presenting with aspects of premature aging, and rapid telomere attrition. The ectopic expression of telomerase in somatic cells results in the extension of telomeres lengths and increased lifespan, bypassing replicative senescence (Vaziri and Benchimol, 1998; Bodnar et al., 1998). Stem cells constitutively express telomerase to maintain telomere lengths and have increased replicative lifespan. A hallmark of cancer cells is the ability of limitless replication. The majority, 80-90%, of cancer cells express telomerase and bypass the replicative barrier and gain replicative immortality (Kim et al., 1994). Some cancer cells use the DNA recombination-based ALT (alternative) pathway to protect telomeres and become immortal (Cesare and Reddel, 2010).

1.2.2 Oncogene-induced senescence

In 1981, the Weinberg lab demonstrated that transfection of DNA from human bladder cancer into NIH/3T3 mouse fibroblasts had a transforming ability and resulted in foci formation (Shih et al., 1981). The human gene permitting foci formation in NIH/3T3 were H-Ras. Subsequent findings showed that family of Ras genes were commonly mutated in cancers and drove tumour formation by accelerating proliferation. Seemingly paradoxical to early work showing that oncogenes drive proliferation, Serrano and colleague (1997) demonstrated that ectopic expression of H-Ras V12 in normal somatic cells leads to senescence, coined as oncogene-induced senescence (OIS). Since the initially discovery of OIS, many more oncogenes have been identified to promote OIS, including BRAF, AKT, E2F1, MYC and cyclin E; additionally, the loss of tumour suppressors PTEN and NF1 leads to OIS. Hyperproliferating signals from

constitutively active oncogenes leads to nucleotide depletion and stalling of the DNA replication fork (Bartkova et al., 2006; Di Micco et al., 2006; Graziano and Gonzalo, 2017). Prolonged stalling of the DNA replication fork results in fork collapse and DNA damage. These processes lead to what is known as replication stress and is likely the signal that directly promote OIS.

1.2.3 Therapy-induced senescence

Cancers are commonly treated with surgery, radiation therapy and/or chemotherapies. Genotoxic radiation and chemotherapies have greater effect on cancer cells than somatic cells because they promote more DNA damage in rapidly proliferating cells; cells that have reduced rates of proliferation do not undergo as much damage. The aim of radiation and chemotherapy is to promote death of cancer cells either through apoptosis; some cells resist death and enter senescence. By causing therapy-induced senescence (TIS), cancer cells would no longer be capable of dividing and tumours would regress. Unfortunately, the effects of chemotherapeutics, and radiation, are not solely limited to cancer tissue; many normal somatic cells undergo TIS. Childhood-cancer survivors who were treated with radiation or genotoxic chemotherapy are more likely to develop age-associated disease, including malignancies, than their cancer-free siblings by 50 years of age (Armstrong et al., 2014). Radiation therapy and several chemotherapies induce genotoxic stress and the DDR. Several factors determine the outcome of therapy, including, drug, dose, duration, and type of cell. The DDR subsequently activates intrinsic mechanisms promoting apoptosis or survival or senescence.

1.2.4 Mechanisms of senescence

Independent of the manner of senescence, whether replicative-, oncogene-, or therapy-induced, a DDR is typically responsible for the initial activation of downstream pathways that regulate senescence. Due to the importance of impeding tumorigenesis, multicellular organism evolved redundancies in initiating and sustaining senescence. There are three main pathways that regulate senescence, the p53 pathway, p16/Rb pathway and the SUV39H1a pathway (Kumari and Jat, 2021). In this section I will focus on the p53 and p16 pathways regulating replicative senescence and therapy-induced senescence.

Telomeres within a population of normal cells vary in length. Single cells analysis of telomere lengths showed that telomere lengths became more heterogenous in length as cells approached their replicative lifespan (Wang et al., 2013). Replicative senescence initiates when either a single telomere or a few telomeres reach a critically short length. When telomeres reach a critically short length, the t-loop is unable to form properly and the exposed unprotected ends of linear DNA leads to a DDR (**Figure 1-1**). Ectopic expression of TRF2 extends the replicative lifespan of somatic cells, suggesting that the shelterin complex plays a role in preventing aberrant telomeric DNA damage signaling, which promotes replicative senescence. Similar to replicative senescence, therapy-induced senescence largely relies on activation of the DDR to trigger senescence. Genotoxic drugs promote replication stress, DNA adducts, DNA intercalation and single stranded- and double stranded DNA breaks throughout the genome. Doxorubicin (Dox), a clinically relevant genotoxic chemotherapeutic, is commonly used to investigate therapy-induced senescence. Dox promotes DNA damage in two distinct mechanisms: 1) Dox inhibits topoisomerase II, preventing

unwinding of supercoiled DNA, which results in replication stress, and prevents DNA repair, 2) metabolism of Dox generates reactive oxygen species (ROS), which damages macromolecules such as lipids and DNA.

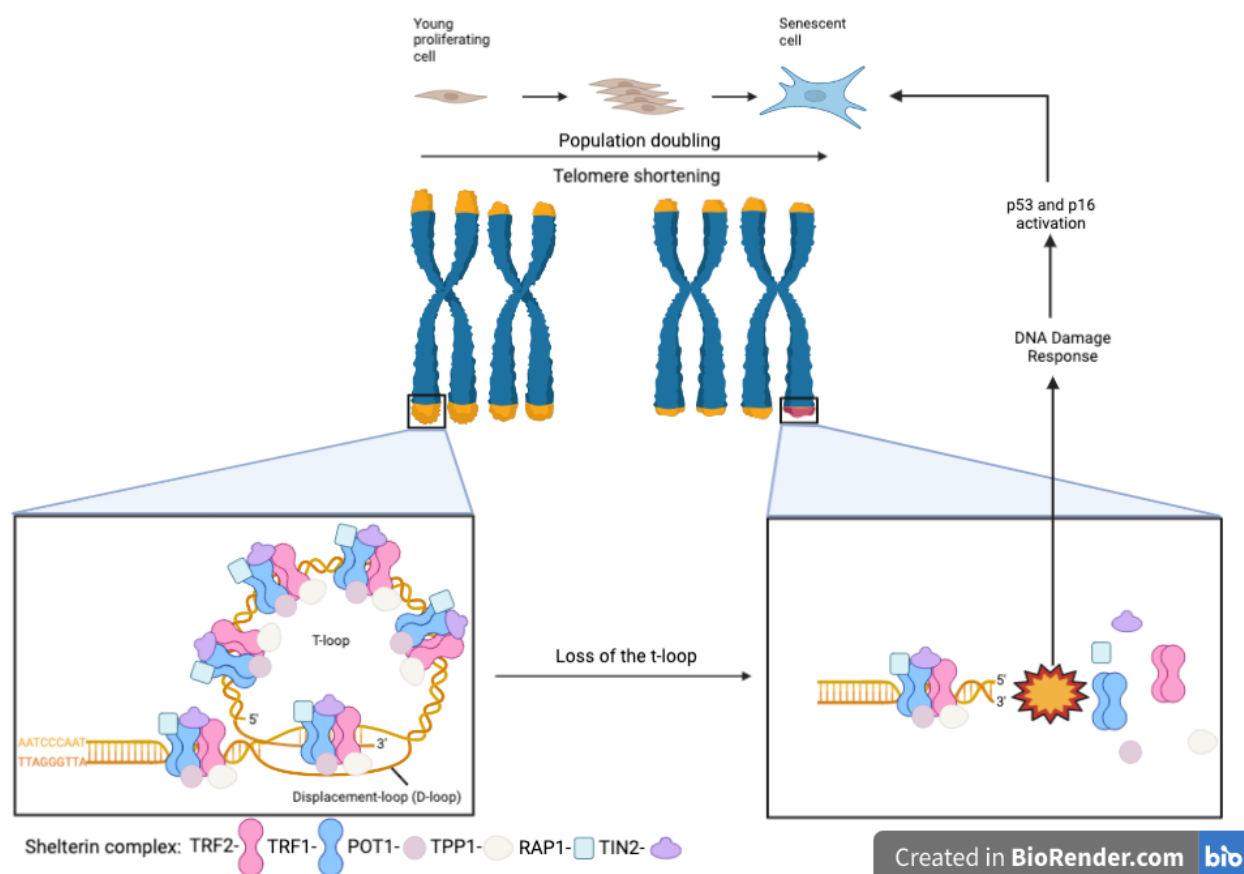


Figure 1-1 Telomere loss model of replicative senescence. Telomeres, an evolutionarily conserved repeating sequence (consisting of TTAGGG repeats in humans) found at the ends of linear eukaryotic chromosomes, protect the non-telomeric gene encoding DNA. The shelterin complex composed on TRF2, TRF1, POT1, TPP1, RAP1 and TIN2, help form a protective t-loop and hide the linear end from being recognized as a DNA double-strand break. Due to the DNA end-replication problem and nuclease resectioning, proliferating cells undergo telomere loss every round of division. When telomere length reaches a critically short length, the t-loop cannot properly form, and the shelterin complex dissociates from the short telomere. In turn, the DNA damage response becomes activated and triggers p53 and p16 activation, which induce replicative senescence.

Damaged DNA needs to be resolved in a quick and efficient manner. An early event in the DDR is the phosphorylation of H2AX serine 139, known as γ H2AX, mediated by ATM or ATR. ATM is recruited to the critically short telomere or site of DNA damage and amplifies the γ H2AX signal (Vaziri et al., 1997; Takai et al., 2003). In turn, γ H2AX signaling enables a feedback loop recruiting and amplifying ATM and ATR activation, which activate CHK1 and CHK2 kinases. The activation and amplification of ATM, ATR CHK1 and CHK2 promote downstream stress response pathways promoting senescence. During senescence, cells display persistent γ H2AX signaling, which is thought to help maintain the senescent phenotype.

1.2.4.1 p53-mediated senescence and regulation

Under normal circumstances, p53 protein level is kept at a low basal level by continuous proteasomal degradation by MDM2 E3 ligase. When cells undergo DNA damage, ATM, CHK1 or CHK2 phosphorylate p53 at serine 15, enabling p53 to dissociate from MDM2 and become stable. Nutlin-3a, a non-genotoxic MDM2 inhibitor, promotes stabilization of p53 resulting in p53-mediated senescence in a cell line specific manner. Activated p53 transcriptionally orchestrates an extensive network of genes that directly and indirectly determine cell fate. Depending on the cell type, duration of stress and severity of insult, p53 will upregulate different genes to promote apoptosis, transient cell cycle arrest or senescence (Hafner et al., 2019).

The classical mechanism of p53-induced senescence is through the upregulation of p21. p21 promotes G1 cell cycle arrest by binding and inhibiting cyclin-dependent kinases (Cdk)-2/4/6, as well as PCNA. p21 kinetics determine cell fate and vary between cells within a population. There

are 3 outcomes of p21 dynamics: 1) initial high expression of p21 that is sustained, resulting in a senescent state, 2) induction of medium levels of p21 expression causes a transient cell cycle arrest and proliferation resumes as p21 levels taper off, 3) most cells initially have low expression of p21 and gradually increase p21 expression, resulting in senescence (Hsu et al., 2019). However, p21 is dispensable for p53-mediated senescence. p53 regulates additional genes to initiate and maintain senescence. During telomere disruption, p53 transcriptionally upregulates BTG2, which promotes senescence. Normal somatic fibroblasts depleted of BTG2 have an extended lifespan. BTG2 promote senescence by sequestering Pin1, a protein that regulates proliferation by stabilizing cyclin D, from the nucleus (Wheaton et al., 2010).

Loss of p53 is frequently seen in multiple types of cancer. By losing p53-mediated senescence, pre-cancerous cells and cancer cells are endowed with the ability to bypass replicative senescence and oncogene-induced senescence. Ectopic expression of p53 short hairpin RNA (shRNA) prior to somatic cells entering replicative senescence, permits somatic cells to bypass the replicative barrier and continue to divide. Somatic cells that have bypassed replicative senescence due to p53 inactivation continue to experience telomere erosion. The loss of multiple telomere ends results triggers wide-spread apoptosis and autophagy-mediated death termed, crisis (Counter et al., 1992; Nassour et al., 2019). Although there is cell line variability, deficiency in p53 leads to reduced levels of therapy-induced senescence.

Regulation of p53 is tightly controlled. For p53 to function as a transcription factor, tetramerization is required. Once tetramerized, p53 binds to p53 response element (a sequence consisting of: PuPuPuC(A/T)(A/T)GPyPyPy N_{0,13} PuPuPuC(A/T)(A/T)GPyPyPy) through its DNA binding

domain (DBD). Ectopic expression of dominant negative p53 (p53DD), a C-terminal p53 isoform containing the tetramerization domain and lacking the DBD, facilitates tetramerization but inhibits transcriptional activation, permitting a similar phenotype to a p53 null background (Shaulian et al., 1991). Mutations within the p53 DBD are commonly found in cancer, as these hotspot mutations inhibit tumour suppressive functions of p53; allowing survival and cell cycle progression through stress.

Alternative regulation of p53 stems from exogenous signals in the environment. Activation of survival pathways by extracellular cytokines, including IL3, IL6, SCF and erythropoietin (EPO) can also prevent p53-dependent apoptosis (Yonish-Raouch et al., 1991; Lin and Benchimol, 1995; Canman et al., 1995; Johnson, Chung and Benchimol, 1993). EPO has been shown to prevent downregulation of MDM2 resulting in destabilization of p53 and modulation of p53 transcriptional activity, preventing p53-mediated apoptosis and instead promoting a senescent-like state (Pham et al., 2019).

p53 is further regulated by post-translational modification. Deacetylation of p53 by ectopic SIRT1 was shown to reduce p21 expression, and reduce p53 mediated cell death (Vaziri et al., 2001). Several subsequent mutagenesis experiments show that mutating lysine (K) to arginine (R) within p53's DBD influences target gene expression and outcome to stress. Mutating K117R in MEFs abolished p53-mediated apoptosis, and prevents p53-mediated transcription of pro-apoptotic genes; p53 K117R cells are still able to enter p53-mediated senescence. MEFs harbouring p53 K117R, K161R, and K162R have defective apoptotic signalling, and fail to enter therapy- and oncogene-induced senescence (Li et al., 2012).

Taken together, current evidence demonstrates that p53 is a key mediator of senescence. p53 activity is regulated on multiple levels, ultimately determining cell fate. The senescence fate is incited based on type, severity, and duration of stress, as well as cell type and extrinsic signaling. p53 protein stability, post-translational modification, and transcriptional co-factors govern which p53-target genes are expressed and the induction of senescence.

1.2.4.2 p16-mediated senescence

Cell cycle progression from G1 to S phase requires induction of multiple S-phase related genes including genes encoding proteins crucial for DNA replication. Transcription factor E2F1 targets several DNA replication genes and promotes their transcription. Activation of E2F1 has been shown to be necessary for G1 to S phase transition. To prevent aberrant entry into S-phase and initiation of DNA replication, E2F1 is regulated by pRb through direct interaction. pRb-E2F1 localizes in the cytoplasm and renders E2F1 transcriptionally inactive, keeping cells in G1. When cells are ready to enter S-phase, pRb is phosphorylated by CDK2/4/6. Hyperphosphorylated pRb dissociates from E2F1, allowing E2F1 to localize to the nucleus and promote transcription of essential S-phase genes. p16 binds and inhibits CDK4/6, and prevents CDK4/6 mediated phosphorylation of pRb. Thus, pRb remains hypophosphorylated and bound to E2F1 (Kumari and Jat, 2021).

p16 initiates senescence in response to stress and can maintain a senescent state independent of p53, however both pathways are often seen acting in unison to reinforce the senescent state. Loss

of p16 is commonly seen during tumorigenesis, typically in melanogenesis and pancreatic cancer development, supporting the notion that p16 prevents tumour development by promoting senescence (Shain et al., 2015; Yachida and Iacobuzio-Donahue, 2013). p16-dependent senescence is seen in a cell line dependent context, as not all cells express p16 (Beauséjour et al., 2003). Recognized as a classical mediator of senescence, the regulation of p16 is poorly understood. Although telomere attrition and DNA damage canonically activates p53, some cells respond by an increase in p16 levels that can maintain senescence independent of p53 activity. Elevated levels of p16 are seen during aging and as cells approach their replicative limit, as well as during genotoxic treatment.

1.2.5 Senescence-associated secretory phenotype (SASP)

Secretion of cytokines, chemokines, metalloproteases, and extracellular matrix proteins is a hallmark of senescent cells. There is considerable heterogeneity in the composition of the SASP, depending on both cell type and tissue, manner of senescence induction, severity of insult, and duration of senescence. The SASP has been shown to influence the tissue micro-environment as well as having systemic effects. SASP can play both positive roles and detrimental roles. The transient existence of senescent cells allows the SASP to recruit immune cells to tissues and allows clearance of damaged cells. Interleukin 6 (IL6), a common SASP marker, can trigger tissue regeneration in a paracrine manner by stimulating stem cell reprogramming (Mosteiro et al., 2018). However, chronic presence of senescent cells disrupts tissue homeostasis by promoting inflammation through IL6 and SASP. Furthermore, despite being a tumour suppressive mechanism, senescence cells have been shown to aid in tumour invasion through SASP (Gorgoulis

et al., 2019). Recently, SILAC-based mass spectrometry revealed that media from senescent cells had an upregulation of coagulation-related genes (Wiley, et al., 2019). Conditioned media from senescent cells promotes coagulation of blood *in vitro* (Lee et al., 2021). Dox-treated mice demonstrated reduced tail bleeding due to faster clotting, than control mice and Dox-treated mice that had senescent cells eliminated (Wiley, et al., 2019).

1.2.5.1 DNA damage response modulated SASP

Not only does DNA damage and the DDR play a pivotal role in the initiation of senescence, DNA damage and the DDR also trigger the SASP. Persistent 53BP1 foci, a marker of DNA damage, is correlated with production of IL6 and SASP. shRNA mediated knockdown of ATM and CHK2 in somatic cells results in a decline of IL6 and IL8 production (Roider et al., 2009). ATM influences SASP targets independent of p53 activity (Coppé et al., 2010). Nutlin-3a (an inhibitor of MDM2) treated fibroblasts enter p53-dependent senescence, independent of DNA damaging signaling, and do not exhibit SASP production (Wiley et al., 2018). Furthermore, therapy-induced senescent fibroblasts treated with nutlin-3a demonstrate a decrease in several SASP genes, suggesting that p53 can negatively regulate aspects of SASP (Wiley et al., 2018).

1.2.5.2 Response to cytosolic DNA influences SASP

Oncogene activation, radiation, and drug treatment promote extensive genomic instability. As a consequence of persistent genomic instability cells may undergo cellular senescence and mitotic errors. Micronuclei (MN) result from mitotic errors and can arise through various mechanisms.

One method is through DSBs persisting in mitotic cells, resulting in chromosomal fragmentation. The fragmented acentric chromosome will lag during mitosis. Another possible mechanism for MN generation is improper kinetochore attachment, resulting in a fully intact chromosome lagging. Alternatively, a dicentric chromosome that is pulled to opposite poles, creating a chromosomal bridge. Bridge resolution may lead to fragmented chromosomes that lag (Fenech and Natarajan, 2011). Lagging chromosomes are sequestered from the primary nucleus and mis-segregated. In turn, the lagging chromosome recruits its own NE, creating a MN within the cytoplasm. Inappropriate NE constitution by failure to incorporate “non-core” NE proteins such as B-type lamins results in MN rupture (Liu et al., 2018). Both MN rupture and CB formation fail to segregate chromosomal DNA from the cytoplasm.

Additional mechanisms underly the accumulation of DNA in the cytosol of senescent cells. During replicative-, oncogene-induced and therapy-induced senescence, the nuclear envelope and chromatin undergo autophagy-mediated degradation, which is thought to help sustain the arrested state by depleting chromatin (Dou et al., 2015; Ivanov et al., 2013). Chromatin is pinched off from the nucleus and resides within the cytosol until degraded or secreted into the extracellular environment. The depletion of lamin B1, a hallmark of senescence, results in decreased structural integrity of the nuclear envelope (Freund et al 2012; Shah et al., 2013; Shimi et al., 2011). Chromatin can herniate from the weakened nuclear envelope and be exposed to the cytosol. More recently, activation of LINE-1 retrotransposon has been shown to be elevated in senescent fibroblasts, fibroblasts from elderly donors and normally aged and progeroid mice (De Cecco et al., 2019; Simon et al., 2019). LINE-1 is reverse-transcribed into ssDNA in the cytosol, while

TREX1, a 3' exonuclease is downregulated during senescence. Together this helps facilitate the accumulation of an RNA:DNA hybrid and ssDNA in the cytosol (De Cecco et al., 2019).

The accumulation of cytoplasmic-DNA initiates the innate immune response mediated through pattern recognition receptors (PRR). Cyclic GMP-AMP synthase (cGAS) a well-defined PRR, binds to cytosolic DNA and activates downstream substrates by the production of 2'3'-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP). The catalytic domain of cGAS, consisting of an A-site and B-site, binds to the sugar-phosphate backbone of DNA. Binding of A-site to DNA facilitates a conformational change that opens that ATP and GTP pocket. The B-site interaction with DNA permits formation of a homodimer complex of cGAS, bound 2 strands of DNA. cGAS preferentially binds to long dsDNAs by forming a ladder-like structure (Decout et al., 2021).

Once bound to DNA, the catalytic subunit of cGAS synthesizes cGAMP. cGAMP activates Stimulator of Interferon Genes (STING) located on the endoplasmic reticulum membrane. Stimulated STING dimerizes and translocated to the Golgi apparatus, where TANK-binding kinase 1 (TBK1) is recruited and undergoes dimerization and autophosphorylation, and phosphorylation of STING (Zhang et al., 2019; Zhao et al., 2019). The phosphorylated STING-TBK1 complex recruits Interferon Regulatory Transcription factor 3 (IRF3), which is phosphorylated and undergoes dimerization. Activated IRF3 is translocated IFN into the nucleus, where it transcriptionally upregulates proinflammatory type-I interferon (IFN) genes and other IFN stimulator genes, such as IL6. Additionally, STING can promote inflammation through a non-canonical pathway. Recruitment of I kappa B Kinase (IKK) to STING leads to the

downstream activation of NF κ B (**Figure 1-2**); STING activation contributes to SASP and “inflammageing” (Ablasser and Chen, 2019).

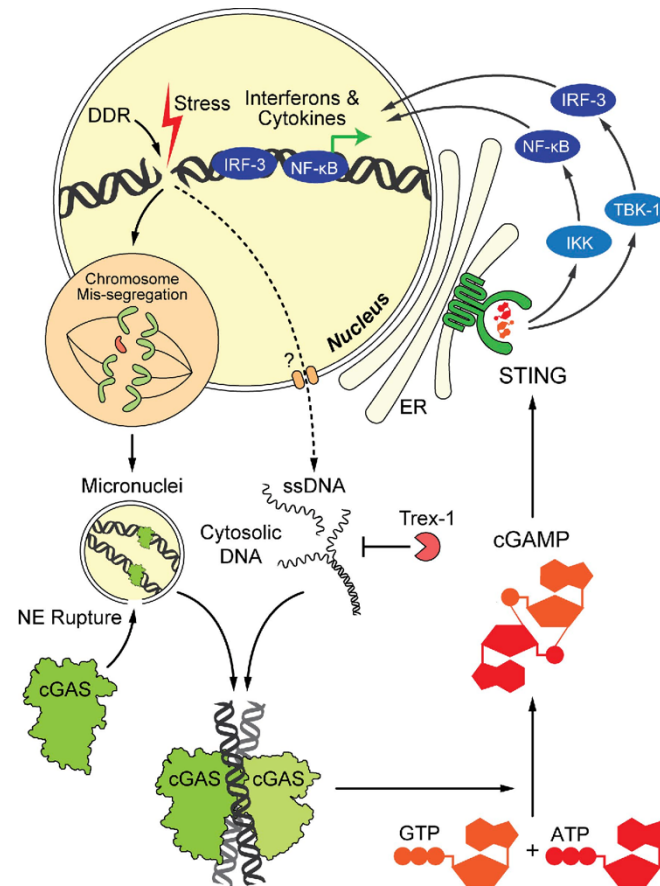


Figure 1-2 cGAS-STING pathway activation in response to cytosolic dsDNA.

Adapted from Li and Chen (2018). Damaged DNA relocating to the cytosol, and mis-segregation of chromatin that form micronuclei undergo cGAS recognition. cGAS dimerizes and generated cGAMP that activates STING located on the ER membrane. In turn, STING activates downstream kinases, IKK and TBK1 that phosphorylate NF κ B and IRF3, respectively. NF κ B and IRF3 re-localize to the nucleus, where they act as transcription factors upregulating proinflammatory type I interferons and cytokines.

Depletion of either cGAS or STING reduces the production of the type-I IFN arm of SASP. Genetic deletion of cGAS in mouse and human cells prevents senescence, whereas shRNA-mediated knockdown permitted cells to enter senescence (Dou et al., 2017; Glück et al., 2017; Yang et al., 2017). DNA damage mediated cGAS activation initiates the production of type-I IFN; the production of some SASP related genes acts in a paracrine and autocrine fashion to reinforce

the senescent state (Kuilman et al., 2008; Acosta et al., 2008). The mechanism in which cGAS regulates senescence is not fully understood, however, it is thought that cGAS regulates senescence by regulating SASP. This remains controversial as STING appears critical for the cGAS mediated type-I IFN response but is not necessary for the induction of senescence. Cells harbouring an inactivating STING mutant or have been depleted of STING have a dampened type-I IFN response to the accumulation of cytosolic DNA but still enter senescence (Dou et al., 2017; Glück et al., 2017; Yang et al., 2017).

1.2.6 Senescence in progerias

Progeria, derived from the latin phrases pro- meaning rapid and geria- meaning age, is the name given to a group of diseases and syndromes that share aspects of accelerated aging in young individuals. There are multiple different types of progerias, none of which completely recapitulates normal aging. Progerias are rare and often due to mutations in DNA damage response genes, DNA repair genes, telomere maintenance genes and genes that ensure proper nuclear organization. Progerias are associated with age-related disease and cancers.

1.2.6.1 Hutchinson-Gilford progeria syndrome

Hutchinson-Gilford progeria syndrome (HGPS) is a rare, heterozygous, autosomal dominant genetic disorder, characterized by rapid segmental aging in children. It is one of several genetic progeroid diseases, some of which include Werner syndrome, Restrictive dermopathy, and Cockayne syndrome (Chen et al., 2003; Csoka et al., 2004; Vidak and Foisner, 2016). HGPS is

caused by a mutation in the LMNA gene that encodes for A-type lamins. Gross phenotypic onset of the disease occurs within the first year of life. Cerebrovascular injury and myocardial infarction, due to severe atherosclerosis, cause premature death of HGPS patients, occurring on average at 13 years of age (Merideth et al., 2008).

An estimated 85% of HGPS patients have a de novo mutation within exon 10, C1824T, of the LMNA gene (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). The de novo mutation (G608G) is silent; however it activates a cryptic splice site within exon 11 that removes 150 nucleotides from the prelamin A mRNA, that are transcribed from exons 11 and 12 (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). Prelamin A goes through an extensive maturation process involving farnesylation and subsequent cleavage of the C-terminal end by ZMPste24. Progerin, the mutant lamin A protein, lacks 50 amino acids near the C-terminal end and prevents ZMPste24 from cleaving the C-terminal end, thus progerin is permanently farnesylated. Progerin causes rapid aging in HGPS patients and progerin production increases in normal patients as they age, suggesting that it may also drive the normal aging process. The remaining 15% of HGPS patients have various other mutations within the LMNA gene or a LMNA processing gene that results in a premature aging phenotype but do not produce progerin, but instead a unique mutant and prelamin protein.

ZMPste24 null mice present with accelerated aging and have an increased accumulation of cells that undergo p53-dependent senescence (Varela et al., 2005). Ectopic expression of progerin in normal fibroblasts leads to premature senescence in a p53-dependent manner (Wheaton et al.,

2017). Progerin-induced senescence is independent of telomere erosion, and promoted by increased replication stress (Wheaton et al., 2017).

1.2.6.2 Dyskeratosis Congentia

Dyskeratosis Congentia (DKC) is a rare genetic disease presented with aspects of premature aging, bone marrow dysfunction and are predisposed to cancers such as leukemia. Mutations in telomere maintenance genes such as DKC1, TERC, TERT NOP10, NHP2 and TIN2 have been identified to cause DKC. Loss of efficient telomere maintenance leads to rapid telomere erosion in both somatic and stem cells. Replicative senescence is triggered prematurely in DKC cells due to the rapid telomere attrition (Mitchell et al., 1999; Wong and Collins, 2006).

1.2.7 Seno-therapy

In 2011, Baker and colleagues engineered a transgenic BubR1 progeroid mouse model capable of clearing p16 positive senescent cells. The system relied on expression of FK506-binding-protein–caspase 8 (FKBP-Casp8) fusion protein through the p16 promoter. Myristylation of FKBP-Casp8 promoted localization to the inner-cytoplasmic membrane, and treatment with AP20187, a synthetic drug, promotes dimerization. Dimerization of FKBP-Casp8 initiated Casp8-mediated apoptosis of p16 expressing cells and reduced *in vivo* levels of senescence (Baker et al., 2011). The clearance of senescent cells in progeroid mice delayed the onset of age-associated phenotypes. The seminal work by Baker et al. (2011), using an *in vivo* model, confirmed Hayflick and Moorhead’s hypothesis that senescence drives aging 50 years later.

Since the discovery that senescent cells directly contribute to age-associated phenotypes, multiple strategies have emerged to eliminate or modulate senescent cells. Senescent cells rely on pro-survival networks to remain viable. Most strategies to eliminate senescent cells act by stimulating the intrinsic apoptotic pathway. Inhibition of p21 and BCLxL with a combination of dasatinib and quercetin (D+Q) was shown to be an effective means of senolysis. Administration of D+Q eliminated senescent cells *in vivo* and extended the lifespan and healthspan of progeroid *Ercc1*^{-Δ} mice (Zhu et al., 2015). Targeting the anti-apoptotic BCL2 family, which are upregulated during senescence, has gained traction. Two small molecule inhibitors ABT-737 and ABT-263 were both shown to have senolytic effects *in vitro* and *in vivo* (Chang et al., 2016; Yosef et al., 2016).

Modulating p53 function so that apoptosis is preferentially induced over senescence has also been explored. FOXO4, a p53 interacting protein, prevents the apoptotic response of p53. shRNA-mediated knockdown of FOXO4 decreases viability of senescent cells. Using FOXO4-DRI, a small molecule that prevents the interaction of FOXO4-p53, p53 protein is redirected to the mitochondria, where p53-mediates a non-canonical form of p53-mediated apoptosis (Mihara et al., 2003; Baar et al., 2017). Treatment with FOXO4-DRI in Dox-treated mice, *Xpd*^{TTD/TTD} progeroid mice and naturally aged mice, promoted elimination of senescent cells and attenuated age-related phenotypes (Baar et al., 2017).

An alternative, novel approach in the clearance of senescent cells uses CAR-T cells that target senescent-specific extracellular antigens. The Lowe lab has recently shown that upregulation of uPAR, a protein that is both secreted and found on the extracellular membrane, can be exploited

to target senescent cells. CAR T cells that recognize uPAR were shown to selectively kill senescent cells *in vitro* and *in vivo*. Four distinct *in vivo* senescence mouse models were shown to benefit by treatment of uPAR CAR T cells (Amor et al., 2020).

Clinical trials are currently being performed to determine whether elimination of senescent cells may be able to delay the onset of, and attenuate age-related disease. Use of senolytics may not be limited to age-associated disease; senolytics may be used as an adjuvant therapy after chemo- or radiation therapy for cancers. Cancers treated with traditional therapy may undergo cell death, but some cells may survive by entering senescence. Recent research has shown that senescence may not be as irreversible as previously thought, and cancer cells that enter therapy-induced senescence may escape and contribute to relapse. Residual senescent cancer cells that persist treatment may be susceptible to adjunctive senolytic treatment and may be able to prevent or delay relapse.

1.2.8 Escape from senescence

Senescence has traditionally been defined as an irreversible state of cell cycle arrest, where cells cannot re-enter the cell cycle. Escape from senescence occurs when a cell enters a non-proliferative senescent state, and then re-enters the cell cycle. Escape from senescence is distinct from senescence bypass; cells that bypass the senescent barrier never enter senescence and continue replicating when they ought to have senesced. Cells that bypass senescence, may continue to divide during stress or enter a transient non-senescent arrested state (Reyes et al., 2018). The distinction between bypass and escape is subtle but key for appropriate interpretation. Although controversial,

a growing amount of evidence is beginning to show that escape from senescence may occur. However, numerous publications that make claims regarding escape from senescence fail to show escape convincingly. In addition, part of the controversy surrounding the reversibility of senescence stems from scientists being reluctant to define cells that re-enter the cell cycle as having been truly senescent, and use terminology such as ‘pseudo-senescence’ or ‘senescent-like’ despite using multiple markers of senescence to identify the cells (Saleh et al., 2019a).

Depending on the type of cancer and age of the patient, the likelihood of entering complete-remission and the duration of remission may vary after therapy. Relapse occurs when rare clones survive therapy, largely attributed to resistance and drug-tolerant initiating cancer stem cells. The emergence of rare proliferating clones after drug treatment results in relapse of the tumour. Cancer cells entering drug-induced senescence may be able to survive therapy and lay arrested for extending periods of time. Rare senescent cancer cells may eventually escape, resume proliferation, and contribute to relapse.

1.2.8.1 Escape from senescence in blood cancers

Escape from senescence has been shown to occur in drug-induced senescent cancer models (Saleh et al., 2019a; Saleh et al., 2019b). Using the E μ -myc transgenic BcL2 overexpression lymphoma model with an inducible SUV39H1a or an inducible p53 plasmid, the Schmitt lab was able to demonstrate that drug-induced senescence was reversible and dependent on either SUV39H1a or p53. While cells entered senescence they underwent a reprogramming and gained senescence-associated stem cell-like properties, referred to as senescence-associated stemness

(SAS). Escaped lymphoma cells were more aggressive, and mice transplanted with previously senescent lymphomas had a shorter survival than mice injected with never-senescent lymphomas (Milanovic et al., 2018). These findings were extended to a murine leukemia model, where it was shown that bulk leukemia cells that escape drug-induced senescence gain *de novo* initiating leukemia stem cell properties.

Duy et al. (2021) demonstrated that AML cells can persist following drug treatment by entering a reversible senescent- diapause-like state *in vitro* and *in vivo*. Entry into the senescent-diapause-like state was ATR dependent. AML cells that resumed proliferation after drug-treatment retained drug sensitivity but were more aggressive. Using bulk and single cell RNA-sequencing (RNA-seq) on matched pairs, transcriptional profiles of AML in patients taken at diagnosis and two to four weeks after therapy, during the refractory period, showed an increase in senescence associated genes during the refractory period.

1.2.8.2 Mechanisms of escape

Escape from replicative senescence was first described in 2003 by the Campisi lab. BJ human fibroblasts and WI-38 fibroblasts were brought to their replicative limits and shown to be a homogenous population of non-dividing cells based on [³H] thymidine-labelling. Despite cells entering replicative senescence, ectopic expression of telomerase was unable to extend telomere lengths of non-replicating fibroblasts and did not re-initiate proliferation. Non-dividing BJ fibroblasts transduced with: 1) SV-40 large T antigen (LgT), a viral protein that binds to and inactivates p53 and pRb, 2) LgTK1, a LgT that inactivates p53 but does not inactivate pRB, or 3)

GSE-22, a peptide that inactivates p53, resulted in cell cycle re-entry. Ectopic expression of CDK4m, a CDK4 mutant that cannot bind to p16 and renders pRB constitutively inactive, in non-dividing BJ fibroblasts resulted in few cells resuming proliferation. Non-dividing WI-38 cells ectopically expressing LgTK1, GSE-22, or CDK4m remained non-dividing. Expression of LgT or both GSE-22 and CDK4m resulted in cell cycle re-enter of WI-38 fibroblasts (Beauséjour et al., 2003). WI-38 fibroblasts required inactivation of both p53 and p16 pathways to resume proliferation after being brought to replicative senescence. BJ fibroblasts only required inactivation of the p53 pathway, potentially due of low expression of p16. These finds indicate that there is cell line variability in the requirements of maintaining senescence.

Using an inducible p21 expression system in p53-null cells, Galanos and colleagues (2016) showed that forced expression of p21 for less than 7 days retained proliferative capability that progressively decreased at day 10, where majority (~65%) cells express lipofuscin, a biomarker of senescence that can be used to screen for senescent cells. Ectopic expression of p21 promoted an increase in Cdc6, a DNA replication licensing protein, and increased aberrant origin firing and replication stress, leading to DNA damage. Elevated levels Cdc6 was shown to be necessary for increased genomic instability. After 20 days of continuous forced p21 expression, a sub-population of p21 positive cells was observed proliferating. The proliferating p21-expressing cells retained genomic instability and micronuclei, as well as gross chromosomal rearrangements. The emerged population behaved more aggressively and demonstrated decreased drug sensitivity.

Following up on this work, Zampetidis et al. (2021) explored the hypothesis that OIS occurs early on during tumorigenesis and rare cells that become genomically unstable during OIS are endowed

with the ability to escape and resume proliferation. Using an inducible Cdc6 quasi-normal epithelial cell model, escape from OIS was shown to be a rare event. DNA damage that occurs during early senescence, due to replication stress, is repaired in an error prone manner. A heterozygous balanced inversion of BHLHE40 (basic helix-loop-helix family member 40, also known as DEC1), a transcription factor that plays a role in circadian rhythm, was shown to be inverted and overexpressed. BHLHE40 transcriptionally targets multiple cell cycle genes and regulators. MDM2, a target of BHLHE40, was shown to be upregulated in escaped cells and although p53 was not mutated in escaped cells, p53 protein expression was suppressed. Expression of BHLHE40 was necessary for sustained viability of escaped cells. CRISPR-Cas9 engineered inversion of the 3.7 Mb contained within the short arm of chromosome 3 promoted overexpression of BHLHE40 and bypass of Cdc6 mediated oncogene induced senescence; the impact on escape of the BHLHE40 inversion mutation was not tested.

The Coqueret group has implicated aspects of the SASP in regulating the emergence of cells after drug-induced senescence. LS714T and MCF7 cell lines were shown to undergo drug-induced senescence (60-80% SA- β -gal positivity), and proliferating colonies were observed several days after drug-treatment. Senescent LS714 and MCF7 cells express and secrete thrombospondin 1 (TSP1), whereas colonies proliferating after drug-treatment did not (Guillon et al., 2019). Stimulation of cells with human recombinant TSP1 reduced the rate of proliferation without promoting senescence and decreased the frequency of emerging colonies. TSP1 interacts with receptor CD47. Cells dividing post-treatment were shown to have decreased expression of CD47 and cells that remained senescent retained CD47 expression. siRNA mediated knockdown of CD47 and TSP1 increased the number of emerging colonies after drug-treatment (Guillon et al.,

2019). Using the same system, the Coqueret group showed that escape from senescence requires Cdk4, a kinase that regulates pRb and E2F. Knockdown of EZH2, a methylase that regulates E2F, was also shown to decrease frequency of emergence. EZH2 was shown to influence AP2M1 expression. AP2M1 was shown to influence SASP and knockdown decreased emergence (Le Duff et al., 2018).

As a new, controversial field, the mechanism underlying escape from senescence are only beginning to be elucidated. To date, the majority of mechanisms described that regulate escape from senescence, directly or indirectly involve loss of p53 or p16 cell cycle arrest pathways.

1.2.8.3 Limitations of demonstrating escape from senescence

Escape from senescence in cancer models has been reported in recent years but remains highly controversial and poorly understood. Numerous publications that make claims regarding escape from senescence fail to show escape convincingly. The experimental design of many findings that conclude escape from senescence have fundamental underlying issues. Previous work has relied on cell population studies and on cell sorting of senescent cells by flow cytometry. Some population-based studies that have a heterogenous partially senescent population do not differentiate between senescent cells that escape senescence, transiently arrested or slow dividing cells that never arrested. The proliferating population after the induction of senescence may easily be explained by the expansion of never senescent cells (Figure 2i). In an attempt to address this issue, some studies have sorted live senescent cells based on fluorescence markers of senescence. However, flow cytometry does not yield entirely pure populations of senescent cells that are

necessary to measure escape. Enrichment of senescent population of 99.9% purity can quickly be overtaken by the 0.01% of cells that are non-senescent and proliferating. By observing the enriched senescent population over time, the fraction of never senescent cells can expand and give the false impression of escape (Figure 2ii). New strategies of tracking individual senescent cells over time are required to definitively show clonal escape from senescence (Figure 2iii) (Saleh et al., 2019b).

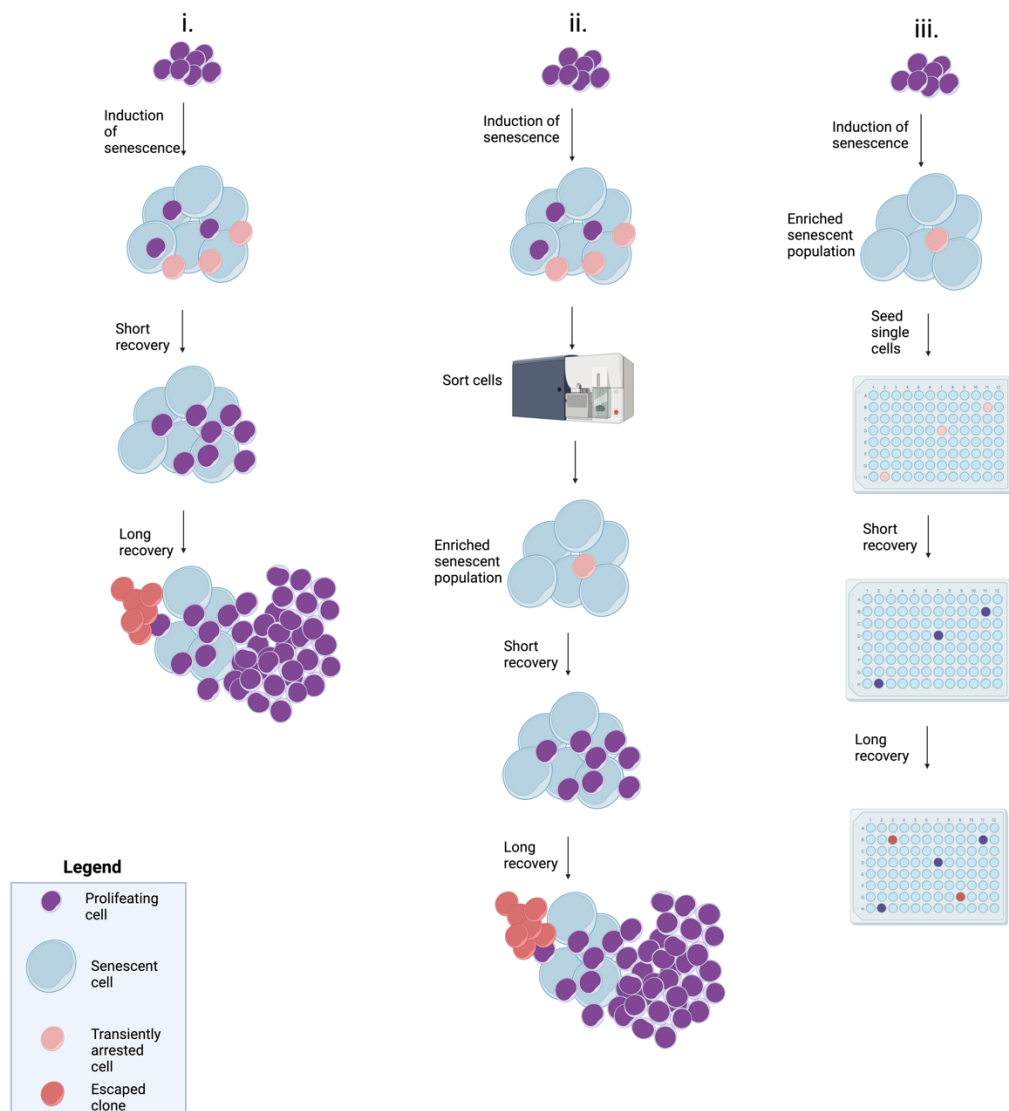


Figure 1-3 Schematic of current methods used to demonstrate escape from senescence.

i) The heterogeneous population of proliferating clones, transiently arrested clones, and senescent cells are allowed to recover. An emerging population of proliferating cells is seen after a short period of time, which continues to expand. Some senescent clones may escape and resume proliferation, however they are indistinguishable from the expanding never senescent population. ii) A pooled heterogeneous population of proliferating clones, transiently arrested clones, and senescent cells are sorted using a fluorescent marker. Introduction of a few non-senescent cells into the enriched senescent population allows the non-senescent cells to expand and quickly overtake the senescent population, giving the false impression of escape from senescence. iii) Individual cells from an enriched population of senescent cells are seeded into individual wells and cells are monitored for growth. Cells observed proliferating shortly after seeding are considered to be never

senescent whereas, cells that are stably arrested for a longer duration and then resume proliferation are considered escaped cells.

1.3 Rationale

Here I provide the rational and evolution of my projects; how my initial research topic focused on aging and DNA damage led me to question the permanence of drug-induced senescence in a cancer model. I initially started my work on understanding the mechanism of replication stress and DNA damage in Hutchinson-Gilford progeria syndrome. Due to technical limitations my project pivoted to determine whether replication stress was responsible for DNA damage and senescence in normal aged cells, where I observed an age associated increase of cytosolic DNA in the form of micronuclei and chromosomal bridges. At the time, recent work demonstrated that cytosolic DNA that accumulated during senescence led to an inflammatory response, and so I focused my hypothesis to address whether the age-associated cytosolic DNA resulted in inflammation in the normal aging process. Prior to the completion of this work several manuscripts were published showing that cytosolic DNA accumulated in cells during normal and premature aging that elicited an inflammatory response. To contribute novel findings to the scientific community, I redirected my question to ask whether the accumulation of cytosolic DNA and chromosomal instability observed during senescence may act to disrupt the mechanism maintaining senescence and facilitate the means by which senescent cells may escape; a question that remains unresolved. In order to begin addressing the aforementioned question, I first had to address whether escape from senescence was possible. In doing so, I used two experimental models and demonstrated that both normal fibroblasts and leukemia cells can escape drug-induced senescence.

Cytosolic DNA accumulates during aging and in premature aging diseases

Contribution of work:

Abby Skosowski an honour's thesis student in Dr. Samuel Benchimol's lab contributed to Figures 2-2A, 2-5, 2-4C and 2-4E.

2.1 Summary

Genomic instability, nuclear envelope defects and chronic inflammation contribute to age-related disease and conditions. Whether these three factors play integrating roles is largely unknown. Here, I make the observation that there is an age-associated increase in micronuclei and chromatin bridges, sources of genomic instability and cytosolic DNA. Decrease in lamin B1 in elderly fibroblasts is associated with an increase in improper micronuclear envelope formation and incomplete encompassment of chromatin bridges. Improper segregation of chromatin from cytosol leads to cGAS recruitment. DNA damage was observed in micronuclei. Consistent with these observations, I observed decreased lamin B1 and increased amount of micronuclei and chromatin bridges in some segmental premature aging diseases such as classical and non-classical Hutchinson-Gilford progeria syndrome (HGPS), and Dyskeratosis Congentia (DKC).

Both micronuclei and chromatin bridges found in HGPS and DKC underwent DNA damage and cGAS recognition. I hypothesize that nuclear envelope defects and the accumulation of micronuclei and chromatin bridges leads to a more robust cGAS-mediated inflammatory response in normal aging and in segmental aging, and may contribute to systemic inflammation.

2.2 Introduction:

Aging leads to loss of cellular function and loss of physiological homeostasis. As a result, age is one of the greatest risk factors for multiple life-threatening diseases including cancer, cardiovascular disease, neurodegenerative disease, and metabolic disorders. Age-associated diseases are responsible for the majority of deaths in the developed world. Chronic inflammation is associated with many age-related diseases and contributes to the etiology of the disease. Despite age being the primary risk factor for death and disease, a fundamental understanding of the aging process is lacking (Lopez Ótin et al., 2013).

Genomic instability represents a hallmark of aging (Lopez Ótin et al., 2013). The genome is under relentless assault from both endogenous and exogenous sources. Thus, constant DNA damage surveillance and efficient repair of the genome is essential for proper function and survival of an organism. Elevated levels of DNA damage have been shown to induce cellular senescence *in vitro*, and aging *in vivo*. Patients in remission, who underwent chemotherapy in the form of genotoxic agents demonstrate accelerated aging than those not exposed to genotoxic agent. Childhood cancer patients treated with genotoxic agents that survive to 50 years of age have a 2.5-fold increase in developing an age-associated diseases, including secondary cancers, than age matched non-treated individuals (Armstrong et al., 2014). Supporting the concept that

DNA damage contributes to aging, multiple progeroid syndromes, including Werner syndrome, Bloom syndrome, Cockayne syndrome, Xeroderma pigmentosum, and Ataxia Telangiectasia are caused by mutations in genes involved in the DDR (Niedernhofer et al., 2018). DNA repair occurs rapidly after the DDR is activated. Non-homologous end joining (NHEJ), and homologous recombination (HR) are the two competitive DNA damage repair pathways responsible for repairing double strand breaks (DSBs), the most lethal form of DNA damage. NHEJ, predominately occurring in G1 phase, is an error prone process that is inherently mutagenic; in contrast, HR, only occurs in S and G2 phase of cell cycle, faithfully repairing DSB by recruiting a homologous strand of DNA and using the homologous strand as a template. Both NHEJ and HR decline with age, with a more dramatic decrease in HR (Li et al., 2016). As a result, aged cells demonstrate prolonged DNA damage signaling and delayed recruitment of DNA repair factors to sites of DNA breaks. As a consequence of persistent genomic instability, cells may undergo cellular senescence, apoptosis or mitotic errors.

Micronuclei (MN) result from mitotic errors and can arise through various mechanisms. One method is through DSBs persisting in mitotic cells, resulting in chromosomal fragmentation. The fragmented acentric chromosome will lag behind while sister chromatids are separated into daughter cells. A second possible mechanism for MN generation is improper kinetochore attachment, resulting in a fully intact chromosome lagging behind while sister chromatids are pulled apart. Alternatively, a dicentric chromosome that is pulled to opposite poles, creating a chromosomal bridge. Bridge resolution may lead to fragmented chromosomes that lag behind (Fenech and Natarajan, 2011). Lagging chromosomes are secluded from the primary nucleus and missegregated. In turn, the lagging chromosome recruits its own nuclear envelope (NE), creating

a MN within the cytoplasm. Inappropriate NE constitution by failure to incorporate “non-core” NE proteins such as B-type lamins results in MN rupture (Liu et al., 2018).

As telomeres shorten with age, the shelterin complex becomes less efficient of protecting the telomere ends. Fusions between telomere ends occur and form dicentric chromosomes. As chromosomes are pulled to opposite sides and their respective daughter nuclei, the dicentric chromosomes are stretched between daughter nuclei, creating a chromatin bridge (CB) and eventually breaking during cytokinesis. The NE forms around both daughter nuclei and surrounds the CB. However, as the CB is stretched further apart the NE on the bridge weakens and eventually fails to compartmentalize the CB (Macejowski et al., 2015).

The generation of micronuclei and chromatin bridges results in two cellular events. The first being that the loss of a properly formed nuclear envelope surrounding MN and CBs results in exposure of self-DNA to the cytosol. Cytoplasmic-DNA initiates an innate immune response mediated through pattern recognition receptors (PRR). Cyclic GMP-AMP synthase (cGAS) the most well defined PRR, binds to cytosolic DNA and activates Stimulator of Interferon Genes (STING). STING activates (Interferon Regulatory Transcription factor 3) IRF3, a transcription factor, that upregulates type I interferon (IFN) genes and other IFN stimulator genes (Ablasser and Chen, 2019).

In this chapter I show that normal elderly fibroblasts exhibit increased levels of MN and CBs compared with fibroblasts from young donors. Age-associated NE defects leads to increased MN rupture and failure to compartmentalize CBs, leading to elevated levels of cytoplasmic-DNA. In

addition, I show that MN increase in a variety of rapid aging diseases such as classical and non-classical typical Hutchinson-Gilford progeria syndrome, and Dyskeritosis Congentia.

Cytoplasmic DNA, in the form of MN and CBs, are recognized by cGAS and undergo DNA damage in both normal and pathological aging. At the time, the question being addresses was whether cGAS would elicit an innate immune response in the elderly cells, HGPS cells and DKC cells. However, while this work was being conducted Lan and colleagues (2019) demonstrated that cytoplasmic DNA found in increased levels in normally aged donor cells and progeria cells promotes a cGAS and STING mediated innate immune response (Lan et al., 2019). Two additional laboratories in 2019 reported that ssDNA accumulation in normal elderly fibroblasts and elderly mice contribute to “inflammaging”, in a cGAS-STING dependent manner (DeMecco et al., 2019; Simon et al 2019).

2.3 Results

2.3.1 Compromised micronuclei nuclear envelope in elderly cells

Elderly cells exhibit loss of NE proteins as well as upregulation of progerin, which contribute to NE instability (McClintock et al., 2007; Scaffidi and Misteli, 2006). Nuclei from elderly fibroblasts exhibit increased nuclear blebbing, increased nuclear size and an array of abnormal nuclei, indicating improper nuclear reformation or loss of NE stability (**Figure 2-1A-C**). To investigate the integrity of the NE in relation to aging, the levels of NE proteins from young and elderly donors were measured by western blotting. Elderly cells expressed decreased levels of lamin B1 and B2 compared to young fibroblasts (**Figure 2-2A**). Lamin A/C levels did not change, consistent with previous reports; however, unlike Scaffidi and Misteli (2006), we failed to detect progerin, with both a progerin specific antibody and Lamin A/C antibody, capable of

detecting progerin, as demonstrated by the inclusion of a Hutchinson-Gilford progeria syndrome positive control (**Figure 2-2B**). To confirm that progerin was not being expressed in a low number of elderly cells that was not possible to be detected by western blotting in whole cell lysates, single cells were analyzed using immunofluorescence microscopy stained with progerin specific antibody. Immunofluorescence microscopy confirmed the absence of progerin in elderly cells (**Figure 2-2C**).

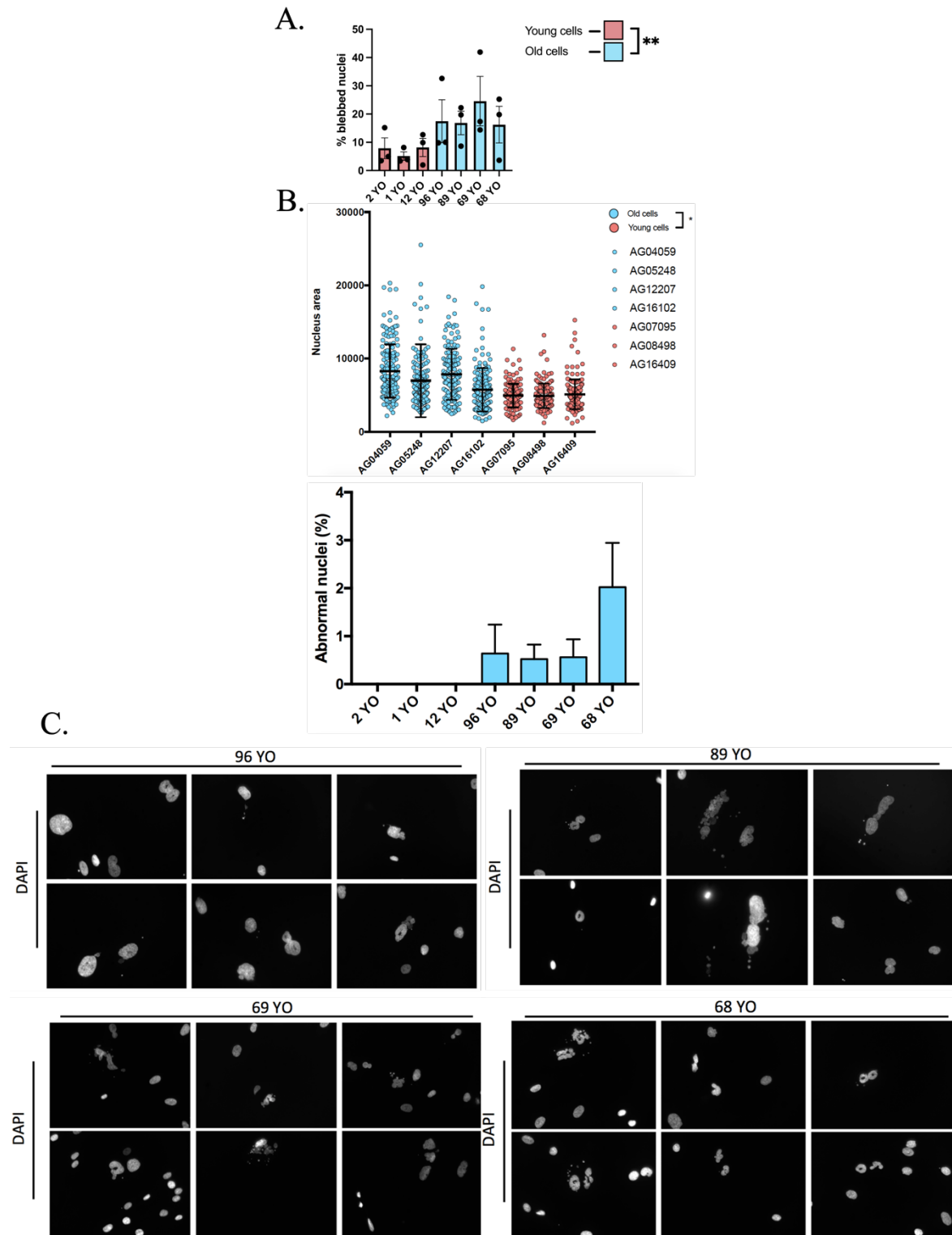


Figure 2-1 Age-related nuclear defects

A. Quantification of blebbed young and elderly fibroblast nuclei. >100 cells from each sample for each independent experiment. Three independent replicates were performed. A two-tailed unpaired t-test was performed on the means of the young cells (n=3) and

old cells (n=4) to determine statistical significance. Error bars represent SEM. $**P<0.01$.

- B. Quantification of young and elderly fibroblast nuclear area. >50 nuclei were measured from each sample for each independent experiment. Three independent replicates were performed. A two-way unpaired t-test was performed on the means of the young cells (n=3) and old cells (n=4) to determine statistical significance. Error bars represent SEM. $**P<0.01$.
- C. Representative immunofluorescent images of abnormally formed nuclei.

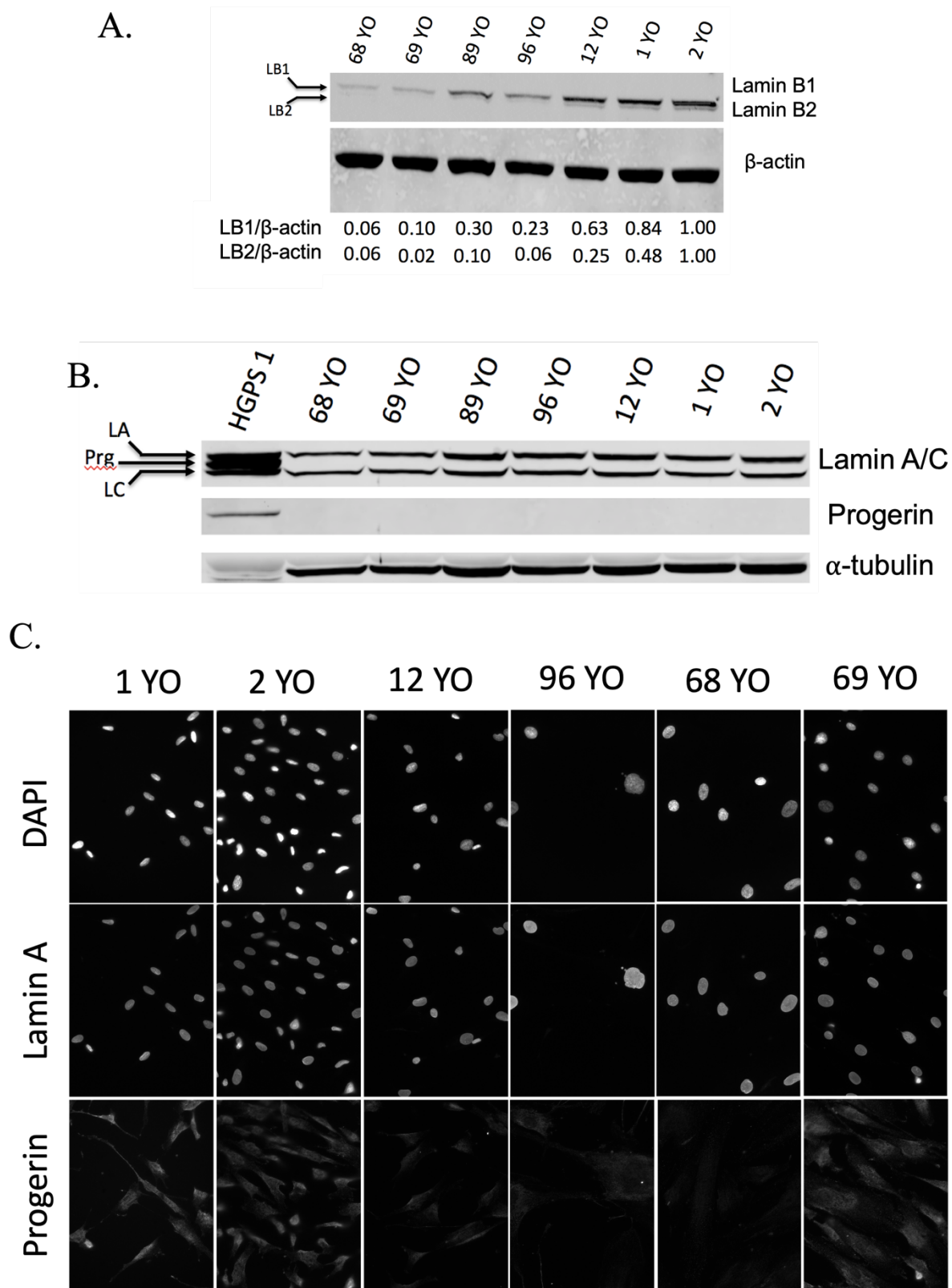


Figure 2-2 Age-related alteration in nuclear envelope composition.

- A. Representative westernblot of lamin B1 and lamin B2 in three normal young donor fibroblasts (12 YO, 2 YO and 1 YO), four normal elderly donor fibroblasts (68 YO, 69 YO, 89 YO, 96 YO). β -actin was used as a loading control. n= 2 independent replicates.
- B. Representative westernblot of lamin A/C and progerin in three normal young donor fibroblasts (12 YO, 2 YO and 1 YO), four normal elderly donor fibroblasts (68 YO, 69 YO, 89 YO, 96 YO). HGPS lysate was used as a positive control for progerin. α -tubulin was used a loading control. n= 3 independent replicates.
- C. Representative immunofluorescence images of three normal young donor fibroblasts (12 YO, 2 YO and 1 YO), four normal elderly donor fibroblasts (68 YO, 69 YO, 89 YO, 96 YO). Cells were stained using DAPI, progerin antibody and lamin A antibody.

Decreased Lamin B1 levels have been shown to be a marker for cellular senescence (Freund et al 2012; Shah et al., 2013; Shimi et al., 2011). Therefore, to verify that the decrease of lamin B1 was not due to an accumulation of senescent cells a senescence-associated beta-galactosidase (SA- β -gal) assay was conducted. Few SA- β -gal (<5%) positive cells were observed in both young and elderly donor samples (**Figure 2-3**).

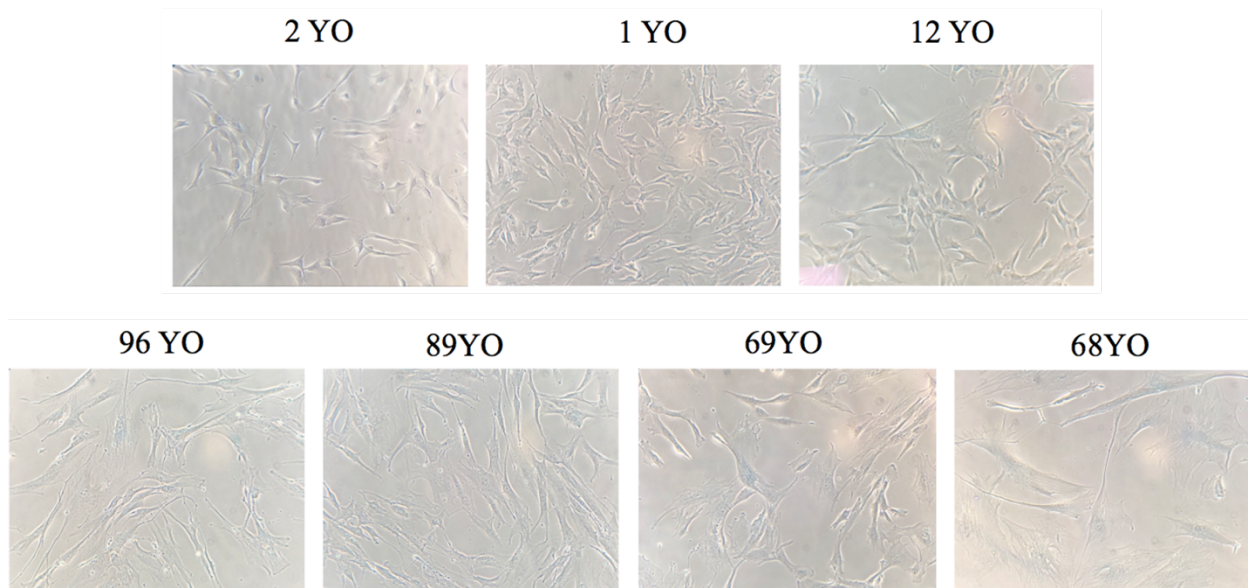


Figure 2-3 Representative images of SA- β -gal staining of proliferating normal young and elderly derived cell lines.

Genomic instability increases with age. Persistent genomic instability in elderly fibroblasts often results in aneuploidy and mitotic defects (Ly et al., 2000; Macedo et al., 2018). To investigate aging-associated genomic instability further, micronuclei were counted in fibroblast strains obtained from 3 normal healthy young donors (1-12 years old) and in fibroblasts obtained from 4 elderly donors (67-96 years old) using immunofluorescence microscopy and DAPI staining. The prevalence of micronuclei increased in elderly fibroblasts (**Figure 2-4A**) consistent with a recent publication by Macedo et al. (2018).

Hatch and colleagues (2013) demonstrated that MN that spontaneously occurred in tumour cell lines have compromised nuclear envelopes (NE) and are deficient in lamin B1. Liu et al (2018) reported that MN that form from lagging chromosomes undergo defective NE assembly in which “non-core” NE proteins including B-type lamins are excluded from the NE. Knockdown of lamin B1 increases the amount of collapsed MN (Hatch et al., 2013). I hypothesized that naturally occurring age-related decreased levels of lamin B1 and loss of NE integrity would lead to an increased frequency of collapsed MN in the elderly cells. To determine the prevalence of collapsed MN in elderly and young cells, two young and two elderly donor derived fibroblasts were randomly selected and stained for retinoblastoma protein (Rb). Rb is a nuclear protein that is normally retained inside intact MN; the absence of Rb in MN is an indicator of MN disruption (Hatch et al., 2013). Elderly cells exhibited a slightly greater number of Rb negative MN than MN in young cells (**Figure 2-4B**).

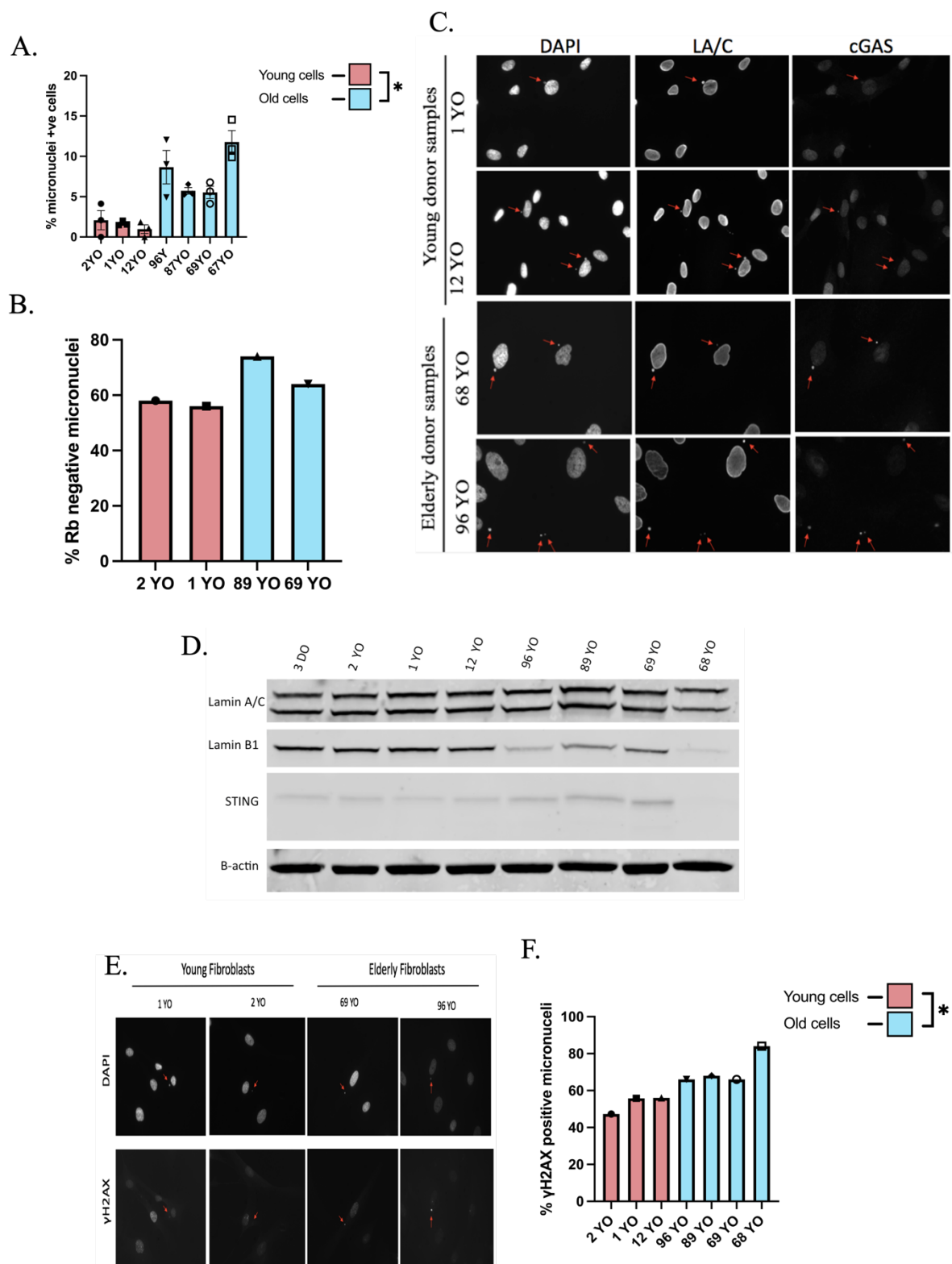


Figure 2-4 Compromised micronuclei nuclear envelope in elderly cells leads to increased cGAS recruitment and increased DNA damage.

- A. Prevalence of micronuclei were determined by counting >100 cells from each sample for each independent experiment. Three independent replicates were performed. A two-way unpaired t-test was performed on the means of the young cells (n=3) and old cells (n=4) to determine statistical significance. Error bars represent SEM. *P<0.05.
- B. Prevalence of ruptured MN were determined by staining cells with Rb and counting >50 micronuclei.
- C. Representative immunofluorescence images of two young and two elderly fibroblasts containing MN stained with Lamin A/C and cGAS. n= 3 independent replicates.
- D. Representative western blot of Lamin A/C, Lamin B1 and STING expression in normal young, and normal elderly fibroblasts. n= 3 independent replicates.
- E. Representative immunofluorescence images of γ H2AX staining in aged and young cells. Red arrows indicate MN. n= 3 independent replicates.
- F. Quantification was performed by counting >50 MN and assessing their γ H2AX status. Related to Figure 1E.

2.3.2 Micronuclei are exposed to the cytosol and undergo DNA damage

Harding et al. (2017) and McKenzie et al. (2017) reported that micronuclei present in cancer cells recruit cyclic GMP-AMP Synthase (cGAS) leading to an innate immune response. cGAS recruitment to MN is likely the result of NE disruption and the exposure of chromatin to the cytoplasm. Both young and elderly fibroblast MN recruited cGAS, however more MN in elderly cells were cGAS positive and, MN in elderly cells had more robust cGAS staining, compared with young donor cells (**Figure 2-4C**).

In response to cytosolic DNA, cGAS activates STING, an endoplasmic reticulum-bound membrane protein. STING in turn activates IRF3, which upregulates the type 1 interferons and the innate immune response (Ablasser and Chen, 2019). Having shown that cGAS is recruited to the MN of elderly cells, I next measured the levels of STING by western blot. STING was upregulated in elderly cells compared to young cells, except for the AG12207 fibroblast cell line taken from a 68 yo donor, which showed no expression of STING (**Figure 2-4D**). These results suggest that STING may be activated in normal elderly fibroblasts.

Collapsed MN undergo extensive DNA damage and have been shown to go through chromothripsis (Castra et al., 2012; Hatch et al 2013; Zhang et al., 2015). Based on the observation that more elderly MN are ruptured, I was interested to determine whether elderly MN undergo more DNA damage. To investigate whether elderly cells contained a higher frequency of MN undergoing DNA damage, immunofluorescence was performed. Elderly fibroblasts stained with γ H2AX antibody demonstrated a higher prevalence of γ H2AX positive compared to young fibroblasts (**Figure 2-4E-F**).

DNA repair has been shown to be impaired in MN (Hatch et al, 2013). To determine whether deficient DNA damage repair was contributing to increased DNA damage in MN, two randomly chosen elderly and two young donor cell lines were subjected to 20 J/m² of UV radiation and allowed to recover for 2, 6, 12, and 24 hours. Primary nuclei of both young and elderly fibroblasts showed robust γ H2AX staining at 2 and 6 hours post irradiation. By 6- and 12-hours post-UV exposure 53BP1 demonstrated similar staining patterns to γ H2AX (**data not shown for 6 and 12 hr**). γ H2AX and 53BP1 foci were resolved in young cell by 24 hours, whereas elderly cells retained γ H2AX and 53BP1 foci, suggesting that elderly cells have impaired DNA repair, consistent with previous findings (**Figure 2-5**). MN of young fibroblasts demonstrated higher levels of γ H2AX post-UV exposure compared to elderly MN, suggesting impaired DNA damage response in MN of elderly cells. By 24 hours after UV exposure, MN from both aged and young fibroblasts recovered to basal levels of γ H2AX (**Figure 2-5**). 53BP1 showed poor localization in

MN of both young and elderly fibroblasts (Figure 2-5).

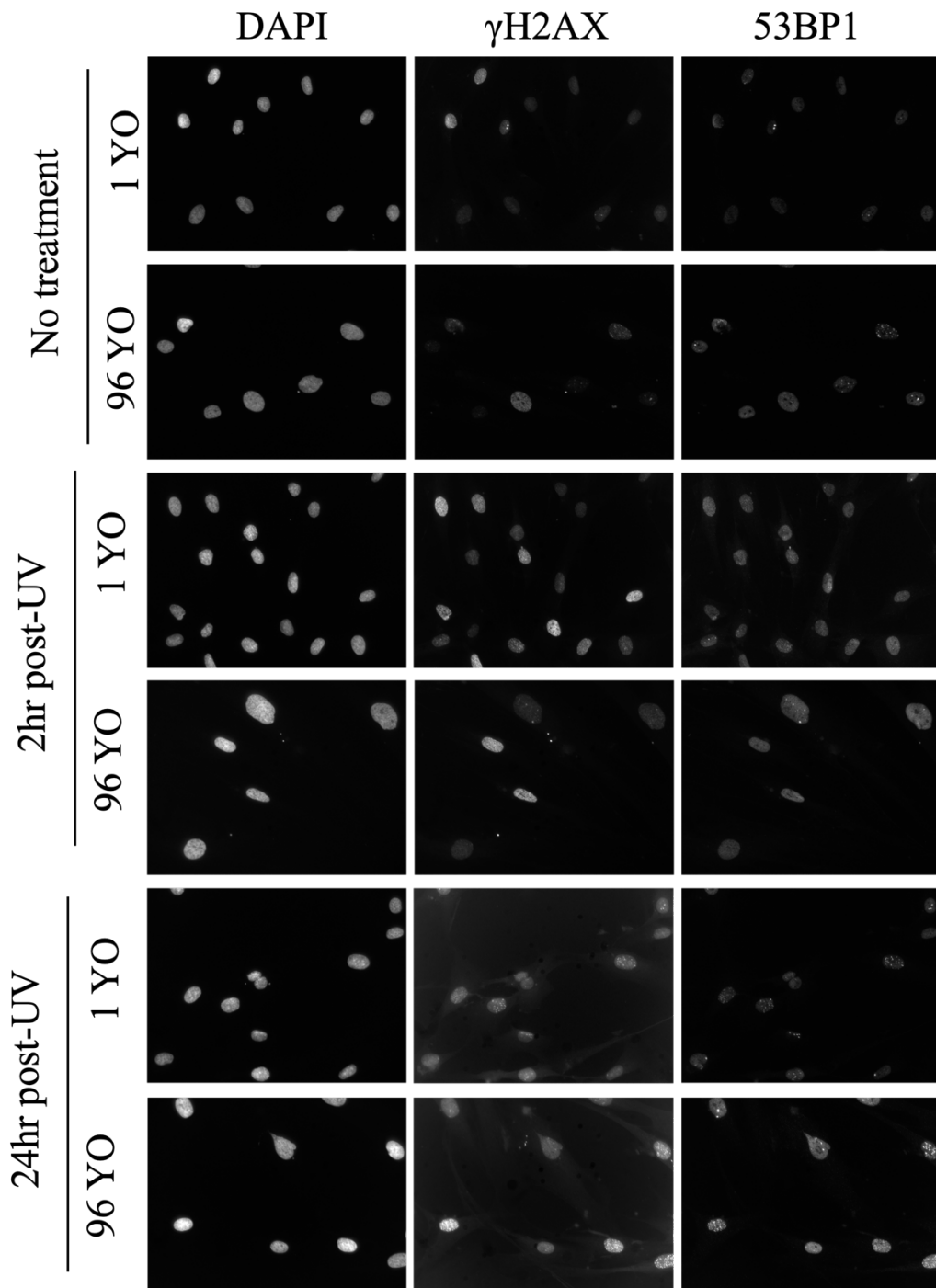


Figure 2-5 Impaired DNA damage response in elderly fibroblasts. Young and elderly fibroblasts were treated with 20 J/m² of UV radiation and harvested 2, 6, 12, and 24 hr after for immunofluorescence microscopy; negative controls were not treated with UV. Representative

images of γ H2AX and 53BP1 foci in 1YO and 96 YO fibroblasts untreated, or recovering UV-treatment for 2 and 24 hr.

2.3.3 Age-associated chromatin bridges evoke cGAS recognition.

A secondary mechanism in which cytosolic DNA can accumulate is by the generation of chromosomal bridges (CBs). The de Lange lab has demonstrated that telomere erosion, resulting from expression of a TRF2 dominant negative mutant, can lead to telomere fusion and the formation of dicentric chromosomes, which are pulled apart during mitosis, creating CBs (Maceijowski et al., 2015). I reasoned that since cells taken from elderly individuals displays shortened telomere lengths (Harley et al., 1990), fusion between eroded telomere-ends may be more common in elderly cells than young cells. To determine whether an age-associated increase in chromatin bridges existed, the number of CBs were identified by DAPI staining using immunofluorescence microscopy and scored in young and elderly donor-derived fibroblasts. CBs are observed at a higher frequency in elderly fibroblasts compared with young fibroblasts (Figure 2-6A).

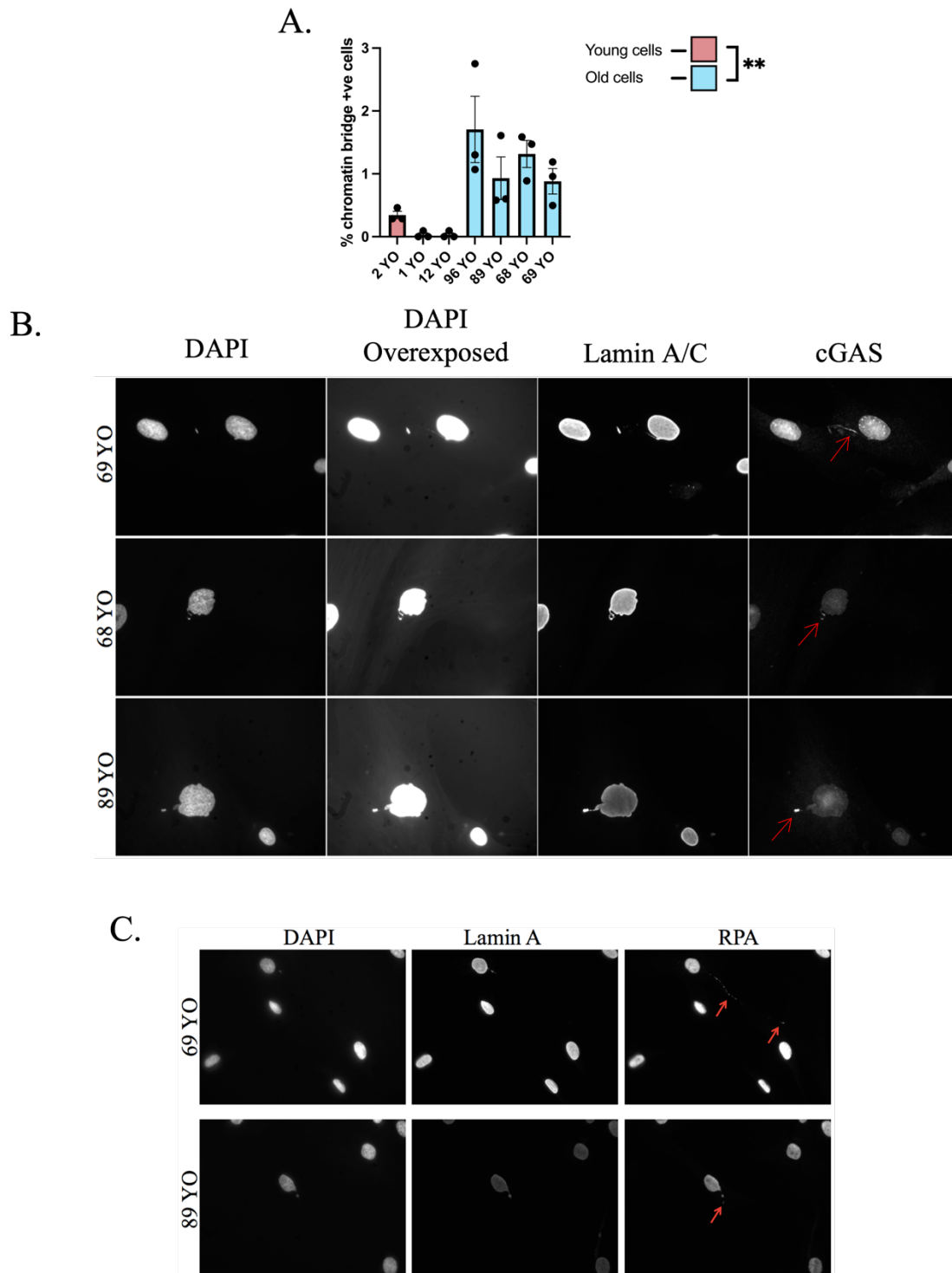


Figure 2-6 Age-associated chromatin bridges evoke cGAS activation.

A. Prevalence of of chromatin bridges were determined by counting >500 cells from each sample for each independent experiment. Three independent replicates were performed. A two-way unpaired t-test was performed on the means of the young cells (n=3

biological replicates) and old cells (n=4 biological replicates) to determine statistically significance. Error bars represent SEM. **P<0.01.

- B. Representative immunofluorescent images of chromatin bridges in 3 elderly fibroblast cell lines immuno-stained with Lamin A/C (LA/C) and cGAS. Red arrows indicate cGAS on the CB. n= 3 independent replicates.
- C. Representative immunofluorescent images of chromatin bridges stained with Lamin A (LA) and RPA32 (RPA) in two elderly fibroblasts cell lines. Red arrows indicate RPA32 on the CB.

Lamins have been shown to protect CB from degradation and hypermutations by cytosolic nucleases and deaminases, respectively (Maceijowski et al., 2015). Failure of the nuclear envelope to fully encapsulate chromatin bridges may also lead to cGAS recognizing the DNA exposed to the cytosol. I hypothesized that reduced levels of Lamin B1 in elderly fibroblasts may result in reduced protection of CB and cGAS recognition, as well as the accumulation of DNA damage on CBs; moreover, a broken CB, by definition contains, a double-stranded break. Similar to collapsed micronuclei, chromatin bridges failed to be fully protected by the nuclear envelope and cGAS was recruited to regions of CBs that had less lamin A/C or were located in the distal broken segment of the CB (**Figure 2-6B**), suggesting parts of some CBs were exposed to the cytosol. Immunofluorescence microscopy revealed that elderly fibroblasts had an accumulation of RPA on CB, a marker of ssDNA (**Figure 2-6C**). Furthermore, CB bridges stained positively for γ H2AX and phosphorylated RPA (pRPA), an additional marker of DNA damage (**Figure 2-7**). Co-immunofluorescent staining of lamin B1 and γ H2AX demonstrated that γ H2AX appeared in regions of CBs that were depleted of lamin B1 (**Figure 2-8**).

69 YO

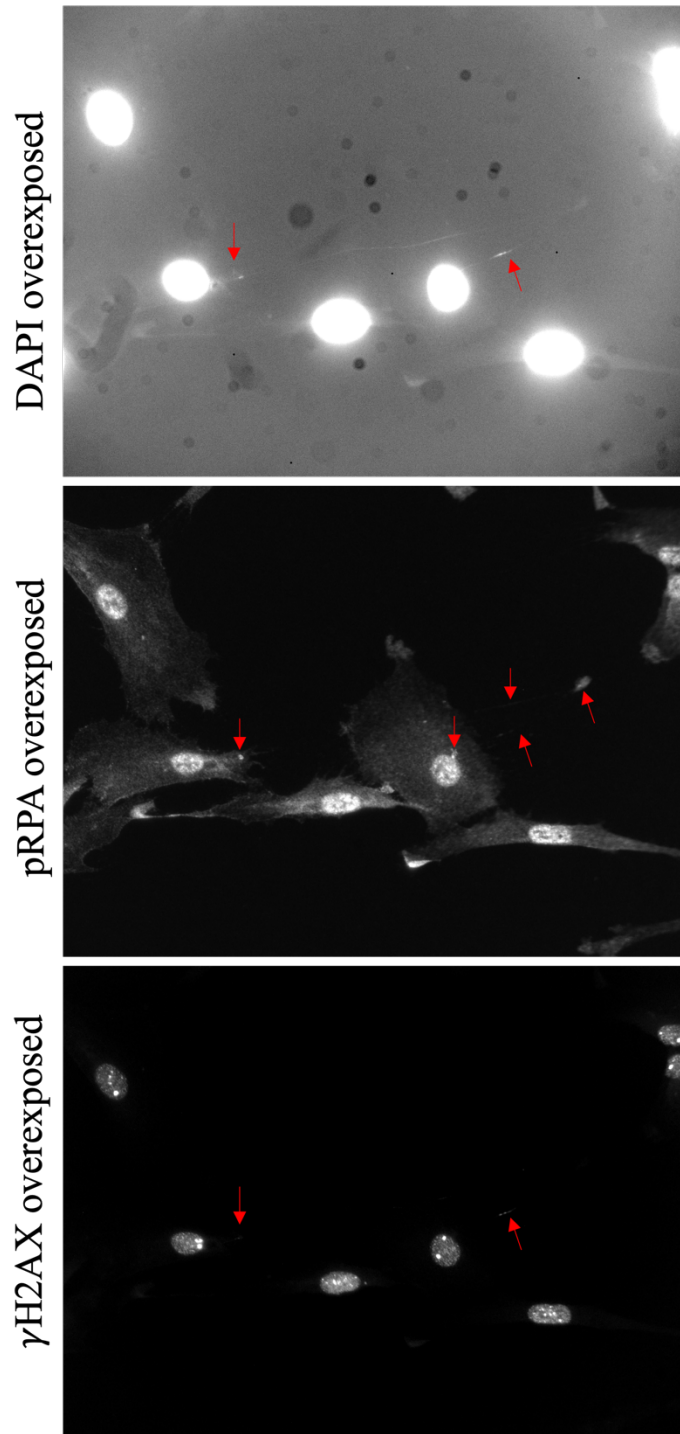


Figure 2-7 Chromatin bridge undergoing DNA damage in elderly donors.

Representative immunofluorescent image of a chromatin bridge forming 3 days post-dox treatment. Related to Figure 2-6B.

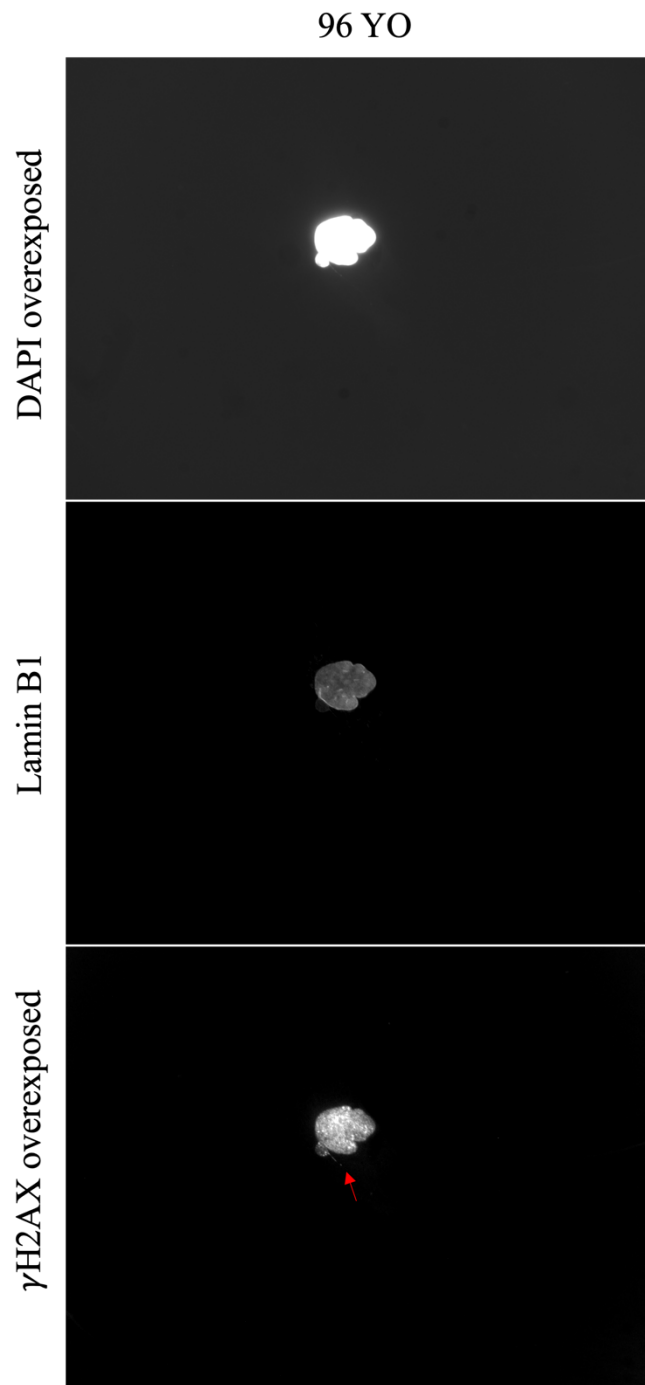


Figure 2-8 Lamin B1 depleted chromatin bridge undergoing DNA damage in elderly donor derived fibroblasts.

Representative immunofluorescent image of a chromatin bridge forming 3 days post-dox treatment. Red arrow indicates region of chromatin bridge that strains positive for γ H2AX.

2.3.4 Multiple rapid aging diseases exhibit cGAS activation in response to accumulation of cytosolic DNA

Classical Hutchinson-Gilford progeria syndrome (HGPS), accounting for 90% of HGPS cases, contains a silent heterozygous G606G mutation in the LMNA gene that results in the production of a lamin A isoform, known as progerin. Other LMNA mutations that cause syndromes presenting as premature aging, are considered to be non-classical HGPS, and produce alternative lamin A and/or C mutants. To validate that the classical HGPS cell lines (HG003 and HG127) produce progerin, whereas the non-classical HGPS cell lines do not, a western blot was performed. Western blot analysis demonstrated that classical HGPS cells produced progerin, whereas the non-classical HGPS and normal young control cell lines did not (**Figure 2-9A**). Furthermore, HGPS cell lines showed a decrease in lamin B1 compared to young normal control cells (**Figure 2-9A**).

There are many commonalities between normal aging and HGPS. To determine whether HGPS cells have elevated level of micronuclei similar to normal aged cells, I performed immunofluorescence on two classical HGPS fibroblasts cell lines and two non-classical HGPS fibroblasts cell lines and compared them to three normal, young donor-derived fibroblasts lines. Consistent with previous finding using classical HGPS cell lines, Cao et al., (2007) and Macedo et al. (2018), micronuclei were elevated in both classical and non-classical HGPS patient derived fibroblasts (**Figure 2-9B**). Micronuclei of HGPS cells showed increased prevalence of γ H2AX, compared to normal young cell lines (**Figure 2-9C**).

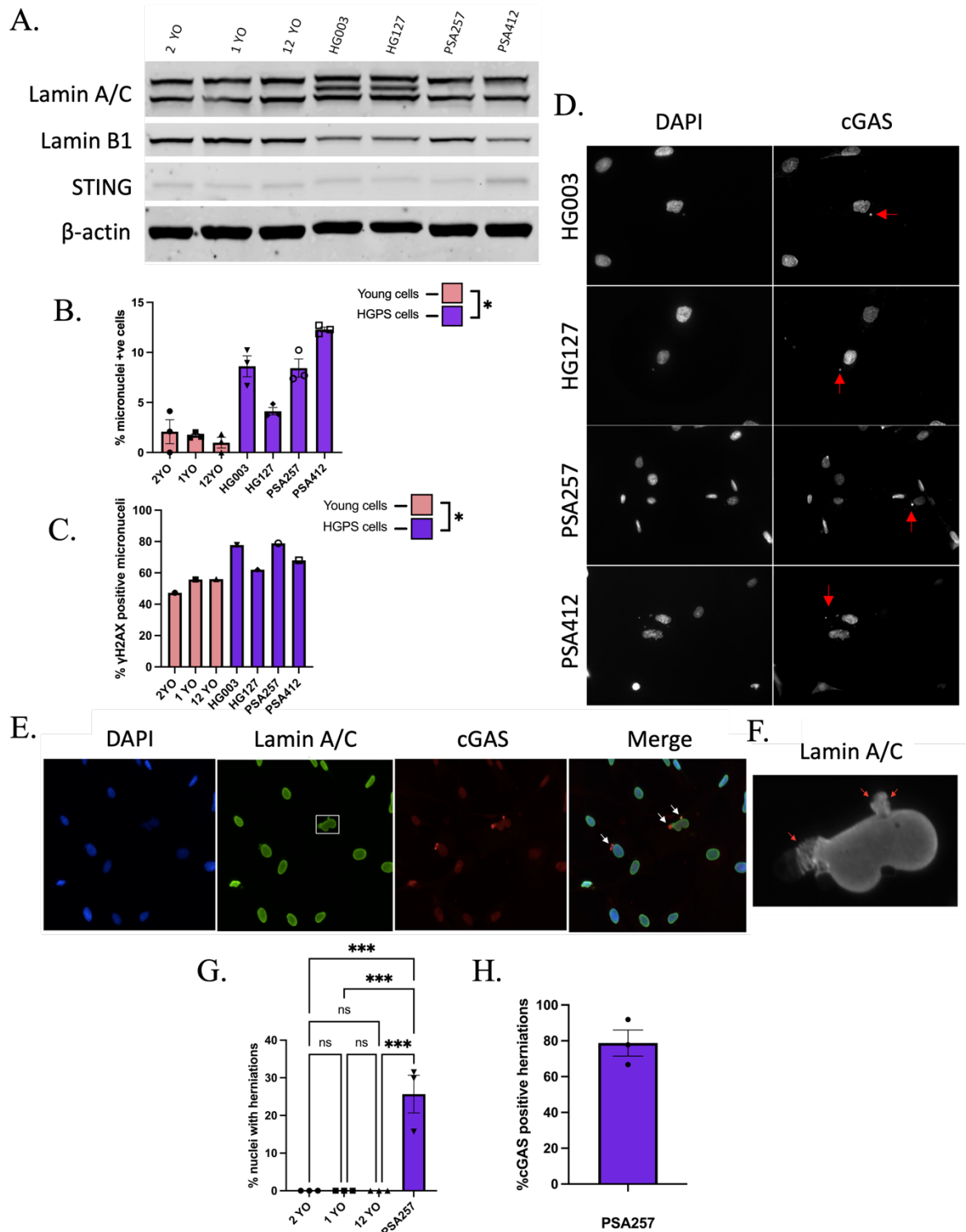


Figure 2-9 Hutchinson-Gilford progeria syndrome exhibit cGAS activation in response to accumulation of cytosolic DNA.

Representative westernblot of 3 normal donor control fibroblasts (2YO, 1 YO and 12 YO), two classical HGPS fibroblasts (HG003 and HG127) and two non-classical HGPS fibroblast strains (PSA257 and PSA412).

- A. Prevalence of micronuclei were determined by counting >100 cells from each sample for each independent experiment. Three independent replicates were performed. A two-way unpaired t-test was performed on the means of the young normal cells (n=3) and HGPS cells (n=4) to determine statistical significance. Error bars represent SEM. *P<0.05.
- B. Prevalence of γ H2AX positive micronuclei in young normal and HGPS fibroblasts. 50 micronuclei were counted from each biological sample. A two-way unpaired t-test was performed on the means of the young normal cells (n=3) and HGPS cells (n=4) to determine statistical significance. Error bars represent SEM. *P<0.05.
- C. Representative immunofluorescent images of HGPS cells showing recruitment of cGAS to micronuclei. The red arrow points out the cGAS positive micronuclei. Three independent replicates were performed.
- D. Representative immunofluorescent images of cGAS positive herniations in PSA257 cell line. Related to Figure 3D, F-H. The white box is magnified in Figure 3F.
- E. Magnified nucleus stained with lamin A/C. Related to Figure 3E.
- F. Quantification of nuclear herniations in 3 normal young fibroblasts controls and the PSA257 non-classical HGPS cell line. >50 nuclei were counted from each sample for each independent experiment. A one-way ANOVA with Tukey's multiple comparison test was performed to determine statistical significance. Error bars represent SEM. ***P<0.001.
- G. Quantification of cGAS positive herniations in PSA257 cells. A total of 61 herniations assessed over 3 independent experiments.

To determine whether MN in HGPS cells underwent rupture and exposure to the cytoplasm, immunofluorescence was performed using a cGAS antibody. Immunofluorescence microscopy demonstrated that cGAS was recruited to MN in both classical and non-classical progeria similar to what is seen in normal elderly fibroblasts (**Figure 2-9D**). Notably, in roughly 15% of PSA257 cells, a non-classical HGPS cell line containing a homozygous Met540Thr lamin A mutation, a distinct nuclear lamin and chromatin herniation phenotype was observed; lamin A/C staining revealed a holed mesh-like herniation from the primary nucleus and NE (**Figure 2-9E-G**). cGAS was recruited in 70% of nuclear herniations, indicating improper chromatin-cytosol separation at herniations (**Figure 2-9H**). Although lamin A/C was partially assembled on chromatin

herniations in PSA257 cells, lamin B1 was absent at chromatin herniations (**Figure 2-10**). Some herniated chromatin displayed γ H2AX foci (**Figure 2-10**).

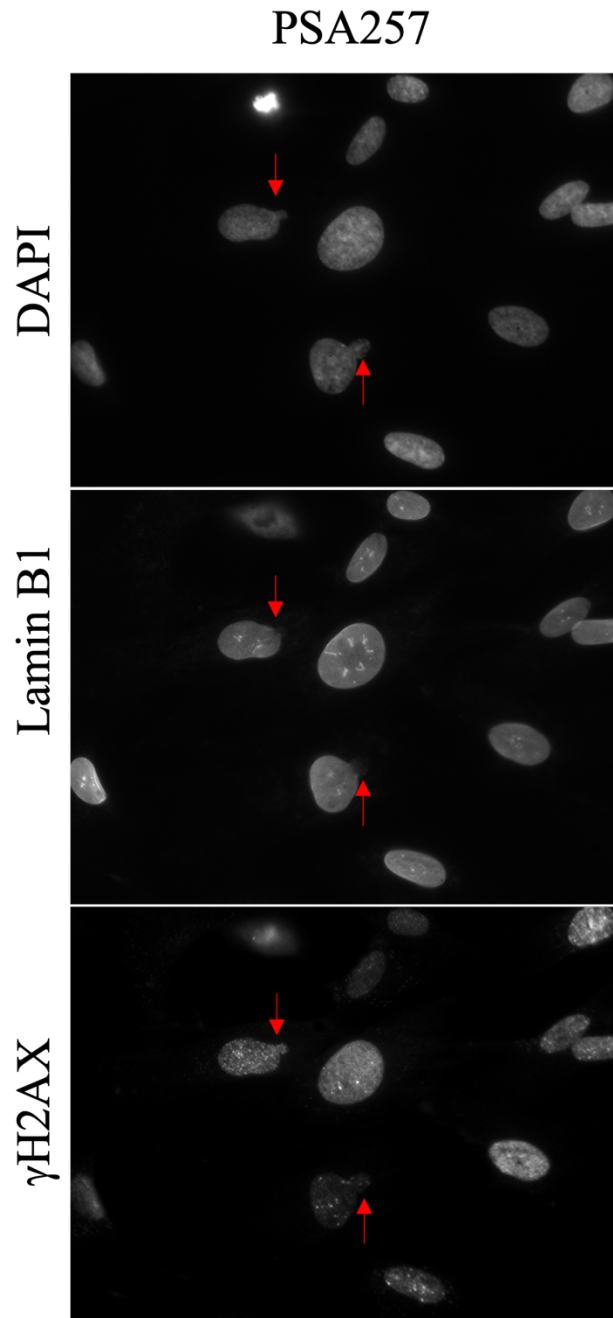


Figure 2-10 Lamin B1 does not surround chromatin herniations.

Representative immunofluorescent image of a chromatin herniation in PSA257, non-classical progeria syndrome cell line. Red arrow indicate region of chromatin bridge that stains positive for γ H2AX.

To investigate whether cGAS recruitment was initiating an innate immune response in HGPS fibroblasts, STING protein levels were assessed by western blot. STING was upregulated in only PSA412, a non-classical HGPS patient fibroblast, compared to young normal donor fibroblasts (**Figure 2-9A**).

I next sought to look whether other diseases that had aspects of accelerated aging also have an increased prevalence of cytosolic DNA in the form of micronuclei or chromatin bridges. Based on the known mechanisms of micronuclei and chromatin bridge formation, I began investigating whether Dyskeritosis Congentia (DKC), and Trisomy 21, also known as Down syndrome (DS) have elevated levels of chromatin bridges and micronuclei. DKC is caused by various mutations in a number of components of the telomerase complex, which has been shown to lead to rapid telomere attrition; I reasoned that loss of telomere maintenance and rapid telomere attrition would increase the likelihood of fusions at the chromosome ends and the formation of dicentric chromosomes. DS is caused by an extra copy of chromosome 21. DS presents as a spectrum of intellectual disability and a set of common physical characteristics. However, DS is also associated with a set of age-related characteristics such as premature skin wrinkling, greying of hair, decline in immune function and Alzheimer's disease (Franceschi et al., 2019). I hypothesized that DS cells may have increased micronuclei similar to aged normal cells due to an increased likelihood of chromosome mis-segregation during mitosis; the additional copy chromosome 21 may pose an issue for the spindle fibers.

To investigate whether DKS and DS cells had elevated levels of micronuclei and chromatin bridges compared to normal young cells, immunofluorescence was performed on two

young normal cell lines, two DKS cell lines, DKS1(GM01174) containing a L27del in DKC, and DKS2 (AG04646) containing a A386T substitution in DKC and two DS cell lines (DS1 and DS2; both trisomy 21). DKC fibroblast cell lines demonstrated elevated levels of MN compared to normal young fibroblasts, whereas DS fibroblasts demonstrated a trend towards increased MN (**Figure 2-11A**). Furthermore, DKC fibroblasts demonstrated higher levels of chromosomal bridges (**Figure 2-11B**). Similar to elderly and HGPS cells, DKC fibroblasts showed recruitment of cGAS to MN and decreased levels of lamin B1; DS cell lines did not (**Figure 2-11C-D**). STING levels appeared to be elevated in DKS cell lines compared to young cells (**Figure 2-11D**).

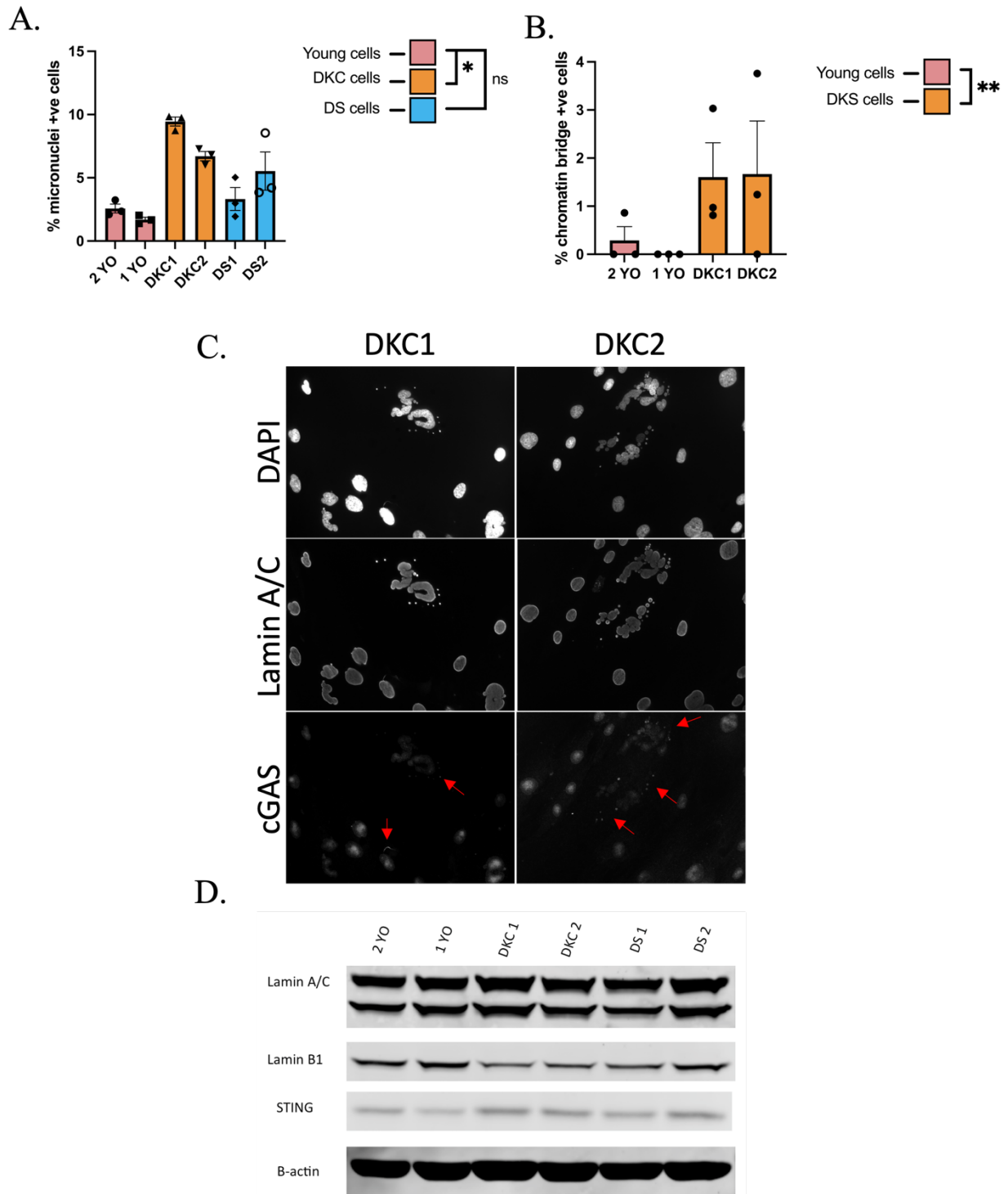


Figure 2-11 Increased levels of micronuclei and chromatin bridges in Dyskeratosis Congenita.

A. Prevalence of micronuclei were determined by counting >100 cells from each sample for each independent experiment. Three independent replicates were performed. A one-way

ANOVA was performed on the means of the young normal cells (n=2), DKS cells (n=2) and DS cells (n=2) to determine statistical significance. Error bars represent SEM. ns represents not significant, *P<0.05.

- B.** Prevalence of chromatin bridges were determined by counting >100 cells from each sample for each independent experiment. Three independent replicates were performed. A two-way unpaired t-test was performed on the means of the young cells (n=2 biological replicates) and DKS cells (n=2 biological replicates) to determine statistical significance. Error bars represent SEM. **P<0.01.
- C.** Representative immunofluorescent images of DKS cells showing recruitment of cGAS to micronuclei and chromatin bridge. The red arrow points out the cGAS positive micronuclei and chromatin bridge. Three independent replicates were performed.
- D.** Western blot of two normal donor control fibroblasts (2YO and 1 YO), two Dyskeratosis Congenita fibroblast strains (DKC1 and DKC2) and two Down Syndrome fibroblast strains (DS1 and DS2). n= 2 independent replicates.

2.4 Discussion

In this chapter, I demonstrate that there are age-related nuclear envelope defects leading to an age associated accumulation of cytosolic DNA. I extend these findings to two other syndromes that display characteristics of premature aging, HGPS and DKS, both showing higher levels of cytosolic DNA. The accumulated cytosolic DNA consists of micronuclei, chromatin bridges and herniated DNA from the primary nucleus. Mackenzie et al. (2017) showed that cGAS was recruited to micronuclei forming in cancer cell lines. In keeping with recent findings, I demonstrate that cGAS is recruited to micronuclei and chromatin bridges; importantly there is an increase of cGAS positive cytosolic DNA in elderly, HGPS and DKC patient cells. I propose a model in which both elevated levels of micronuclei and chromatin bridges, and the compromised nuclear envelope formation surrounding micronuclei and chromatin bridges increases the recruitment of cGAS to cytosolic DNA and mediates an inflammatory response in normal aging, and progerias. My study aimed to link aging with increased activation of the cGAS-STING arm of the inflammatory response through the accumulation of cytosolic DNA.

The cGAS-STING pathway is an important innate immune response that is activated by cytosolic DNA and results in the expression of inflammatory cytokines and interferon genes. Motivated by reports demonstrating that micronuclei activate cGAS-STING mediated inflammation, I aimed to determine whether the accumulation of cytosolic DNA resulted in a cGAS-STING elicited a pro-inflammatory response in normal and pathological aging. While generating stable young, elderly, HGPS and DKC shRNA-mediated cGAS and STING knockdown cell lines, three papers appeared in 2019 that largely addressed this question (Decco et al. 2019; Lan et al. 2019; Simon et al. 2019). Lan and colleagues demonstrated that cells derived from healthy elderly individuals, HGPS and Ataxia Telangiectasia patients had increased levels of cytosolic dsDNA, which led to a cGAS-STING mediated innate immune response. Furthermore, an age-related decrease of Dnase2a was observed and the ectopic expression of Dnase2a resulted in a decreased cytosolic dsDNA and diminished inflammatory response (Lan et al., 2019).

Chronic inflammation contributes to many age-associated diseases such as atherosclerosis, type 2 diabetes, Alzheimer's disease, Parkinson's disease, and cancer. With an aging population, chronic inflammation will play an even greater role in diminishing healthspan. Modulation cGAS-STING innate immune response may be a new strategy to dampen inflammation associated with senescence and aging.

Although micronuclei and chromatin bridges have been shown to promote cGAS-STING activity, which has been shown to play a role in maintaining senescence (Duy et al., 2017; Yang et al., 2017; Glück et al., 2017), micronuclei and chromatin bridges may play a more sinister role

on disease etiology. Micronuclei and chromatin bridges have both been shown to be mechanisms of chromothripsis, a chromosome pulverizing event in which multiple genes are hypermutated, lost, or rearranged in a single instance (Maceijowski et al., 2015; Liu et al., 2015).

Chromothripsis is thought to be a tumour initiating event and chromothripsis signatures are seen in a variety of cancers (Cortés-Ciriano et al., 2020). In theory, chromothripsis may be a single event sufficient of transforming a normal cell into a cancerous cell. A single event that results in multiple mutations challenges the stepwise progression model of cancer, which takes years for a normal cell to fully transform (Forment et al., 2012; Vendramin et al., 2021). The age associated increase in cancer has been attributed to the successive accumulation of mutations over an individual's lifespan, in a stepwise manner. The positive correlation between the increased risk of cancer with age and age-related increase of micronuclei and chromatin bridges raises the questions regarding the clonal evolution of cancer in elderly individuals. Furthermore, despite genomic and chromosomal instability promoting senescence, could rare instances of chromosome mis-segregation lead to a loss of senescence maintenance genes, permitting escape from senescence.

2.5 Materials and methods

2.5.1 Cell lines and cell culture

HGADFN003 (HG003), HGADFN127 (HG127), PSADFN257 (PSA257), PSADFN317 (PSA317) and PSADFN412 (PSA412) were obtained from the Progeria Research Foundation. AG07095 (2 YO), AG08498 (1YO), AG16409 (12 YO), AG04059 (96 YO), AG05248 (87 YO), AG12207 (68 YO), AG16102 (69 YO), AG04823 (DS1), AG06922 (DS2), GM01774 (DSK1), and AG04646 (DSK2) were obtained from the Coriell Institute for Medical Research. All cells

were maintained in Alpha Minimum Essential Medium supplemented with 10% fetal bovine serum at 37°C at 5% CO₂. Cells were split by rinsing cells with 1xPBS, pH 7.4, and incubating cells with 0.05% trypsin until all cells lifted from the tissue culture flask. Trypsinization was then stopped by adding complete media, followed by pelleting cells by centrifugation.

Table 1- List of donor derived cell lines.

Cell line	Age of Donor	Tissue	Known Pathology	Mutation
HGADFN003	2 years old (y) 0 months (m)	Skin- Fibroblast	Hutchinson-Gilford Progeria Syndrome (HGPS)	LMNA Exon 11, heterozygous c.1824C>T (p.Gly608Gly)
HGADFN127	3 y 9 m	Skin- Fibroblast	HGPS	LMNA Exon 11, heterozygous c.1824C>T (p.Gly608Gly)
PSADFN257	1 y 10 m	Skin- Fibroblast	Non-Classical HGPS	LMNA Exon 10, homozygous c.1619 T>C (p.Met540Thr))
PSADFN317	3 y 9 m	Skin- Fibroblast	Non-Classical HGPS	ZMPste24 Exon 6, heterozygous c.743C>T (p.Pro248Leu); Exon 10, heterozygous c.1349G>A (p.Trp450Stop))
PSADFN412	7 y 1m	Skin- Fibroblast	Non-Classical HGPS	LMNA Exon 11, heterozygous c.1762T>C (p.C588R))
AG07095	2 y	Skin- Fibroblast	Seemingly Normal	N/A
AG08498	1 y	Skin- Fibroblast	Seemingly Normal	N/A
AG16409	12 y	Skin- Fibroblast	Seemingly Normal	N/A
AG04059	96 y	Skin- Fibroblast	Seemingly Normal	N/A
AG05248	87 y	Skin- Fibroblast	Seemingly Normal	N/A
AG16102	69 y	Skin- Fibroblast	Seemingly Normal	N/A
AG12207	68 y	Skin- Fibroblast	Seemingly Normal	N/A
AG06922	2 y	Skin- Fibroblast	Down Syndrome	Trisomy 21
AG04823	5 y	Skin- Fibroblast	Down Syndrome	Trisomy 21

AG04646	11 y	Skin- Fibroblast	Dyskeratosis Congentia, X-linked	DKC1 A386T
GM01774	7 y	Skin- Fibroblast	Dyskeratosis Congentia, X-linked	DKC1 L37del

2.5.2 Drug treatment

Cells were treated with 20 J/m² of UV-radiation in the absence of media, on ice. Immediately after UV exposure, cells were harvested or allowed to recover to media for 2, 6, 12, 24 hr.

2.5.3 Immunofluorescence

Cells were grown to 60-75% confluency on glass coverslips 24 hr prior to harvesting. Cells were washed with 1xPBS, pH 7.4 on ice, followed by 2% formaldehyde fixation for 15 min at RT. Fixed cells were washed 3 times with 1xPBS, pH 7.4 and subsequently permeabilized with 0.5% Triton-X-100 in 1xPBS, pH 7.4 for 30 min on ice. Cells were then incubated with blocking buffer (consisting of 5% bovine serum albumin in 0.2% Triton-X-100 in 1xPBS, pH 7.4) for 1 hr on ice. Following blocking, primary antibodies were diluted in blocking buffer at 1:200-1:1000, and incubated with cells overnight at 4°C with parafilm placed overtop the coverslip to ensure even distribution of the antibody and prevent dehydration. The following primary antibodies were used: lamin A/C (E-1, sc376248), lamin A (H-102, sc-20680), progerin (13A4, ab66587), lamin B1 (Invitrogen ,10H34L18), γ H2AX (cell signalling, D7T2V), cGAS (cell signalling, D1D3G), RPA2 (Invitrogen, 9H8), phopho-RPA2 serine 33 (NB100-544). Cells were washed 3 times with cold 1x PBS, pH 7.4. for 5 min per wash, followed by incubation with secondary antibodies, Alexa 488 and Alexa 555, diluted 1:1000 in blocking buffer for 1 hour on ice in the

dark and covered with parafilm. Cells were washed 3 times with cold 1x PBS, pH 7.4. for a total of 15 min and then incubated in DAPI solution (DAPI diluted 1:10,000 in ddH₂O) for 10 minutes. Cells were washed 3 times with cold 1x PBS, pH 7.4. for a total of 15 min and then mounted on cover slides using ProLong Gold antifade medium. Slides were allowed to cure overnight in the dark and subsequently imaged on Olympus IX71.

2.5.4 Measuring micronuclei, chromatin bridges, herniations and abnormal nuclei

Random fields of view were observed by immunofluorescent microscopy and micronuclei and chromatin bridges were scored. Micronuclei were scored based on whether a cell contained at least one DAPI positive body smaller than the primary nucleus. Individual cells were discriminated based on background staining. Total micronuclei positive cells were divided by the total number of cells counted. Chromosomal bridges were detected by DAPI and scored as follows: an intact chromatin bridge (a string of DAPI positive stain attaching two primary nuclei to each other) was assigned a score of 1; a broken chromatin bridge (a string of DAPI that lead from one nucleus to the cytosol or extracellular space) was assigned a score of 0.5. Intact and broken chromatin bridges were summed and divided by total number of cells counted. Abnormal nuclei were detected by DAPI and confirmed with lamin A/C staining. Abnormal nuclei were scored based on their irregular non-symmetrical shape or presence of holes that were lamin A/C negative. **Figure 2-1C** shows examples of some abnormal nuclei. Herniations were detected by DAPI staining, and defined by their irregular outpouching from the oval/circular shaped primary nucleus, abnormal nuclei were excluded from the scoring. Total herniation positive nuclei were summed and divided by total nuclei.

2.5.5 Measuring nuclear area

DAPI staining was used to identify nuclei. DAPI stained images were processed on imageJ, where nuclear circumference was manually outlined. The total pixel area within the outline was used to determine the area of the nuclei using imageJ.

2.5.6 Western blotting

Cells were pelleted and washed with 1xPBS. Cells were then lysed in boiling 1x SDS (sodium dodecyl sulfate) lysis buffer (1% SDS, 10% glycerol, 88 mM Tris- HCl pH 6.8, in water) and boiled at 97°C for 10 min. A Pierce BCA Protein assay (Cat#23225) was used to measure protein concentration of lysates as instructed by manufacturer. Once protein concentrations were measured, 0.1% bromophenol blue and 0.1M dithiothreitol (DTT) was added to the lysed sample. 50 µg of total protein lysate per sample was loaded on an SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked for 1 hour at room temperature (RT) with blocking buffer consisting of 5% milk in 1x PBS 0.1% tween-20 (PBST), followed by an overnight incubation with primary antibodies diluted in blocking buffer at 4°C. The following are the primary antibodies used: lamin A/C (E-1, sc376248), progerin (13A4, ab66587), lamin B1 (Invitrogen ,10H34L18), cGAS (cell signalling, D1D3G), STING (Invitrogen, 2H1L5), and β-actin (sc-47778), α-tubulin (Calbiochem, DM1A). Membranes were washed with PBST and incubated for 1 hr at RT in HRP, Cy3, or Cy5 secondary antibody diluted in blocking buffer. Membranes were then washed with PBST. Pierce ECL Western blotting substrate for 1 min on membranes incubated with HRP secondary antibody, and subsequently imaged on MicroChemi System (DNR Bio Imaging System) and

analyzed on GelCaptureChemi program. Membranes incubated with Cy3 or Cy5 secondary antibody were imaged on a Typhoon imager.

2.5.7 Senescence-associated- β -galactosidase staining

SA- β -gal staining was performed as stated in Dimri et al., 1995. Briefly, cells were harvested and washed with 1x phosphate buffer solution (PBS) pH7.4 followed by fixation in 3% formaldehyde for 20 minutes. Cells were washed with 1x PBS followed by suspended and incubated overnight at 37°C β -gal staining solution. SA- β -gal solution was made directly before using and consisted of: 1 mg 5-bromo4-chloro-3-indolyl P3-D-galactoside, 150 mM NaCl, 40 mM citric acid pH 6.0, 40 mM sodium phosphate pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl₂.

Heterogeneity in leukemia cells that escape drug-induced senescence

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The following manuscript has been submitted for publication and is under review at Cell Death and Disease.

Contribution of work:

Kyra Kerkhofs in Mark Bayfield's lab performed the polysome profiling experiment and related western blotting (Figure 3-4E-F), and assisted with RNA-sequencing analysis by generating Figures (3-3A-C).

Sahib Singh Madahar contributed to qRT-PCR experiments (Figure 3-3K-L), and OCI-M2 response to DNR (Supplementary Figure 3-3).

3.1 Summary

Erythropoietin (EPO) suppresses drug-induced apoptosis in EPO-receptor positive leukemia cells and allows cells to persist after drug treatment by promoting cellular senescence. A small proportion of senescent cells can re-enter the cell cycle and resume proliferation after drug treatment. Using a single cell assay to track individual cells that exit a drug-induced senescence-like state, we show that cells exhibit asynchronous exit from a senescent-like state and display different rates of proliferation. Escaped cells retain sensitivity to drug treatment but display inter-clonal variability. We also find heterogeneity in gene expression with some of the escaped clones retaining senescence-associated gene expression. Senescent leukemia cells exhibit changes in gene expression that affect metabolism and senescence-associated secretory phenotype (SASP) related genes. We show that the senescent gene signature is a prognostic marker of poorer overall survival in AML and multiple other cancers. Senescent leukemia cells depend on lysosome activity and chloroquine, an inhibitor of lysosome activity, has senolytic activity. Our study indicates that the serious risks associated with use of erythropoietin stimulating agents (ESAs) in anemic cancer patients may be attributed to their ability to promote drug-tolerant cancer cells through the senescence program.

3.2 Introduction

Acute myeloid leukemia (AML) is a clonal malignancy arising in myeloid progenitor cells. AML remains the most lethal form of blood cancer, with a mortality of nearly 70% in patients 65 years and older. For a majority of patients who respond well initially, the disease recurs within 3 years of diagnosis (Döhner et al., 2015). Combination chemotherapy often involving cytarabine and

daunorubicin (DNR) are commonly used to treat AML. Chemotherapy-induced genotoxic damage to cells results in cell death and in cellular senescence.

Senescence has traditionally been defined as a stress-induced, permanent state of cell cycle arrest, where the cells remain metabolically active and are resistant to apoptosis. Senescent cells undergo reprogramming and display a senescent-associated secretory phenotype that can regulate intrinsic and extrinsic pathways and influence tissue homeostasis (Gorgoulis et al., 2019; Hernandez-Segura et al., 2018; Lee and Schmitt 2019). Many endogenous and environmental stresses can promote senescence and, in most cases, cellular senescence depends on activation of p53/p21 axis and/or the RB/p16 pathway. Previously, we reported that Erythropoietin (EPO) protects murine myeloid leukemia DA3/EPOR cells from p53-mediated apoptosis, and instead promotes a senescent state (Pham et al., 2019). Although senescence has been thought to be a favourable outcome of chemotherapy, recent reports suggest that cancer cells are able to escape from therapy-induced senescence. Hence, cellular senescence may allow cancer cells to survive during drug treatment and escape from senescence may contribute to tumour cell expansion and patient relapse (Duy et al., 2021; Saleh et al., 2019). Moreover, tumour cells that escape senescence have been shown to acquire a more malignant phenotype (Duy et al., 2021; Milanovic et al., 2018).

Cancer relapse following chemotherapy is often associated with the acquisition of mutations in a subpopulation of cancer cells that confer drug resistance. Alternative epigenetic mechanisms to drug tolerance are emerging and have clinical significance (Cara and Tannock, 2001; Kuczynski et al., 2013; Sharma et al., 2010). Mechanisms surrounding non-genetic drug tolerance are largely unknown. Recently, a stress-induced embryonic dormancy state known as embryonic

diapause (from here on referred to as diapause) was found to contribute to drug resistance in colon cancer, adenocarcinoma and AML (Duy et al., 2021; Dhimolea et al., 2021; Rehman et al., 2021).

Here we demonstrate that EPO protects DA3/EPOR leukemia cells from drug-induced cell death by promoting and maintaining senescence. Importantly, drug-induced cellular senescence is reversible in DA3/EPOR upon drug withdrawal. Drug-recovered cells that escape drug-induced senescence are heterogenous; displaying asynchronous release from senescence, varying rates of proliferation, differential responses to second round drug-treatment, and differences in gene expression. During senescence, DA3/EPOR cells undergo a partially reversible reprogramming, gaining a diapause-like phenotype and increasing expression of coagulation related genes. Despite inter-clonal heterogeneity, RNA-seq revealed that the bulk of drug-recovered cells mirrored the naïve, never drug-treated DA3/EPOR cells. We demonstrate that high expression of the senescent signature is associated with decreased survival in AML and multiple other cancers. We show that human and murine leukemia cells undergoing therapy-induced senescence depend on lysosome function and that inhibition of lysosome function with chloroquine (CQ) promotes senolysis.

EPO and erythropoiesis-stimulating agents (ESAs) have been used to manage chemotherapy-induced anemia in cancer patients (Aapro et al., 2019). Several studies have reported that treatment of anemic cancer patients with ESAs increases tumour progression, tumour recurrence, thrombosis and death (Glaspy, 2009; FDA, 2017). These serious safety concerns have restricted the use of ESAs in patients receiving chemotherapy. Our study indicates that the serious risks

associated with use of ESAs in anemic cancer patients may be attributed to their ability to promote persistent cancer cells through the senescence program.

3.3 Results

3.3.1 DA3/EPOR leukemia cells persist drug treatment and enter senescence

To determine whether drug-induced senescence is a reversible phenotype in transformed cells, we employed the DA3/EPOR murine myeloid leukemia cell line (Miura et al., 1991). Previously, we demonstrated that EPO protected DA3/EPOR cells from p53-induced death upon exposure to genotoxic agents and promoted a senescence-like state (Pham et al., 2019). EPO supplemented DA3/EPOR cells demonstrated a ~35-fold reduced sensitivity to Dox-induced cell death compared with cells treated with Dox alone (**Figure 3-1A**). We treated DA3/EPOR cells with Dox in the presence or absence of EPO for 24 hr and cultured the cells in EPO without Dox for 3 days. On day 4 (3 days after Dox removal), we observed that 96-99% of surviving DA3/EPOR cells (Dox+/EPO+) and about 20% of drug-treated cells not supplemented with EPO (Dox+/EPO-) entered senescence on the basis of SA- β -gal staining, a well-established biomarker of cellular senescence (**Figure 3-1B; Supplementary Figure 3-1A-C**). To determine if EPO was required to maintain the senescence phenotype, we removed EPO from the senescent cells on day 4 and cultured the cells for 2 additional days. Naïve DA3/EPOR cells in the absence of EPO remained viable, albeit had a reduced rate of proliferation; in the presence of EPO, DA3/EPOR cells have a population doubling time of approximately 11 hours. Withdrawal of EPO from senescent DA3/EPOR cells led to an approximate 5-fold decrease in cell viability on day 6 indicating that EPO sustains the senescence program and viability (**Figure 3-1C**). Dox+/EPO+ treated cells exhibited increased cell size and accumulated in the G1 and G2/M phases of the cell

cycle (**Supplementary Figure 3-1B, D-E**), two additional features associated with cellular senescence. Next, we measured the proliferative capacity of DA3/EPOR cells treated with Dox in the presence and absence of EPO by counting viable cells every 2 days, over an 8-day course. Dox+/EPO+ cells exhibited a slight decrease in viable cell number for 4 days as expected for a population of primarily senescent cells. An increase in cell number, however, was detected on day 6 and continued into day 8 concomitant with a decrease in the proportion of SA- β -gal positive senescent cells (**Figure 3-1D; Supplementary Figure 3-1C**).

DA3/EPOR cells were treated with Dox and EPO for 72 hr and proliferation was assessed every 3 days. These cells entered a prolonged non-proliferative state and exhibited 97% SA- β -gal positivity comparable to that seen after 24 hr of Dox/EPO treatment (**Figure 3-1E; Supplementary Figure 3-1F**). Under these conditions, cell proliferation was delayed until 12 days post drug treatment (**Figure 3-1F**).

3.3.2 Drug-induced senescence is independent of p53 and p16 in DA3/EPOR cells

DNA damage-induced senescence requires activation of the p53/p21 axis and/or the p16/Rb pathway. To determine whether cells entered a p53-dependent form of senescence, a dominant negative p53 (p53DD) construct was expressed in DA3/EPOR cells (**Supplementary Figure 3-2A**). p53DD is a C-terminal truncated isoform of p53 containing the tetramerization domain but lacking the DNA-binding domain that stabilizes and transcriptionally inactivates wild-type p53 (Shaulian et al., 1992). Three independent DA3/EPOR-DD clones retained the ability to enter Dox-induced senescence similar to empty vector control cells (DA3/EPOR pcDNA) (**Figure 3-1G; Supplementary Figure 3-2B**). To assess the role of CDKN2a (p16) in senescence, levels of

p16 were measured by qRT-PCR. DA3/EPOR expressed very low levels of p16, and no increase in expression was detected when these cells entered senescence (**Figure 3-1H**). In comparison, mouse embryonic fibroblasts (MEFs) express basal levels of p16 and these levels increase during Dox-induced senescence (**Figure 3-1H**). These results indicate that DA3/EPOR cells enter senescence through a p53- and p16-independent pathway.

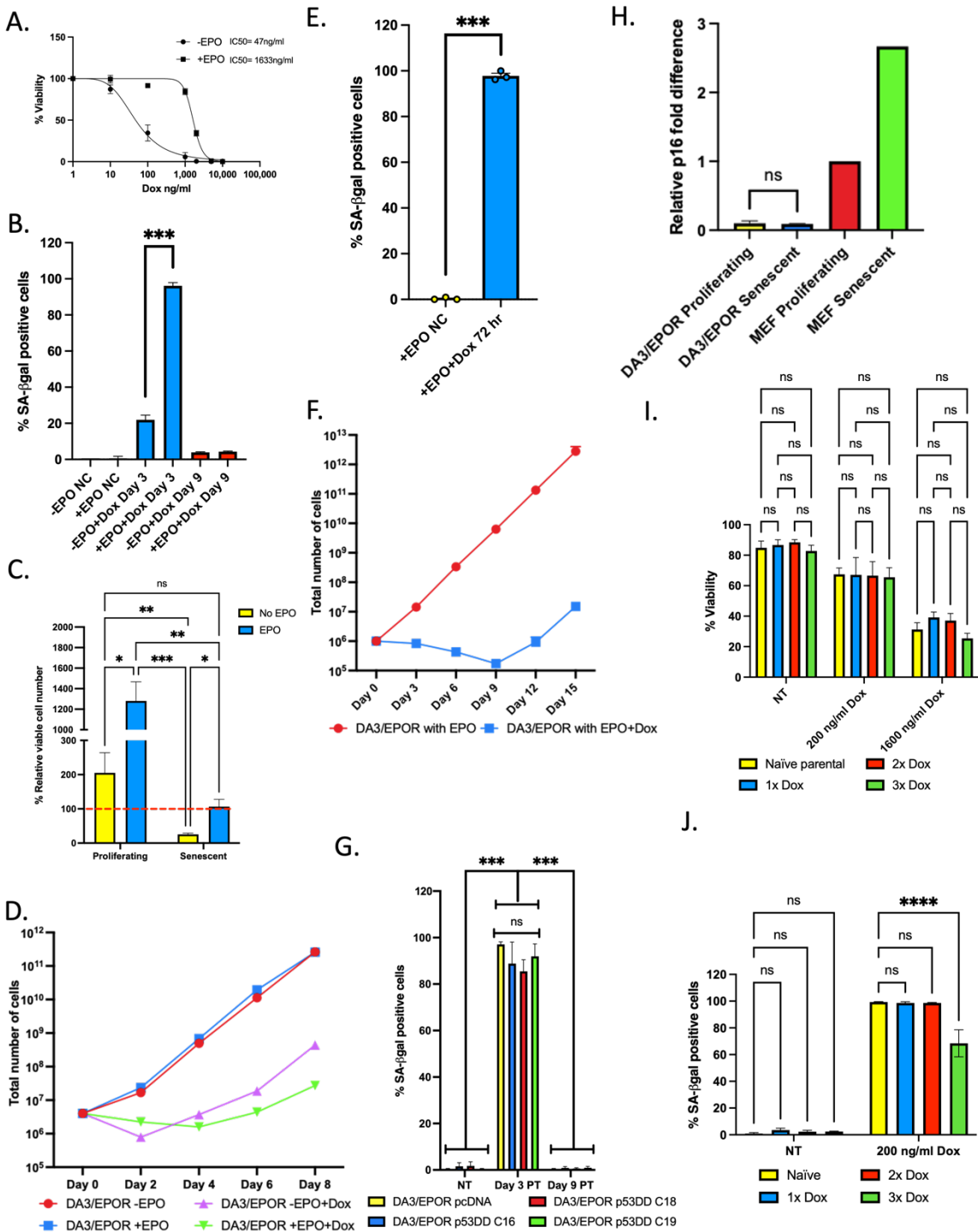


Figure 3-1 DA3/EPOR cells enter a drug-induced senescent state.

A. Percentage of viable DA3/EPOR cells treated for 72 hr with increasing amounts of doxorubicin (Dox) in the presence or absence of erythropoietin (EPO). Error bars

represent SEM, n=3 biological replicates; a minimum of 100 cells were counted for each biological replicate.

- B. Percentage of senescent DA3/EPOR cells after treatment with Dox for 24 hr. SA- β -gal staining of DA3/EPOR cells was performed 3 and 9 days post-treatment. DA3/EPOR cells were treated with or without 200 ng/ml Dox for 24 hr in the absence or presence of EPO. After drug treatment, all cells were maintained in complete, EPO containing media. Related to Supplementary Figure 1A. Mean $\pm$ SEM, n = 3 independent experiments; Two-way student T-test. A minimum of 100 cells were counted for each independent experiment.
- C. Percentage of viable senescent and proliferating DA3/EPOR after being maintained in media with or without EPO for 48 hr. Viability cells was normalized to the initial number of cells seeded. Mean $\pm$ SEM, n = 4 independent experiments; two-way ANOVA.
- D. Proliferation of DA3/EPOR cells treated with or without 200 ng/ml Dox in the absence or presence of EPO. After 24 hr drug treatment, all cells were maintained in complete, EPO containing media. Cells were counted every 2 days. Related to Supplementary Figure 1A. Mean $\pm$ SEM, n = 3 independent experiments.
- E. Percentage of senescent DA3/EPOR cells after treatment with Dox for 72 hr. Cells were treated with Dox for 72 hr and stained for SA- β -gal 24 hr after Dox treatment. Related to Supplementary Figure 1C. Mean $\pm$ SEM, n = 3 independent experiments; Two-way student T-test. A minimum of 100 cells were counted for each independent experiment.
- F. Proliferation of DA3/EPOR treated with or without 200 ng/ml Dox for 72 hr in the presence of EPO. Cells were counted every 3 days.
- G. SA- β -gal staining of DA3/EPOR cells expressing p53DD (clones C16, C18 and C19) and empty vector (pcDNA). SA- β -gal staining was performed 3 and 9 days post-treatment of control and Dox treated cells. Mean $\pm$ SEM, n = 3 independent experiments; one-way ANOVA. A minimum of 100 cells were counted for each independent experiment.
- H. p16 RNA expression in proliferating and senescent DA3/EPOR cells determined by qRT-PCR. Untreated and 3 day post-Dox treated MEFs were included as a positive control for p16 expression. Mean $\pm$ SEM, n = 3 independent experiments for proliferating and senescent DA3/EPOR cells, n = 1 for proliferating and senescent cells MEFs; two-way student test.
- I. Percentage of viable DA3/EPOR and drug-recovered DA3/EPOR cells with increasing concentrations of Dox for 48 hr relative to control treated DA3/EPOR. Drug-recovered DA3/EPOR cells were generated by treating naïve DA3/EPOR cells with 200 ng/ml Dox for 24 hr in EPO and allowing the population to recover. Drug-recovered cells that went through 1, 2, and 3 rounds of dox treatment are indicated by 1x Dox, 2x Dox and 3x Dox, respectively. Cells were stained with trypan blue 48 hr drug treatment and cells were counted using a hemocytometer. Mean $\pm$ SEM, n = 3 independent experiments; two-way ANOVA.
- J. SA- β -gal staining of DA3/EPOR and drug-recovered DA3/EPOR populations performed 3 days and 9 days after treatment with Dox. Drug-recovered cells that went through 1, 2, and 3 rounds of dox treatment are indicated by 1x Dox, 2x Dox and 3x Dox, respectively. Mean $\pm$ SEM, n = 3 independent experiments; two-way ANOVA. A minimum of 100 cells were counted for each independent experiment.

Statistical significance is shown by ns, not significantly different, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.3.3 DA3/EPOR cells persist drug treatment

Cancer cells may survive drug treatment for a number of reasons including presence of genetic variants, non-genetic mechanisms that are associated with slow proliferation, checkpoint activation resulting in transient cell cycle arrest, and cellular senescence. The term persistent cancer cells has been used to describe cells that survive chemotherapy and exhibit a reversible slow growing state that is not driven by any genetic variations (Shen et al., 2020). To determine whether the drug treatment resulted in the selection of a drug-resistant clone, the drug-recovered population was re-treated with drug for two additional rounds. Drug-recovered populations and naïve cells were treated with increasing concentrations of Dox for 48 hrs and cell viability was measured. No differences in cell viability was observed between naïve parental and drug-recovered cells (**Figure 3-1I**). To determine whether drug-recovered DA3/EPOR cells retained sensitivity to enter drug-induced senescence, we treated drug-recovered populations with Dox and EPO for 24 hr and measured senescent cells 3 days after treatment using SA- β -gal staining. Drug-recovered DA3/EPOR cells all showed an increase in SA- β -gal positivity, although the drug-recovered population that went through 3 rounds of treatment showed reduced amount of SA- β -gal cells compared to naïve cells being treated with Dox (**Figure 3-1J**).

Cell cycle analysis using flow cytometry demonstrated that naïve DA3/EPOR and drug-recovered cells treated with EPO and Dox arrested in the G1 and G2/M phases of the cell cycle (day 3) and re-established a normal cell cycle profile (**Supplementary Figure 3-1E**). Cell size

increased 3 days post-treatment for both naïve and drug-recovered DA3/EPOR cells (**Supplementary Figure 3-1D; data not shown**). Naïve and drug-recovered cells had comparable sub-G1 populations after drug treatment (**Supplementary Figure 3-1E**). Collectively, these data show that DA3/EPOR cells resume proliferation after drug treatment. These cells retain drug sensitivity and the ability to enter drug-induced senescence, and thus, the senescent-like state facilitates transient drug-tolerance.

3.3.4 Drug-induced senescence in DA3/EPOR cells is reversible

To investigate whether Dox-induced senescence is reversible, we treated DA3/EPOR cells with Dox+EPO for 24 hr, waited another 24 hr for the cells to recover in the absence of drug, and seeded single cells into 96 well plates while monitoring wells for cell proliferation every 3 days (**Figure 3-2A**). As noted earlier, under these same conditions, 96-99% of surviving cells enter senescence on the basis of SA- β -Gal staining. We reasoned that if Dox-treated single cells proliferate within 3 days post-seeding, then these cells likely never entered senescence; instead, they may have undergone checkpoint activation and entered a transient cell cycle arrest or they may have been tolerant to drug and never exited the cell cycle. On the other hand, if Dox-treated cells show delayed growth and proliferate only after 3 days post-seeding, then these cells likely entered senescence and subsequently escaped. 68.2% of wells seeded with untreated control DA3/EPOR cells contained proliferating cells within 3 days of seeding, while only 0.7% of wells seeded with Dox-treated cells contained proliferating cells within 3 days of seeding (**Figure 3-2B**). Dox-treated cells exhibited delayed growth on days 6 and 9 post seeding. Notably, of the 38 wells seeded with Dox-treated cells that showed growth, 30/38 individual cells (78.9%)

showed delayed growth on days 6 and 9, while 9/402 untreated individual cells (2%) showed delayed growth on day 6 with almost all of the untreated cells showing growth within 3 days of seeding (**Figure 3-2C**). These data indicate that a small fraction of cells that enter drug-induced senescence exhibit asynchronous exit from senescence.

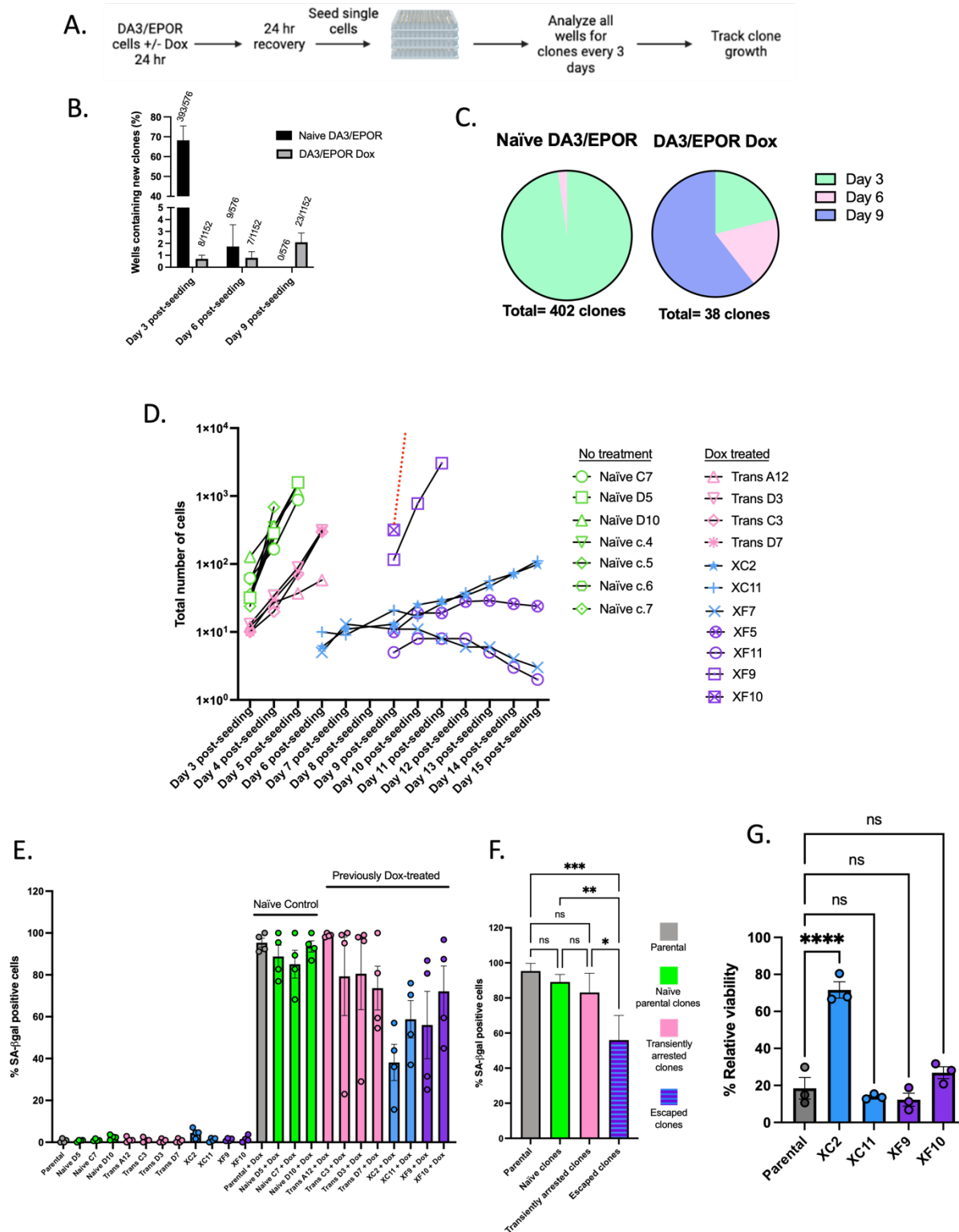


Figure 3-2 DA3/EPOR cells that escape senescence exhibit heterogeneity.

A. Experimental scheme. Dox-treated and untreated single cells were seeded into 96 well plates and monitored every 3 days for new expanding clones. Expanding clones were

defined by ≥ 5 cells/ well. Newly formed colonies were counted every 3 days and expanding colony size was counted to determine proliferation rates. Related to Supplementary Figure 2A.

B. Percentage of wells containing new colonies. Mean $\pm$ SEM, n = 3 independent experiments.

C. Pie chart showing proportions of when new expanding clones were detected (related to 2A).

D. Rates of proliferation of randomly selected expanding colonies.

E. SA- β -gal staining of parental DA3/EPOR, 3 naïve clones cells, 8 expanded drug-recovered clones (4 of which went through a transient arrest and 4 that escaped senescence). Mean $\pm$ SEM, n = 4 independent experiments.

F. Statistical analysis of SA- β -gal staining of the Dox-treated naïve (n= 3 biological samples), transiently arrested (n= 4 biological samples), and escaped clones (n= 4 biological samples) compared with Dox-treated parental cells. Related to Figure 2D. Mean $\pm$ SEM; one-way ANOVA.

G. Relative viability of 4 escaped clones compared to parental DA3/EPOR cells treated with Dox in the absence of EPO. Mean $\pm$ SEM, n = 3 independent experiments; one-way ANOVA.

Statistical significance is shown by *P<0.05, **P<0.01, ***P<0.001.

Clonal expansion from single cells revealed extensive heterogeneity in the growth rate of escaped cells; some escaped clones expanded rapidly while others were unable to sustain proliferation (**Figure 3-2D**) compared with naïve (never treated cells) and drug-treated cells that grew within 3 days of seeding. Proliferation rates of two escaped clones, XC2 and XC11, remained slow for >30 days in culture post-dox treatment, and then increased to a similar rate as naïve cells. Notably, drug-treated clones that appeared within 3 days of seeding consisted of smaller colonies (fewer cells per well) compared with the naïve untreated cells supporting the view that these cells arose from transiently arrested cells that did not senesce (**Figure 3-2D**). The proliferation rates of naïve untreated clones and transiently arrested drug-treated clones were fairly uniform suggesting that the heterogeneity observed in the escaped clones likely arose de

novo as a result of drug-induced entry and exit from senescence and did not pre-exist in the parental population.

We compared the drug sensitivity of cells derived after expansion of individual escaped DA3/EPOR cells with cells derived from naïve and transiently arrested individual cells. Escaped clones (XC2, XC11, XF9 and XF10) showed variable drug sensitivity both in terms of drug-induced senescence (**Figure 3-2E-F**) and drug-induced cell death (**Figure 3-2G**) while the naïve and transiently arrested clones exhibited uniform drug sensitivity similar to parental cells.

To explore further the heterogenous behaviour of escaped clones, three independent naïve DA3/EPOR clones (C7, D5 and D10) were subjected to Dox treatment and investigated using the single cell assay. The 3 clonal populations entered senescence similar to the parental population (**Figure 3-2E**). Escape from drug-induced senescence occurred asynchronously in the 3 clonal populations (**Supplementary Figure 3-3A-B**). Clonal expansion from single cells revealed extensive heterogeneity in the growth rate of escaped cells (**Supplementary Figure 3-3C**). Like the naïve parentals, all (3/3) naïve clones demonstrated the ability to persist following drug treatment through transient cell cycle arrest, permanent senescence, and a reversible senescent state. A small proportion of senescent cells acquired the ability to escape senescence in a stochastic manner. Together these results support the idea that heterogeneity in cell behaviour arises after drug treatment and de novo in the escaped cells.

3.3.5 OCI-M2 human leukemia cells persist during daunorubicin treatment and enter senescence

OCI-M2 human leukemia cells, derived from a patient with erythroleukemia, were treated with the clinically relevant drug DNR for 24 hr. DNR-treated OCI-M2 cells entered senescence 3 days after drug treatment based on high levels of SA- β -gal positivity and increased cell size (**Supplementary Figure 3-4A**). The proportion of senescent cells decreased over time. DNR-treated OCI-M2 cells showed sustained proliferative arrest and delayed cell death up to 7 days post-treatment (**Supplementary Figure 3-4B**). Drug-recovered OCI-M2 cells were able to resume proliferation after 7 days post-DNR treatment (**Supplementary Figure 3-4B**). The ability of the mutant p53 (V274D)-expressing cell line, OCI-M2, to enter senescence supports our earlier finding using the DA3/EPOR DD clones that drug-induced senescence occurs independently of p53. Drug-recovered OCI-M2 cells re-treated with DNR demonstrated the ability to enter senescence to comparable levels as naïve OCI-M2 cells (**Supplementary Figure 3-4C**); hence, drug-recovered OCI-M2 cells are not resistant to drug-induced senescence. Unlike drug-recovered DA3/EPOR cells, drug-recovered OCI-M2 cells demonstrated increased viability to DNR 3 days and 7 days post-treatment compared with naïve cells (**Supplementary figure 3-4D**).

To determine whether the OCI-M2 cells could escape DNR-induced senescence, we performed single cell analysis on DNR-treated OCI-M2 cells and monitored individual clones for proliferation. Of the wells that showed proliferating clones, 98.4% of naïve untreated OCI-M2 cells formed colonies within 3 days post-seeding (**Supplementary Figure 3-5A-B**). Drug-recovered OCI-M2 cells demonstrated heterogeneity; 4 of the 25 clones underwent transient or no arrest (cell expansion appearing by day 3 post-seeding), and 19/25 clones escaped DNR-induced senescence (cell expansion appearing by day 6 or day 9 post seeding) (**Supplementary**

Figure 3-5A-B). Furthermore, escape from senescence occurred in an asynchronous manner (**Supplementary Figure 3-5A-B**). Most naïve OCI-M2 cells showed uniform rates of proliferation, whereas escaped OCI-M2 cells exhibited variable rates of proliferation (**Supplementary Figure 3-5C**) similar to what was observed in the DA3/EPOR cells. These data indicate that OCI-M2 cells undergo drug-induced senescence and that a small proportion of cells escape senescence asynchronously and exhibit differences in proliferation rates.

3.3.6 Transcriptional Reprogramming During Senescence

To gain insight into the mechanism of drug-induced senescence and to evaluate differences in gene expression between drug-recovered cells and naïve proliferating cells, RNA-seq was performed on naïve, senescent and drug-recovered DA3/EPOR cells. We collected the senescent cells on day 3 after Dox/EPO treatment; the drug-recovered cells were collected 9 days after Dox/EPO treatment. Drug-recovered population (cells that survive drug treatment and resume proliferation) consists of a mixture of cells including cells that may never have senesced as discussed above. We found that the transcriptional profiles of senescent cells were highly distinct from naïve and drug-recovered cells; drug-recovered cells resembled the naïve proliferating population (**Figure 3-3A-C**). The volcano plot, displaying gene expression of drug-recovered proliferating cells compared with senescent cells mirrors the volcano plot of naïve proliferating cells with senescent cells, suggesting that the drug-recovered signature is similar to the naïve proliferating gene signature (**Figure 3-3C**).

Gene Set Enrichment Analysis (GSEA) showed a significant enrichment for senescent genes in the Dox-induced senescent cells and not in the drug-recovered cells (**Figure 3-3D**). Additionally,

RNA-seq demonstrated that senescent DA3/EPOR cells displayed an upregulation of multiple senescent associated secretory phenotype (SASP) genes, which decreased in drug-recovered cells (**Supplementary Figure 3-6A**; Coppé et al., 2010). p16 was not detected in any of the samples by RNA-seq (data not shown) consistent with our earlier results (**Figure 3-1H**).

GSEA demonstrated significant enrichment for gene signatures associated with various pathways. Figure 3-3E-F show the top ten enriched and downregulated Hallmark and KEGG pathways in senescent DA3/EPOR cells compared to proliferating controls. To validate RNA-seq, several KEGG and Hallmark categories were further investigated. We included a later time point (20 days PT) to investigate the stability of the transcriptional changes of drug-recovered cells compared to proliferating cells. Consistent with the upregulation of Cellular Adhesion Molecules during senescence, qRT-PCR demonstrated that senescent DA3/EPOR cells had elevated levels of Intercellular Adhesion Molecule 1 (ICAM1) and Integrin β 2 (ITGB2) compared with naïve and drug-recovered cells (**Figure 3-3G**). DA3/EPOR cells began to form aggregates during Dox treatment and remained aggregated throughout the senescent period. Cell aggregates dispersed as cells resumed proliferation (**Figure 3-3H**). Dox-treated DA3/EPOR cells were able to reform aggregates after being separated through pipetting (**Figure 3-3H**). These data indicate that aggregation is associated with the senescence phenotype.

GSEA demonstrated that the coagulation and complement hallmark pathways were highly upregulated during senescence in DA3/EPOR cells. Three components of the coagulation and complement gene sets, were selected for qRT-PCR analysis using RNA isolated from naïve, senescent, and drug-recovered cells. Expression of SerpinB2 (PAI2), vWF (Von Willebrand

factor) and C3 (complement III) mRNA increased during senescence in DA3/EPOR cells (Figure 3-3I). SerpinB2 and vWF mRNA levels returned to basal levels with prolonged time in culture. Expression of C3 remained stably elevated in drug-recovered cells on day 9 and day 20 post-treatment, indicating that although the majority of genes in the drug-recovered cells return to basal levels, there are some phenotypic distinctions between drug-recovered and naïve cells (Figure 3-3I).

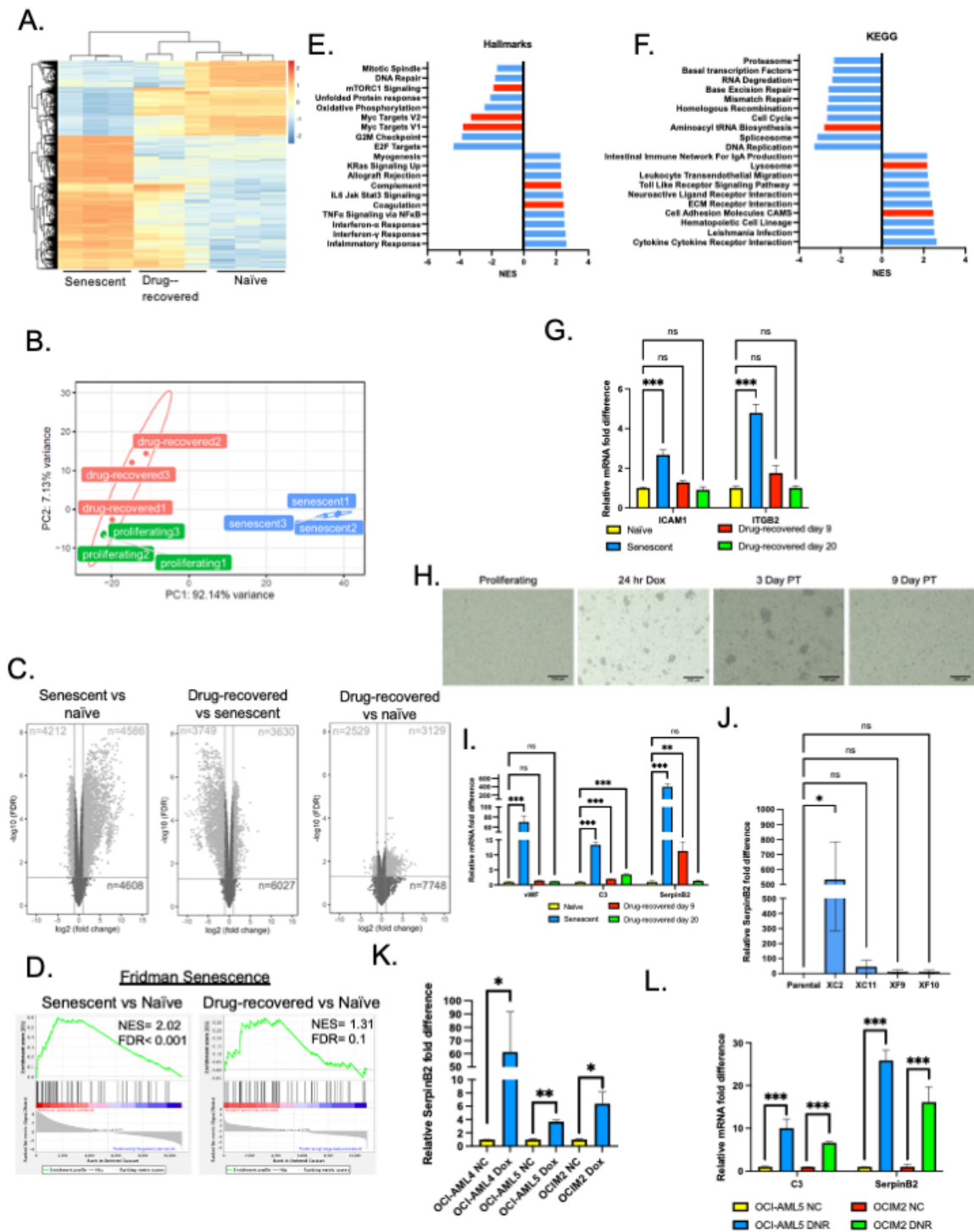


Figure 3-3 Senescent-associated changes in gene expression.

A. Heatmap of proliferating, senescent (day 3 PT), and drug-recovered (day 9 PT) DA3/EPOR cells using the top 1000 differentially expressed genes in senescent DA3/EPOR cells compared to proliferating control DA3/EPOR cells.

- B. Principal component analysis (PCA) grouping proliferating control (green), day 3 senescent (blue) and day 9 drug-recovered cells (red) in biological triplicate.
- C. Volcano plots showing relative gene expression of RNA-seq data. Genes with a fold change of >2 and an FDR < 0.05 were represented as light grey dots; genes with a fold-change less than 2 and non-significant genes were displayed as dark grey dots. Total number of significantly upregulated and downregulated genes are stated in the respective quadrates. Total number of insignificant genes are stated in the bottom right quadrant.
- D. GSEA plot of Fridman's senescence in senescent DA3/EPOR cell compared to proliferating controls, and drug-recovered DA3/EPOR cells compared to proliferating controls.
- E. Normalized enrichment scores (NES) of the top 10 most upregulated and downregulated Hallmarks of senescent DA3/EPOR cells compared to proliferating control cells. All Hallmarks have an FDR of >0.001 . Hallmarks highlighted in red were validated.
- F. NES of the top 10 most upregulated and downregulated KEGG pathways of senescent DA3/EPOR cells compared to proliferating control cells. All pathways have an FDR of >0.001 . KEGG pathways highlight in red were validated.
- G. qRT-PCR analysis of ICAM1 and ITGB2 mRNA levels in proliferating, senescent, drug-recovered(9 days post-treatment), and later drug-recovered (20 days post-treatment) DA3/EPOR cells. Mean $\pm$ SEM, $n = 3$ independent experiments; one-way ANOVA.
- H. Representative images of proliferating, 24hr Dox treated, senescent (3 days PT), and drug-recovered (9 days PT) DA3/EPOR cells. After 24 hr Dox treatment, cells were washed with 1xPBS and cells clumped together were mechanically separated.
- I. qRT-PCR analysis of complement III (C3), von Willebrand factor (vWF), and SerpinB2 mRNA levels in proliferating, senescent, drug-recovered (9 days post-treatment), and later drug-recovered (20 days post-treatment) DA3/EPOR cells. Mean $\pm$ SEM, $n = 3$ independent experiments; one-way ANOVA.
- K. qRT-PCR analysis of SerpinB2 mRNA levels in Dox-induced senescent OCI-AML4, OCI-AML5 and OCI-M2 cell lines, relative to their proliferating controls. Mean $\pm$ SEM, $n = 3$ independent experiments; one-way ANOVA.
- L. qRT-PCR analysis of C3 and SerpinB2 mRNA levels in DNR-induced senescent OCI-AML5 and OCI-M2 cell lines, relative to their proliferating controls. Mean $\pm$ SEM, $n = 3$ independent experiments; Student t-test.

Statistical significance is shown by ns, not significantly different, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The drug-recovered population is heterogenous, consisting of cells proliferating after a transient cell cycle arrest, cells that have escaped senescence, and cells that remain in senescence. To determine whether the RNA-seq and qRT-PCR of the drug-recovered population reflected cells that truly escaped from drug-induced senescence, we performed qRT-PCR on four escaped

clones and assessed the levels of SerpinB2, which was upregulated in drug-recovered cells. Here again, we see inter-clonal heterogeneity in the expression of SerpinB2; escaped clone XC2 displayed significantly elevated SerpinB2 relative to parental control cells and to the 3 other independent escaped clones (**Figure 3-3J**).

To explore the possibility that SerpinB2, vWF and C3 are potential biomarkers of therapy-induced senescence in human leukemia, 3 human AML cell lines, OCI-AML4, OCI-AML5 and OCI-M2 were treated with Dox for 24 hr and increase of SA- β -gal positive cells were detected 3 days after drug treatment (**Supplementary Figure 3-6B**). Consistent with the senescent phenotype, OCI-AML4, OCI-AML5, and OCI-M2 exhibited increased cell size (**Supplementary Figure 3-4C**). qRT-PCR analysis demonstrated that SerpinB2 mRNA levels increase significantly during Dox-induced senescence in all 3 human AML cell lines (**Figure 3-3K**). Previous reports have suggested that SerpinB2 is a marker of senescence and may aid in maintenance of the senescent state (Hsieh et al., 2017). Dox-induced senescent OCI-AML5 cells had elevated levels of C3; expression of C3 in Dox-induced senescent OCI-AML4 and OCI-M2 trended upward (**Supplementary Figure 3-6D**). vWF was not detected in human AML cell lines (data not shown). OCI-AML5 and OCI-M2 cells were also treated with the clinically relevant drug DNR for 24 hr. DNR-treated OCI-AML5 and OCI-M2 cells entered senescence 3 days after drug treatment based on high levels of SA- β -gal positivity and increased cell size (**Supplementary Figure 3-6E-F**) and expressed increased levels of SerpinB2 and C3 mRNA (**Figure 3-3L**). We propose that SerpinB2 and C3 be considered as biomarkers of therapy-induced senescence in AML.

3.3.7 Senescent DA3/EPOR Cells Mimic Diapause

Chemotherapy-treated colon carcinomas, adenocarcinoma, and acute myeloid leukemias were recently shown to enter a reversible diapause-like cell state that enabled cell survival during drug treatment (Duy et al., 2021; Dhimolea et al., 2021; Rehman et al., 2021). Diapause is a reversible, evolutionary conserved, embryonic stress state, which enhances cell survival in response to environmental stress. Using GSEA and a diapause gene set, we observed significant enrichment for diapause-associated genes in senescent DA3/EPOR cells (**Figure 3-4A**; Duy et al., 2021; Boroviak et al., 2015).

Senescent DA3/EPOR cells were enriched for multiple diapause associated hallmark pathways (**Figure 3-4B**; Hussein et al., 2020). Myc and mTOR inhibition promote a dormant state that mimics diapause (Bulut-Karslioglu et al., 2016; Scognamiglio et al., 2016). Both Myc and mTOR pathways were downregulated in senescent DA3/EPOR cells (**Figure 3-3E**). Diapaused epiblasts exhibit decreased proliferation, Myc expression, translation-associated genes and active translation (Boroviak et al., 2015). To evaluate whether senescent, non-proliferating DA3/EPOR cells were in a diapause-like state, levels of Myc expression and translation were assessed. Senescent cells demonstrated decreased c-Myc protein levels, which increased to normal levels after proliferation was resumed on day 9 (**Figure 3-4C**). GSEA demonstrated that Gene Ontology (GO) translation initiation and termination, as well as tRNA metabolic processes and ribosome biogenesis were downregulated in senescent DA3/EPOR cells compared with naïve cells (**Figure 3-4D**). Polysome analysis by sucrose gradient centrifugation, revealed that senescent DA3/EPOR cells had reduced global translation that returned to normal levels in the drug-recovered cells (**Figure 3-4E**). Consistent with reduced translation, senescent cells

displayed an altered distribution of ribosomal proteins RPL9 and RPS6 in the polysome fractions, with relatively higher levels in the free subunit and monosome fractions (**Figure 3-4F**). Together, these data point towards an association between drug-induced senescence and the diapause state in DA3/EPOR cells.

We next asked whether further perturbation to the mTOR pathway in senescent DA3/EPOR cells would decrease survival. DA3/EPOR cells were treated with increasing concentrations of rapamycin (Rapa), an mTOR inhibitor, for 48 hr. Rapa decreased senescent DA3/EPOR cell viability in a dose dependent manner, whereas proliferating DA3/EPOR cells remained viable with a reduced rate of proliferation (**Figure 3-4G**).

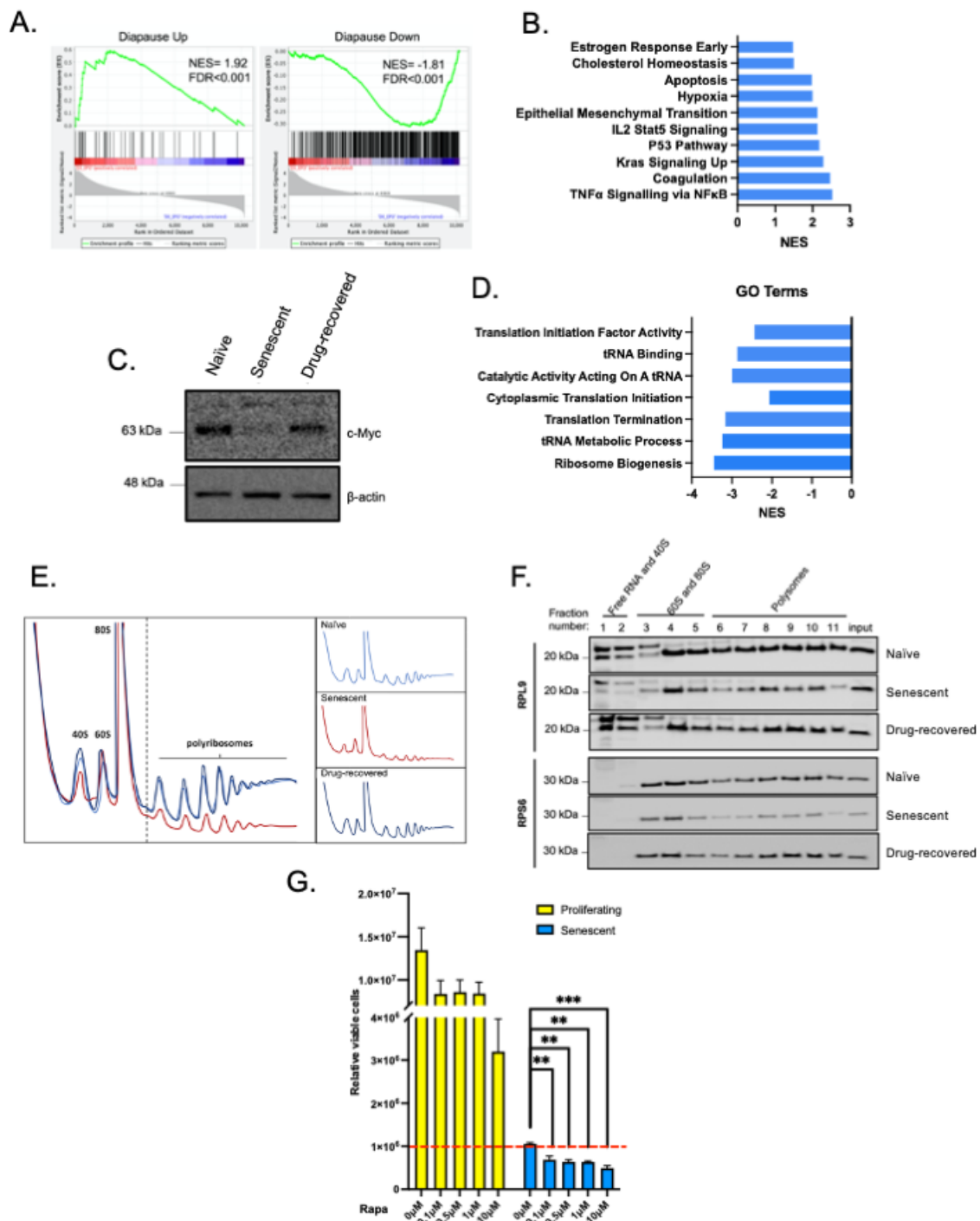


Figure 3-4 Drug-induced senescence is associated with a diapause-like state.

- A. GSEA plots of senescent DA3/EPOR cells compared to control cells using a curated diapause gene set of genes up and down regulated in a diapause state (Diapause gene set was derived from Boroviak et al., 2015; and is identical to the Diapause gene set used in Duy et al., 2021; Supplementary Table 2).

- B. NES of Hallmark gene sets found in a diapause state. All Hallmarks had an FDR<0.01 (Hussein et al., 2020).
- C. Representative western blot of c-Myc in proliferating, senescent, and drug-recovered DA3/EPOR cells (n= 2 biological replicates).
- D. NES of Gene Ontology (GO) related to translation of senescent DA3/EPOR cells compared to proliferating controls.
- E. Representative traced polysome profiles of proliferating, senescent, and drug-recovered (Day 9) DA3/EPOR cells. Overlayed traced polysomes profiles on the left; individual traced polysome profiles on the right.
- F. Fractions of polysomes were taken, and protein was isolated. RPL9 and RPS6 levels were analyzed by western blot. n= 3 independent experiments.
- G. Relative viability of senescent and proliferating DA3/EPOR was assessed by comparing the total number of viable cells 48 hr after treatment with increasing concentration of rapamycin (Rapa), to the initial number of cells seeded. The dashed red line indicated the initial number of cells seeded prior to treatment. Mean $\pm$ SEM, n = 3; one-way ANOVA. Statistical significance is shown by **P<0.01, ***P<0.001.

3.3.8 Lysosomal Activity Protects Senescent Leukemia Cells

KEGG pathway analysis of the RNA-seq data indicated that the lysosome pathway was one of the highest enriched pathways during senescence and that the proteasomal pathway was downregulated (**Figure 3-3F**). Senescent DA3/EPOR cells display an increased level of SA- β -gal activity, an indicator of lysosomal mass (**Figure 3-1B**; Kurz et al., 2000). In addition, senescent cells stained more intensely with the LysoTracker probe than proliferating cells (**Figure 3-5A**). The proteasome and lysosome are integral for protein turnover and cellular homeostasis and we asked whether the lysosomal pathway might be enriched to compensate for loss of the proteasome pathway.

To test whether enhanced lysosomal activity promoted survival in senescent DA3/EPOR cells, cells were treated for 24 hr with NH₄Cl, a lysosome inhibitor. Senescent DA3/EPOR cells demonstrated increased sensitivity to NH₄Cl compared with proliferating control cells (**Figure 3-5B**). Non-senescent cells exhibited proliferation, albeit slowed, but the senescent population

experienced a decrease in cell number from the initial number of viable cells seeded. Next, we treated DA3/EPOR cells with chloroquine (CQ), another known lysosome inhibitor, for 72 hr and observed striking senolytic activity (**Figure 3-5C**). We confirmed that reduced number of proliferating cells treated with CQ was due to a decrease in the rate of proliferation, and that CQ has specific lytic activity in senescent DA3/EPOR cells (**Figure 3-5D**).

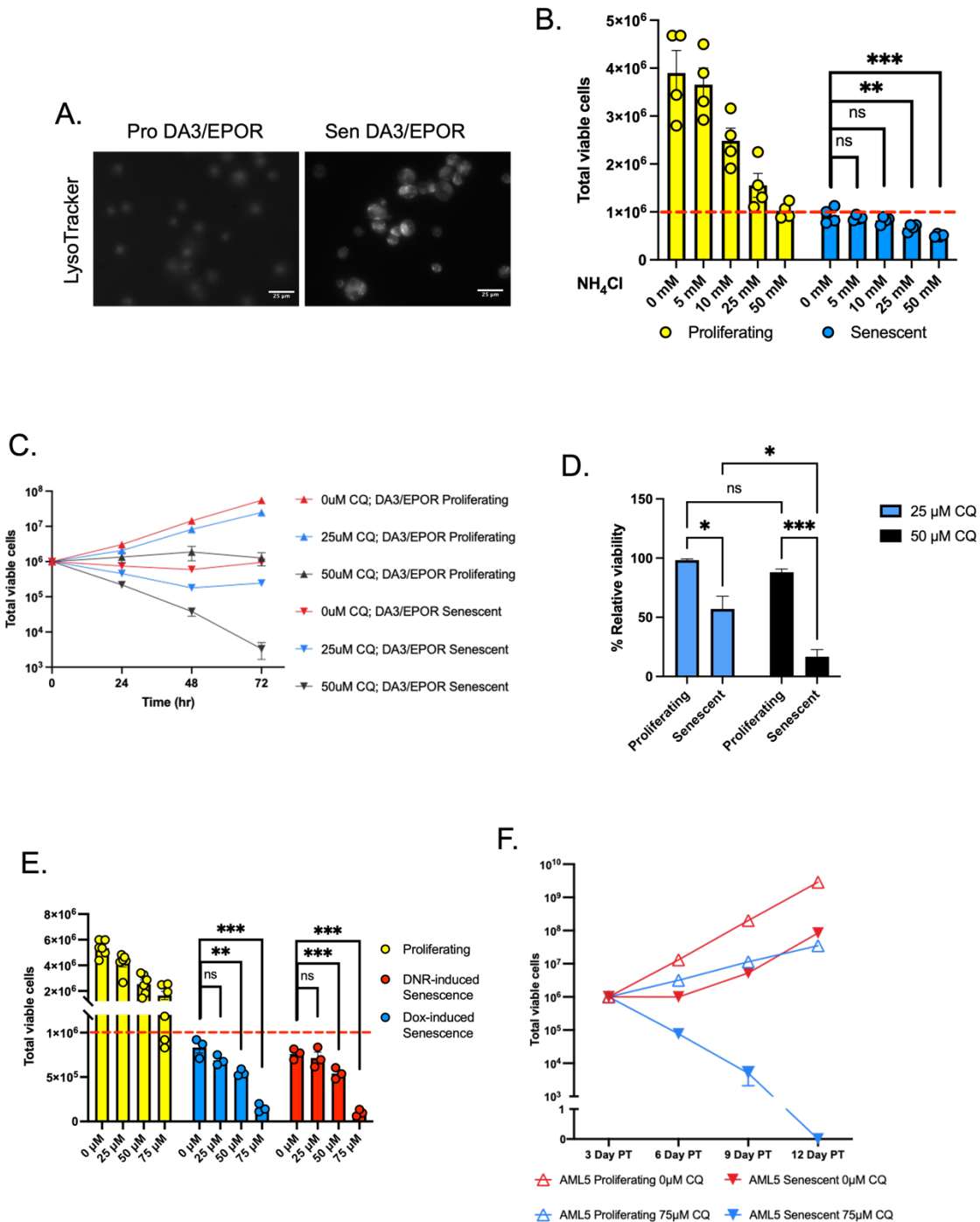


Figure 3-5 Upregulation of lysosomal pathway promotes survival of drug-induced senescent cells.

A. Representative immunofluorescence images of proliferating and senescent DA3/EPOR cells stained for 30 minutes with LysoTracker Red DND-99.

B. Viable proliferating and senescent (3 days post-dox treatment) DA3/EPOR cells were treated with increasing concentrations of NH_4Cl or vehicle control for 24 hr. The dashed

red line indicates the initial number of cells seeded prior to treatment. Cell numbers that fall below the dashed red line reflect cytotoxicity. Mean $\pm$ SEM, n = 4 independent experiments; one-way ANOVA.

C. Senescent and proliferating DA3/EPOR cells were treated for 72 hr as indicated and viable cells counted every 24 hrs. Senescent cells were obtained 3 days after Dox treatment. Mean $\pm$ SEM, n = 3 independent experiments.

D. Relative viability of senescent and proliferating DA3/EPOR cells treated with 25 μ M and 50 μ M CQ compared to control treated (0 μ M CQ). Mean $\pm$ SEM, n = 3 independent experiments; two-way ANOVA.

E. Senescent and proliferating OCI-AML5 cells were treated with increasing concentrations of chloroquine (CQ) or vehicle control for 48 hr. The dashed red line indicates the initial number of cells seeded prior to treatment. Cell numbers that fall below the dashed red line reflect cytotoxicity. Mean $\pm$ SEM, n = 6 independent experiments for proliferating cells, n = 3 for senescent cells obtained after Dox treatment, n = 3 for senescent cells obtained after DNR treatment; one-way ANOVA.

F. Proliferating and senescent (3 days post-DNR treatment) OCI-AML5 cells were treated with 75 μ M CQ and viable cells were counted every 3 days. Mean $\pm$ SEM, n = 3 independent experiments.

Statistical significance is shown by ns, not significantly different, *P<0.05, **P<0.01, ***P<0.001.

To examine whether CQ has similar senolytic activity on human AML cells, we treated OCI-AML5 cells with Dox or DNR to induce senescence and treated the cells with increasing concentrations of CQ. CQ exhibited senolytic activity on the senescent population (**Figure 3-5E**). OCI-M2 cells are highly sensitive to CQ and as a result, we were unable to evaluate senolytic activity in these cells (**Supplementary Figure 3-7**). We observed that OCI-AML5 cells induced to enter senescence with DNR (0.1 μ M, 24 hr) are able to resume proliferation 6-9 days after drug treatment. We targeted the senescent leukemic cells with 75 μ M CQ and effectively eliminated this population of cells. Proliferating OCI-AML5 cells treated with CQ had a reduced rate of proliferation and remained viable (**Figure 3-5F**).

3.3.9 Senescence gene signature is associated with poor survival in AML and multiple other cancers

Given the potential role of senescent cells in facilitating AML relapse (Duy et al., 2021; Saleh et al., 2019), we investigated whether the senescence signature that we found in DA3/EPOR (top 100 genes upregulated during Dox-induced senescence recognized by Gene expression profiling interactive analysis (GEPIA2) (Tang et al., 2019) could be associated with clinical outcome in AML patients. We utilized the Cancer Genome Atlas (TCGA) AML cohort and segregated the patients in two groups defined as residing within the top and bottom quartiles of the senescence signature. Kaplan-Meier analysis showed that patients with a high signature score had significantly worse overall survival than patients with a low signature score (**Figure 3-6A**). Furthermore, we analyzed the same cohort of AML patients for the senescence signature derived independently in five other studies (Fridman and Tainsky, 2008; Purcell et al., 2014; Hernandez-Segura et al., 2017; Casella et al., 2019; Duy et al., 2021). Notably, these senescence signatures were derived using human fibroblasts; except for Figure 3-6F, which was derived from drug-induced senescent AML cells. Patients were segregated into two groups, defined as having a high or low senescence signature. Kaplan-Meier analysis showed that AML patients with a high signature score had significantly worse overall survival than patients with a low signature score (**Figure 3-6B-F**).

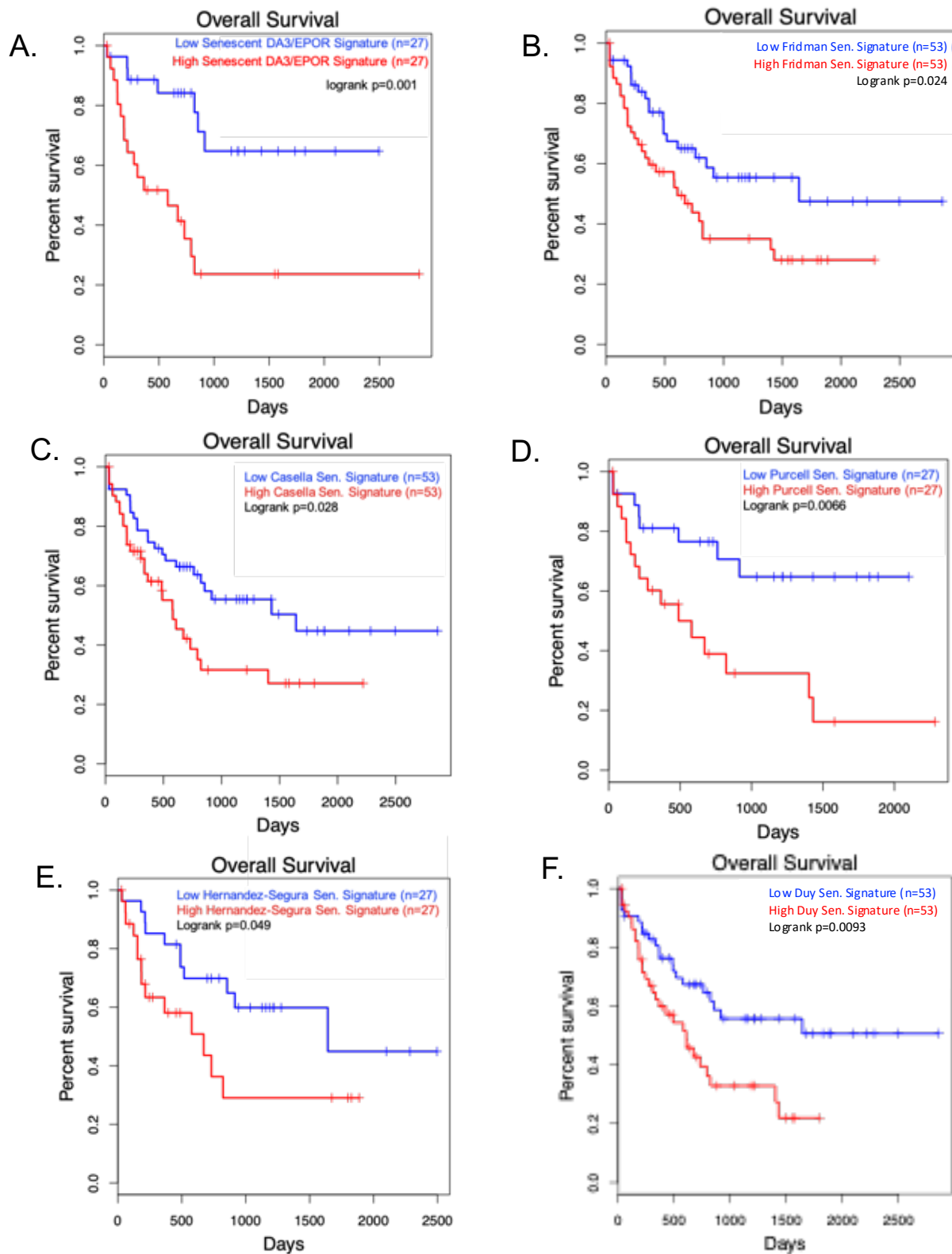


Figure 3-6 Senescence signature is associated with reduced AML patient survival.

A-E. Kaplan-Meier survival curves were generated on GEPIA2 using publicly available TCGA data.

- A. Overall survival of AML patients segregated based on top and bottom quartiles of high and low expression of the DA3/EPOR senescent signature. The DA3/EPOR senescent signature consisted of the top 100 upregulated genes (recognized by GEPIA2) during senescence in DA3/EPOR cells compared to proliferating controls (Figure 3D; Supplementary Table 1).
- B. Overall survival of AML patients segregated based on median high and low expression Fridman's senescent signature (Supplementary Table 1).
- C. Overall survival of AML patients segregated based on median high and low expression Casella's senescent signature (31). Antisense and LINC RNAs were excluded from the analysis (Supplementary Table 1).
- D. Overall survival of AML patients segregated based on top and bottom quartiles of high and low expression of the Purcell senescent signature. CCDC80, CLDN1, GMPR, ITGB3, NEGR1, ODZ2, and SCN3A were excluded from the analysis, as they were upregulated in control cells (Supplementary Table 1).
- E. Overall survival of AML patients segregated based on top and bottom quartiles of high and low expression of the Hernandez-Segura senescent signature (Supplementary Table 1).
- F. Overall survival of AML patients segregated based on median high and low expression Duy's senescent signature (Supplementary Table 1)..

We extended our analysis to various cancers of the TCGA and found that a high senescence signature (Fridman and Tainsky, 2008) corresponds to poor overall survival in some but not all cancers (**Supplementary Figure 3-8A-B**).

3.4 Discussion

In this study, we used an experimental cell model to show that chemotherapy induces reversible senescence in myeloid leukemia, allowing some cells to resist drug treatment and resume proliferation when drug is removed. How escaped cells differ from senescent and naïve cells is largely unknown. We show that the naïve parental DA3/EPOR population and naïve clones derived from single cells can enter drug-induced senescence. A small fraction of cells that enters drug-induced senescence exhibit asynchronous exit from senescence. Clonal expansion from single cells revealed extensive heterogeneity in the growth rate of escaped cells. Moreover,

escaped clones exhibit different sensitivities to a second round of drug treatment. We also find inter-clonal variability in the expression of a senescence-associated gene, SerpinB2.

Based on the single cell assays, autonomous escape from senescence appears to be a rare event occurring in an asynchronous manner several days post-treatment. In contrast, the pooled drug-recovered population of DA3/EPOR cells resumes proliferation within 4 days post treatment.

The drug-recovered population that resumes proliferation is likely to be composed of cells that never arrested, cells that went through a transient arrest and cells that escaped senescence.

However, we cannot overlook the possibility that pooled drug-treated population act in a paracrine like manner to facilitate the escape of senescent cells.

Three highly expressed genes in senescent cells include vWF, C3, and SerpinB2, components of the coagulation and complement hallmark gene sets. Complement effectors directly enhance coagulation and complements inhibit anti-coagulant factors. C3, a gene playing a central role in the complement cascade and a part of the coagulation gene set, was the only validated gene to remain stably upregulated in the drug-recovered population, demonstrating that the drug-recovered population retains some senescence-associated phenotypes and are distinct from naïve proliferating controls. Moreover, the interactions of complement with other inflammatory mediators can increase the thrombogenicity of blood (Markiewski et al., 2007). Using C3^{-/-} and C5^{-/-} mice models, Subramaniam and colleagues (2017) reported that complement proteins participate in venous thrombosis after ligation of the inferior vena cava (Subramaniam et al., 2017). The activation of SASP in senescent cells may also contribute to thrombosis (Wiley et al., 2019). Thrombosis occurs in up to 10% patients with newly diagnosed AML with most thrombotic events occurring before the start of the second course of chemotherapy (Libourel et

al., 2016). In addition, 28% of patients with multiple myeloma undergoing chemotherapy experience deep vein thrombosis (Zangari et al., 2001). The possible contribution of drug-induced senescent cells to thrombosis in patients undergoing chemotherapy is intriguing and warrants further investigation.

Consistent with our previous study, we find that EPO mediates survival of DA3/EPOR cells and allows cells to persist through drug treatment (Pham et al., 2019). Senescent DA3/EPOR cells enter dormancy with reduced translation and c-myc expression that resembles a diapause state. Transcriptional profiles of senescent DA3/EPOR cells show parallels with diapause epiblasts. A diapause-like state has been recently shown to promote a reversible state of drug persistence in colon cancer and AML (Duy et al., 2021; Rehman et al., 2021). The molecular mechanisms that enable cancer cells to persist through drug treatment are largely unknown. The escaped clones retained sensitivity to drug treatment and retained the ability to re-enter senescence, indicating that resistance to drug treatment was a transient response. We propose a non-genetic mechanism in which cells gain transient resistance. We show that DA3/EPOR cells enter a drug-induced senescent-like state that mimics diapause. Survival of cells post-therapy in the senescent, diapause-like state partially depends on continued EPO treatment. Traditionally, senescence has been viewed as a tumour suppressive barrier that halts the progression of tumour formation. We propose that tumour cells can exploit the senescent phenotype to persist the duration of chemotherapy. Persistent cancer cells can utilize multiple, non-mutually exclusive, evolutionarily conserved stress responses to survive therapy (Shen et al., 2020).

The mechanism underlying the deleterious effects observed in cancer patients receiving EPO-stimulating agents to treat anemia is not well understood. EPO, acting through the canonical

EPO-R or through ephrin-type B receptor 4 (EphB4), an alternative EPO-R, may account for the increased tumour growth and progression seen in cancer patients treated with ESAs (Pradeep et al., 2015). EPO-R and EphB4 are expressed in a wide range of tissues, hematopoietic and nonhematopoietic cancers. A remarkable degree of heterogeneity in EPOR expression is found in AML (**Supplementary Figure 3-9**). Our findings suggest that ESAs may contribute to tumour progression and decreased patient survival by promoting and sustaining cellular senescence in response to chemotherapy.

AML cells emerging from therapy-induced senescence may contribute to patient relapse, seen in 40-50% of AML patients (Thol and Ganser, 2020). Clinical studies report that chemotherapy promotes senescence in AML (Duy et al., 2021) and in other cancers (Fitsiou et al., 2021). Heterogeneity within the drug-recovered population may contribute to increased aggression in relapsed patient (Marusyk et al., 2020). Although senescent cells are resistant to Dox-induced apoptosis, senolytic drugs can be used to selectively eliminate senescent cells (Zhu et al., 2015). Here we show that lysosome activity is upregulated during therapy-induced senescence and this promotes the survival of DA3/EPOR cells; lysosome inhibition and cytotoxic drug treatment display synthetic lethality in certain mouse and human leukemic cell lines. CQ, an FDA approved anti-malaria drug, can be used to inhibit lysosome function and may act as a senolytic for therapy-induced senescence cells in AML patients undergoing chemotherapy. Eliminating therapy-induced senescent cells may decrease the detrimental effects of SASP and the frequency of AML relapse.

3.5 Material and methods

3.5.1 Cell Culture

DA3/EPOR cells and derivative clones (5, 17) were maintained at 37°C with 5% CO₂ in RPMI 1640 media supplemented with 10% Fetal Bovine Serum (FBS) and 1 U/ml recombinant human erythropoietin (EPO). OCI-AML4, OCI-AML5, and OCI-M2 cells were obtained from Dr. Mark Minden (University Health Network, Toronto); these cells were maintained at 37°C with 5% CO₂ in α -MEM with 10% FBS.

3.5.2 Drug Treatment

DA3/EPOR cells were washed with phosphate buffer solution (PBS) followed by treatment with doxorubicin (Dox) (200 ng/ml, or at the specified concentrations), in the absence or presence EPO (1 U/ml). Human AML cell lines, OCI-M2 and OCI-AML5, were treated with 200 ng/ml Dox or 0.1 μ M or 0.25 μ M Daunorubicin (DNR), respectively, for 24 hr. Chloroquine (CQ) was freshly prepared in sterile ddH₂O prior to treating cells; Ammonium Chloride (NH₄Cl) was solubilized in sterile ddH₂O; sterile ddH₂O served as a vehicle control for CQ and NH₄Cl. Cells were treated with CQ and NH₄Cl at the indicated concentrations for the desired times.

Rapamycin (Rapa) was prepared in DMSO and used to treat cells at the specified concentrations and time, as indicated on the figure; DMSO served as a vehicle control.

3.5.3 Cell Proliferation and Viability

Total cells were counted on dates indicated on individual experiments. Cells were pelleted by centrifugation at 1000 rpm and resuspended in PBS. Cells were mixed with 1:1 mixture of PBS pH 7.4 and 0.4% trypan blue stain (Thermo). All viable cells were counted by hemocytometer;

all 4 quadrants of the hemocytometer were counted and averaged. The number of live cells per ml was determined as: (average live cell number) $\times 2 \times 10^5$.

3.5.4 Single cell cloning

DA3/EPOR cells were treated with Dox or vehicle control (ddH₂O) for 24 hr and allowed to recover for 24 hr in drug-free media. A serial dilution was performed and single cells were seeded into wells of a 96-well plate. All wells were assessed for colony formation every 3 days. Colonies were defined as ≥ 5 cells in a well. Cells in wells containing colonies were manually counted directly under the microscope or on imageJ.

3.5.5 SA- β -gal staining

Briefly, cells were harvested and washed with PBS pH 7.4 followed by fixation in 3% formaldehyde for 20 min. Cells were washed with PBS, suspended and incubated overnight at 37°C in freshly made b-gal staining solution consisting of: 1 mg 5-bromo-4-chloro-3-indolyl P3-D-galactoside, 150 mM NaCl, 40 mM citric acid pH 6.0, 40 mM sodium phosphate pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl₂.

3.5.6 Cell cycle analysis by Flow cytometry

Cells were pelleted by centrifugation and washed with PBS and fixed with cold 75% ethanol before storing at -20°C until use. Fixed cells were pelleted and washed with PBS and suspended in Staining Buffer (0.2% Triton X-100, 1 mM ethylenediaminetetraacetic acid [EDTA], in PBS) followed by incubation with RNaseA (100 μ g/ml, ThermoScientific) at 37°C for 30 min.

Cells were stained with 50 µg/ml propidium iodide (PI) for 1 hr. PI fluorescence was measured using a BD FACSCalibur flow cytometer (Becton Dickinson). PI fluorescence, cell size and granularity, and data analysis was performed using the BD CellQuest Pro software. Cell size and granularity was measured based on forward scatter and side scatter.

3.5.7 LysoTracker

LysoTracker Red DND-99 was used according to the manufacturer's instructions. Briefly, 1x LysoTracker Red DND-99 was incubated with live cells for 30 min at 37°C with 5% CO₂. Cells were pelleted, washed with PBS and incubated with DAPI for 10 min. Cells were resuspended in ProLong Gold anti-fade medium and placed on cover slides and allowed to cure overnight.

3.5.8 Cell size

Cells were mixed at a 1:1 ratio with 0.4% trypan blue and pl 46 aced on a hemocytometer. Multiple random fields of views were imaged, and pixels were manually calculated on ImageJ. Pixel lengths were converted to µm by calculating the amount of pixels of a known reference length. Trypan blue positive (dead) cells and cells with irregular (non-spherical) shapes were excluded from the analysis.

3.5.9 Quantitative real-time PCR (qRT-PCR)

Cells were pelleted and stored at -80°C until use. Total RNA was isolated by TRIzol (Life Technologies) according to the manufacture's instructions. RNA was solubilized in RNA cleaning buffer consisting of: 75% ethanol, 75 mM NaOAc, 50 ug/ml glycogen, and stored at -

80°C for 1 hr. RNA was pelleted, washed with 75% ethanol and resuspended in nuclease free H₂O. RNA concentration and purity was measured using a NanoDrop One spectrophotometer (Thermo Scientific). cDNA synthesis was performed with 2 µg of total RNA using the High Capacity RNA-to-cDNA Kit (ThermoFisher Scientific) and subjected to PCR using the SsoFaster EvaGreen Supermix Kit (BioRad) on a DNA Engine Opticon 2 Real-Time PCR Detector (BioRad). Samples were run in technical triplicate. Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method; C_t values were normalized to mouse Rps18 or human GAPDH. Primer efficiencies were determined for all previously unpublished primers prior to use in qRT-PCR. Statistical analysis was performed on the $\Delta\Delta C_t$ values.

The following oligonucleotides were used as qRT-PCR primers:

Mouse:

C3 F: 5'AAG CAT CAA CAC ACC CAA CA

C3 R: 5'CTT GAG CTC CAT TCG TGA CA

ICAM1 F: 5'GGTCCTTGCCTACTTGCTG

ICAM1 R: 5'CTGTGCTTTGAGAACTGTGG

ITGB2 F: 5'GTCCTTCCGACAGTTTCTCTC

ITGB2 R: 5'GGAGTCATGGAGTGTGGTATC

P16 F: 5'AAT CTC CGC GAG GAA AGC

P16 R: 5'GTC TGC AGC GGA CTC CAT

Rps18 F: 5'GTG TTG AGG AAA GCA GAC AT

Rps18 R: 5'CAG TCT GGG ATC TTG TAC TG

SerpinB2 F: 5'CAC CAC AGG GGG ATT TG

SerpinB2 R: 5'ACC CTT CGG GTA GCA GGT TT

vWF F: 5'CAG CAT CTC TGT GGT CCT GA

vWF R: 5'GAT GTT GTT GTG GCA AGT GG

Human:

C3 F: 5'AGTCTCCTGCTTTAGTGATGC

C3 R: 5'GCCTTTGTTCTCATCTCGCT

GAPDH F: 5' GGA GCG AGA TCC CTC CAA AAT

GAPDH R: 5' GGC TGT TGT CAT ACT TCT CAT GG

SerpinB2 F: 5'- GTT CAT GCA GAT CCA GA

SerpinB2 R: 5'- CGC AGA CTT CTC ACC AAA CA

vWF F: 5'ACTCACACAAAGTCTTCTCACA

vWF R: 5'GCTCATGCAACATCTCCTCTG

3.5.10 RNA-seq analysis

DA3/EPOR cells were harvested and frozen at -80°C, 3- or 9-days after Dox treatment (24 hr); untreated control cells were harvested on the 3rd day along with the treated samples. Total RNA was isolated using Monarch Total RNA Miniprep Kit (New England BioLabs) as described by the manufacturer. RNA was isolated for all samples on the same day. RNA-seq was performed in biological triplicate by Genome Quebec, using the Illumina NovaSeq 6000 at a depth of 25 million pair-ended reads that were 100 bp in length. Genome Quebec performed quality control and ensured that all samples had a >6.8 RIN value prior to performing a poly-A tail selection and converting the RNA into cDNA. RNA-seq alignment was performed on an Amazon EC2

instance Linux terminal. Although the Quality Control (QC) did not detect any Illumina adapter sequences, the first 13 bp were trimmed using Flexbar. HiSAT2 was then used to align reads to the MM10 indexed genome. HTSEQ was used to generate read counts, which were then used for differential expression. Differential expression was performed on Rstudio using EdgeR. Gene Set Enrichment Analysis (GSEA) was used to investigate differentially regulated pathways and gene sets. GSEA used EdgeR normalized read counts. Rstudio was used to generate heatmaps and perform principal component analysis (PCA). RNA expression data was generated using GEPIA2 using publicly available data from TCGA and GTEx. RNA-seq data can be accessed at: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE186200>

3.5.11 Western Blotting and Antibodies

Cells were pelleted and washed with PBS. Cells were then lysed in 1 x SDS (sodium dodecyl sulfate) lysis buffer (1% SDS, 10% glycerol, 88 mM Tris- HCl pH 6.8, in water) and boiled at 97°C for 10 min. A Pierce BCA Protein assay (Cat#23225) was used to measure protein concentration of lysates according to the manufacturer's instructions. Once protein concentrations were measured, 0.1% bromophenol blue and 0.1M dithiothreitol (DTT) was added to the lysed sample. 50 µg of total protein lysate per sample was separated by SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked for 1 hr at room temperature (RT) with PBST blocking buffer (5% milk in PBS and 0.1% Tween 20), followed by an overnight incubation with primary antibodies diluted in blocking buffer at 4°C. The following primary antibodies were used: FL393 (sc-6243, Santa Cruz Biotechnology), RPL9 (Abcam, ab182556), RPS6 (Abcam, ab40820) and β -actin (sc-47778). Membranes were washed with PBST and incubated for 1 hr at RT in HRP secondary

antibody diluted in blocking buffer. Membranes were washed with PBST and incubated with Pierce ECL Western blotting substrate for 1 min. Western blots were imaged on a MicroChem System (DNR Bio Imaging System) and analyzed on a GelCaptureChemi program or using film.

3.5.12 Polysome Analysis

Polysome profiling was performed by fractionation as described (<https://bio-protocol.org/e833>). Briefly, DA3/EPOR cells were incubated with 100 µg/mL cycloheximide for 5 minutes prior to lysis. Cells were washed twice in PBS containing 100 µg/mL cycloheximide and lysed in hypotonic lysis buffer (5 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 1.5 mM KCl, 1X Halt™ & Protease Inhibitor Cocktail (ThermoFisher Scientific, c 138 atalogue number 78425), 100 µg/mL cycloheximide, 2 mM DTT, 200 Units/mL SUPERase In™ RNase Inhibitor (ThermoFisher Scientific, catalogue number AM2696), 0.5% (v/v) Triton X-100 and 0.5% (w/v) sodium deoxycholate). Cell lysates were prepared by centrifugation for 5 minutes at 20,000g at 4°C and 20 A260nm was loaded onto a 7-step sucrose gradient prepared in sucrose buffer (20 mM HEPES (pH 7.6), 100 mM KCl, 5 mM MgCl₂ and 100 µg/mL cycloheximide) followed by centrifugation at 30 000 rpm for 3 hr in a Beckman SW41 rotor at 4°C (acceleration: max., deceleration: no brake). The absorbance was measured at 254 nm in a continuous flow and fractions were collected. Protein samples were precipitated in 10% trichloroacetic acid overnight at -20°C. The protein pellet was collected by centrifugation for 10 minutes at 10 000 g at 4°C, followed by two acetone washes and resuspension in 2X Laemmli buffer. Protein samples were separated by SDS-PAGE and immunoblotted.

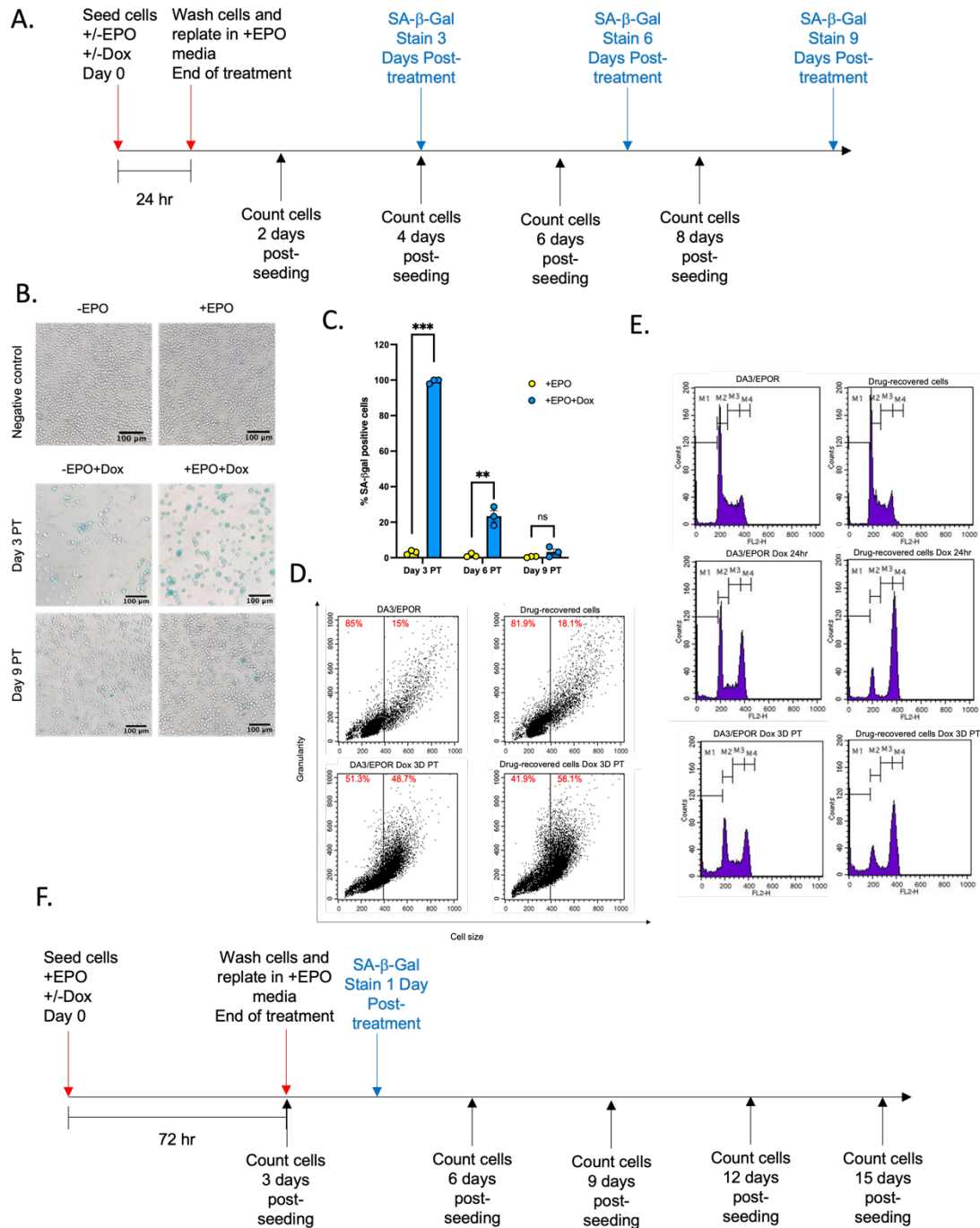
3.5.13 Survival analysis

Kaplan-Meier survival curves were generated using TCGA expression, survival data and Gene Expression Profiling Interactive Analysis 2 (GEPIA2). Kaplan-Meier survival curves were plotted for groups of patients based on quartile or median expression.

3.5.14 Statistical analysis

Graphpad Prism 9 was used to determine statistical significance. Two-way Student's *t*-test (to compare means of two samples different samples), by one-way ANOVA (to compare means of more than two different samples) or by two-way ANOVA (to compare the means of samples influenced by two independent variables) was performed to determine P values. One-way ANOVA was followed by either Dunnett's method (to compare multiple means to a single control mean), or Tukey's method (to compare all means to each other). Data graphed on GEPIA2 was statistically analyzed using default GEPIA2 settings and tests.

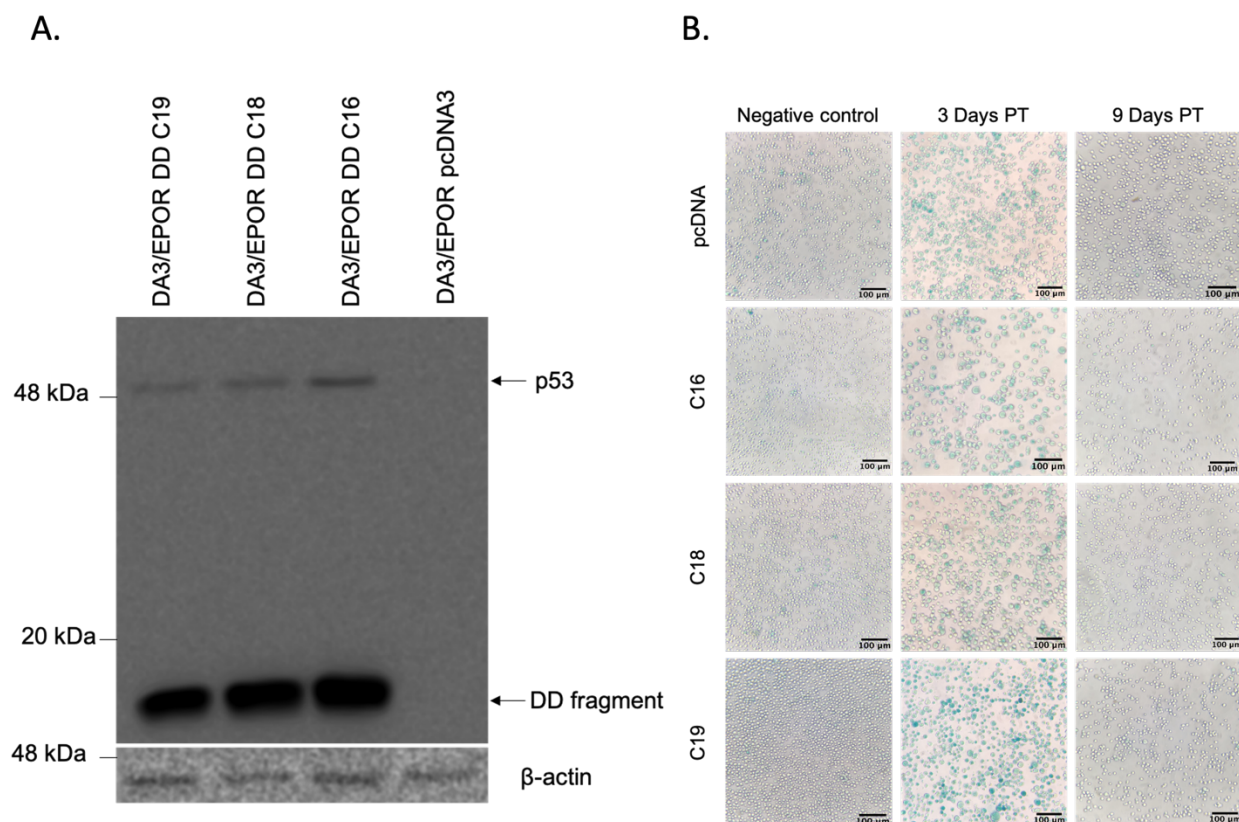
3.5 Supplementary material



Supplementary Figure 3-1

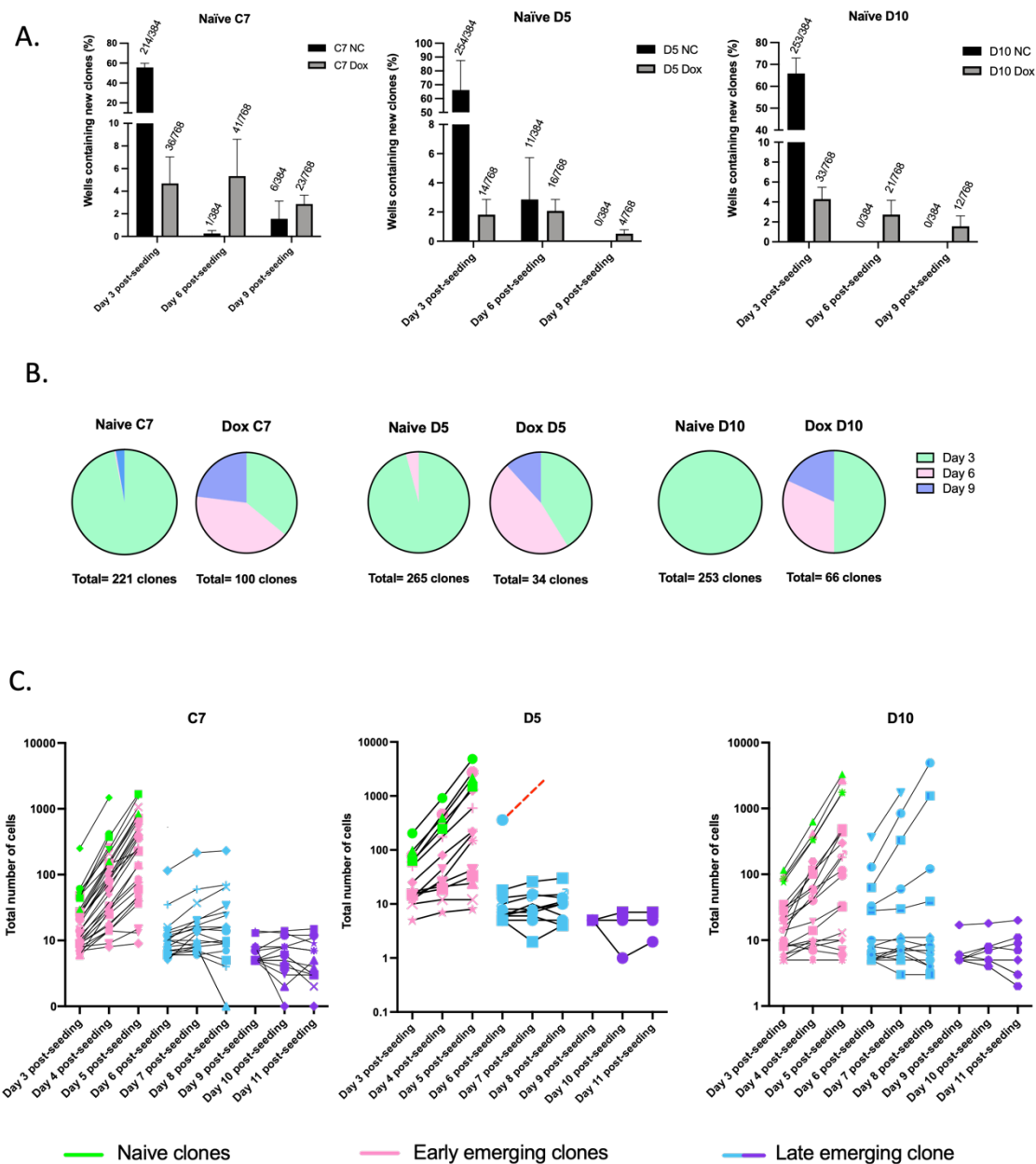
- A. Schematic of DA3/EPOR cells being treated with Dox and EPO for 24 hr. Related to Figure 1A, C.
- B. Representative images of SA- β -gal stain of DA3/EPOR cells 3 and 9 days post-treatment. DA3/EPOR cells treated with or without EPO in the presence of absence of Dox for 24 hours. Related to Figure 1A. Scale bars are shown in the bottom right of each panel.

- C. Quantification of SA- β gal stain of DA3/EPOR cell 3, 6 and 9 days post-24 hour Dox treatment (senescent), and negative control (proliferating) cells maintained in media containing EPO. Error bars represent SEM, n=3 biological replicates; a minimum of 100 cells were counted for each biological replicate.
- D-E. FACS analysis of DA3/EPOR cells and drug-recovered DA3/EPOR cells treated with Dox for 24 hr in complete media. Cells were harvested immediately after 24 hr treatment or allowed to recover for 3 days post-treatment in complete media. n=2 independent experiments.
- D. FACS analysis demonstrating granularity and cell size.
- E. Cells were stained with propidium iodide to measure DNA content and cell cycle profiles.
- F. Schematic of DA3/EPOR cells being treated with Dox and EPO for 72 hr. Related to Figure 1D-E.



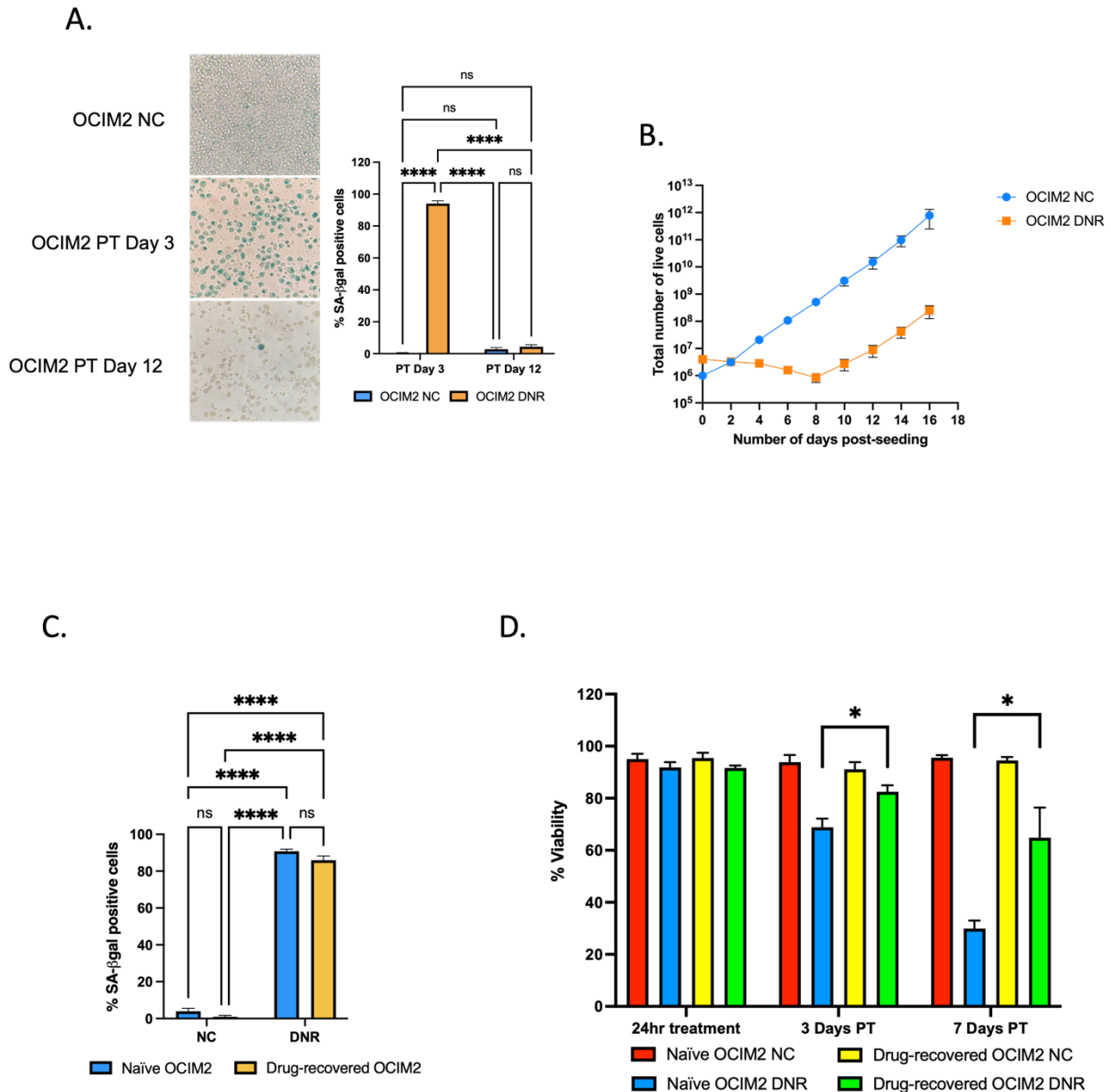
Supplementary Figure 3-2

- A. Western blot of DA3/EPOR DD expressing clones. FL393 p53 polyclonal antibody shows stabilization of full length wt-p53 and high expression of DD fragments in C16, C18 and C19 clones. β-actin was used as a loading control. n= 2 independent experiments.
- B. Representative images of SA-β-gal stain of DA3/EPOR p53 dominant-negative expressing clones C16, C18 and C19, and pcDNA empty vector control, 0, 3 and 9 days post-24 hr Dox treatment. Related to Figure 1F. Scale bars are shown in the bottom right of each panel.



Supplementary Figure 3-3

- Percentage of wells containing new colonies of naïve clones. Mean $\pm$ SEM, $n = 3$ independent experiment.
- Pie chart showing proportions of when new expanding clones were detected (related to supplementary figure 3A).
- Rates of proliferation of randomly selected expanding colonies. Related to supplementary figure 3-3A. $n=3$ independent experiments.

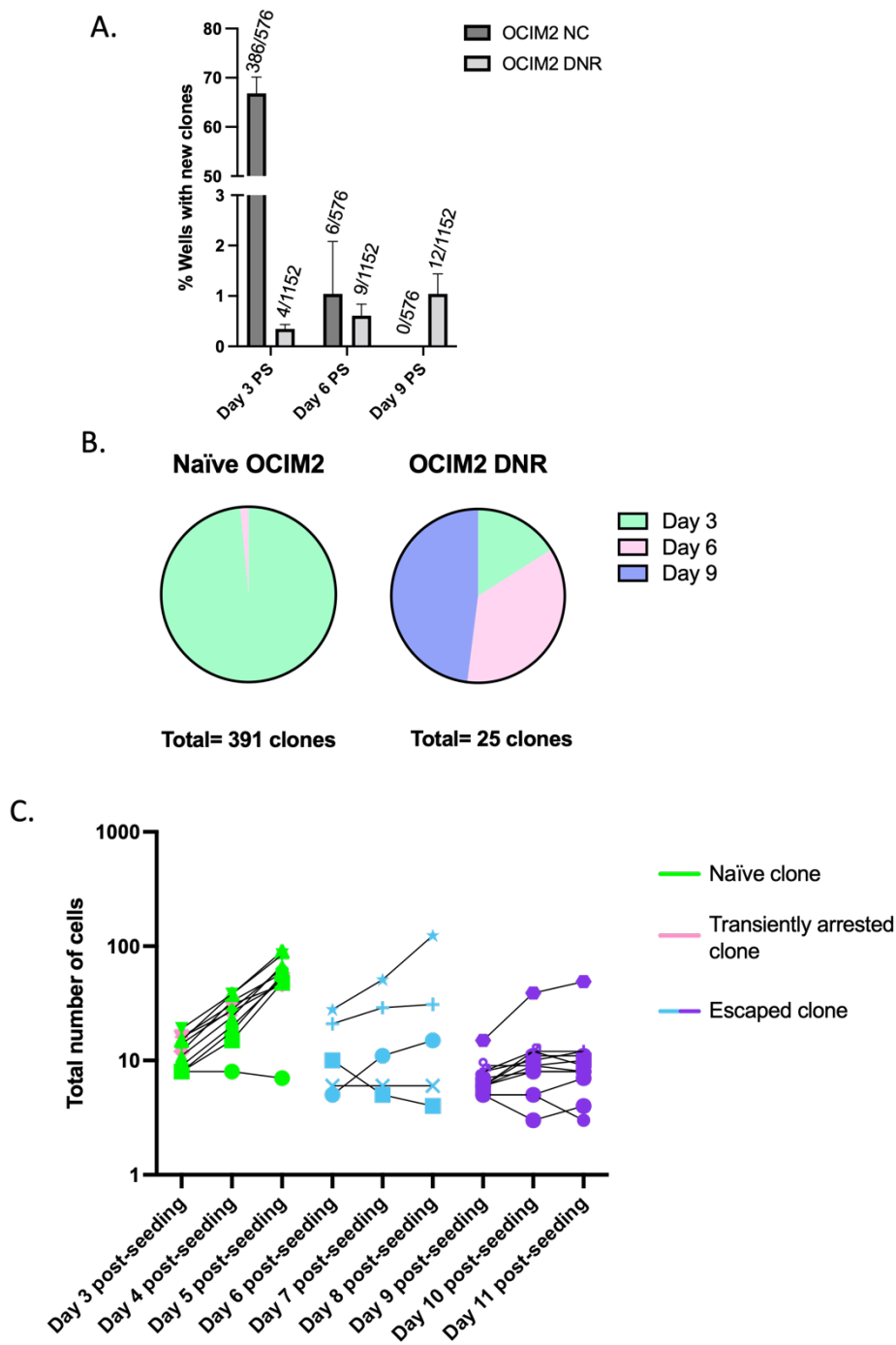


Supplementary Figure 3-4

- Representative images and quantification of SA-β-gal stain of OCI-M2 cell 3, 9 and 12 days post-24 hour 0.25μM DNR treatment and negative control (naïve) cells. Mean ± SEM, n = 3 independent experiments; two-way ANOVA. A minimum of 100 cells were counted for each independent experiment.
- Proliferation curves of OCI-M2 cells treated with 0.25μM DNR for 24 hr or vehicle control (ddH₂O). After 24 hr treatment, cells were maintained in drug-free media. Cells were counted every 2 days. Mean ± SEM, n = 3 independent experiments.
- Quantification of SA-β-gal stain of naïve and drug-recovered OCI-M2 cell 3 days post-24 hr DNR treatment. A minimum of 100 cells were counted for each sample. Mean ±

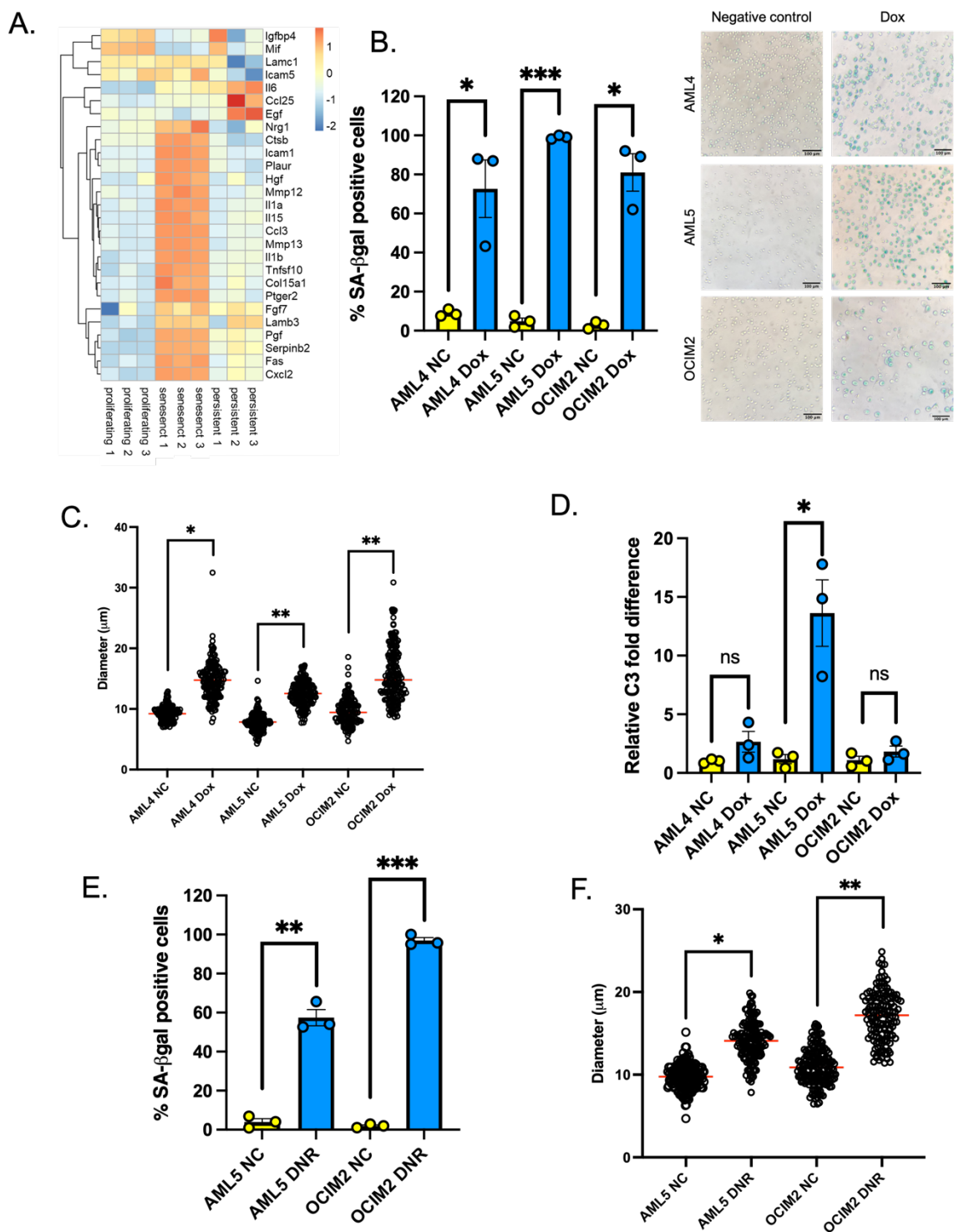
SEM, n= 4 independent experiments; two-way ANOVA. A minimum of 100 cells were counted for each independent experiment.

- D. Naïve and drug-recovered OCI-M2 cells were treated with 0.25 μ M DNR for 24 hr or vehicle control (NC). Viability was measured after 24 hr treatment, 3 days post-treatment and 7 days post-treatment. Mean $\pm$ SEM, n = 3 independent experiments; two-way ANOVA. A minimum of 100 cells were counted for each independent experiment. Statistical significance is shown by ns, not significant, *P<0.05, ****P<0.0001



Supplementary Figure 3-5

- Percentage of wells containing new colonies of naïve clones. Mean $\pm$ SEM , n=3 independent replicates for each naïve clone.
- Pie chart showing proportions of when new expanding clones were detected (related to supplementary figure 3-5A).
- Rates of proliferation of randomly selected expanding colonies. Related to supplementary figure 3-5A.

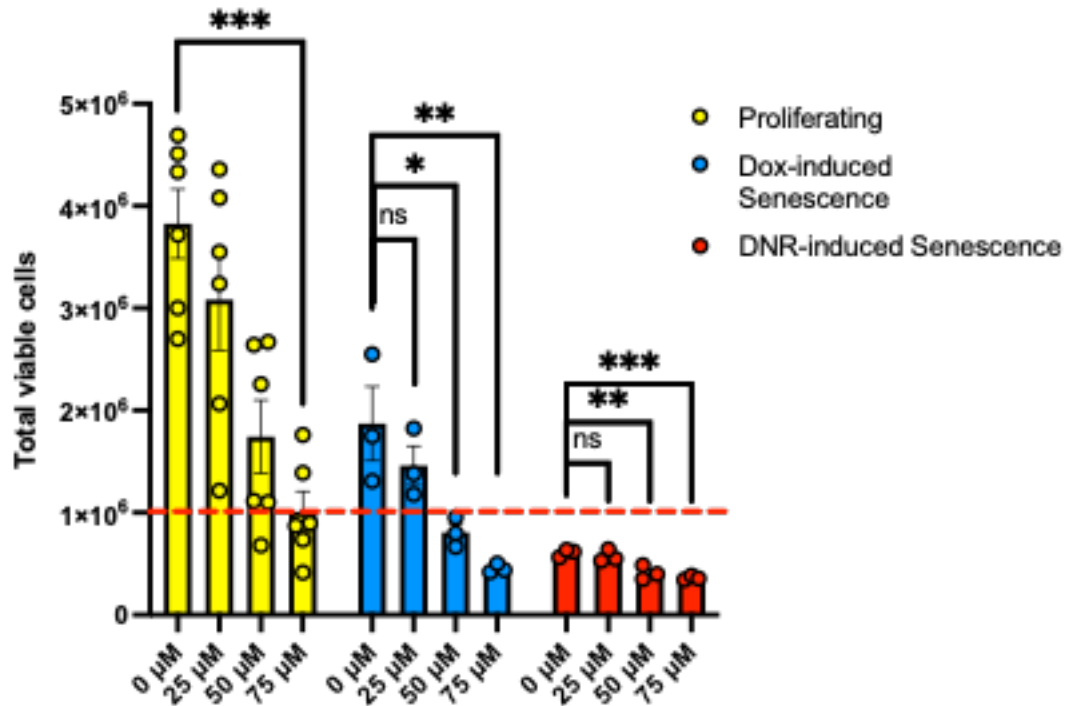


Supplementary Figure 3-6

A. Heat map displaying RNA-seq regulation of Coppé's senescence-associated secretory gene set (Coppé et al., 2010) in proliferating, senescent and drug-recovered DA3/EPOR cells.

- B. Representative images and quantification of SA- β gal stain of human OCI-AML4, OCI-AML5 and OCI-M2 cells 72 hours post-24 hour Dox treatment (senescent), and negative control (proliferating) cells. Student t-test was performed to determine statistical significance (n= 3 biological replicates). Scale bar shown in bottom right.
- C. Diameter of NC and Dox treated OCI-AML4, OCI-AML5 and OCI-M2 cells measured from 3 independent experiments (OCI-AML4 Proliferating n= 162, OCI-AML4 Senescent n=167; OCI-AML5 Proliferating n=160, OCI-AML5 Senescent n=161; OCI-M2 Proliferating n=156, OCI-M2 Senescent n=179). A minimum of 50 cells were measured for each independent experiment. The red line indicates the median diameter. Student t-test was performed using the means of each independent replicate; n= 3 biological replicates.
- D. Relative mRNA expression of C3 by qRT-PCR of Proliferating and Dox-treated OCI-AML4 and OCI-M2 cells. Mean $\pm$ SEM, n = 3 independent experiments; Two-way student T-test.
- E. Quantification of SA- β gal stain of human OCI-AML5 and OCI-M2 cells 72 hours post-24 hour DNR treatment (senescent), and negative control (proliferating) cells. Mean $\pm$ SEM, n = 3 independent experiments; Two-way student T-test. A minimum of 100 cells were counted for each independent experiment.
- F. Diameter of NC and DNR treated OCI-AML5 and OCI-M2 cells measured from 3 biological experiments (OCI-AML5 Proliferating n= 167, OCI-AML5 Senescent n=158; OCI-M2 Proliferating n= 167, OCI-M2 Senescent n= 147). A minimum of 100 cells were measured for each independent experiment. The red line indicates the median diameter. Student t-test was performed using the means of each independent replicate; n= 3 biological replicates.

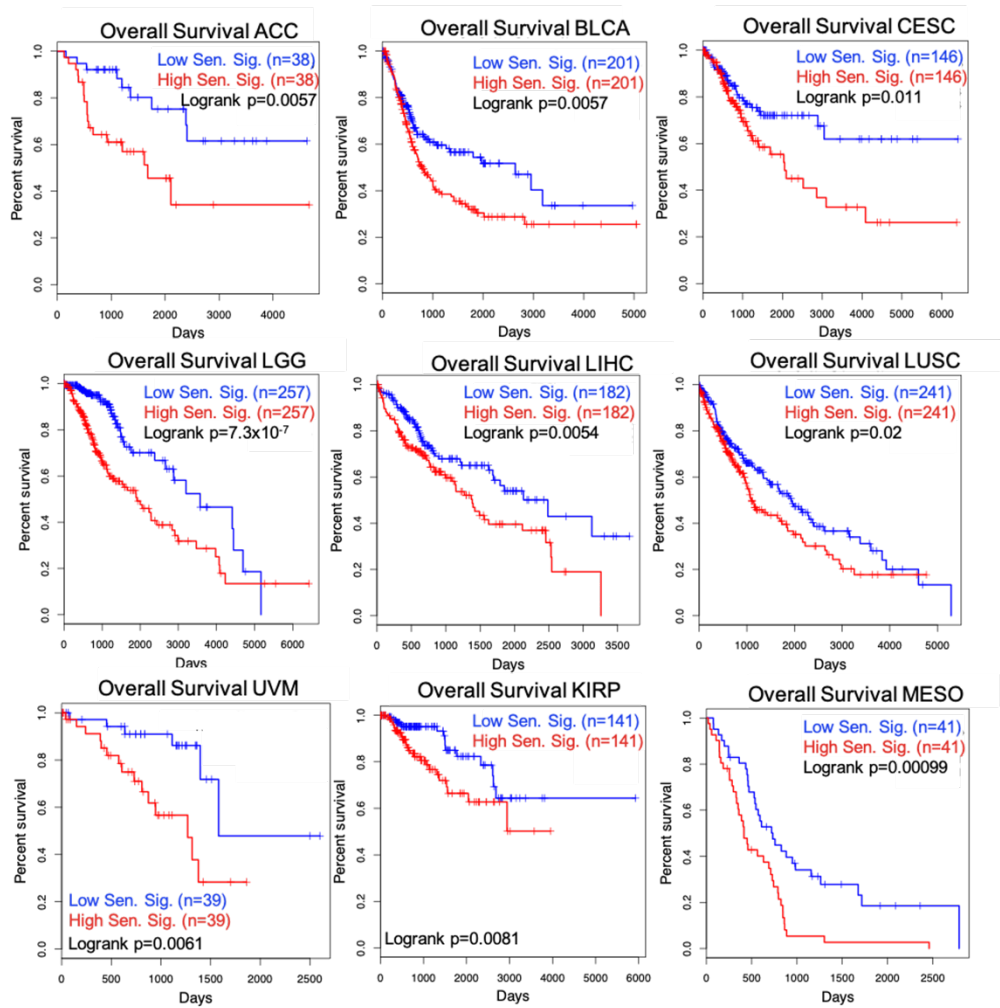
Statistical significance is shown by ns, not significant, *P<0.05, **P<0.01, ***P<0.001.



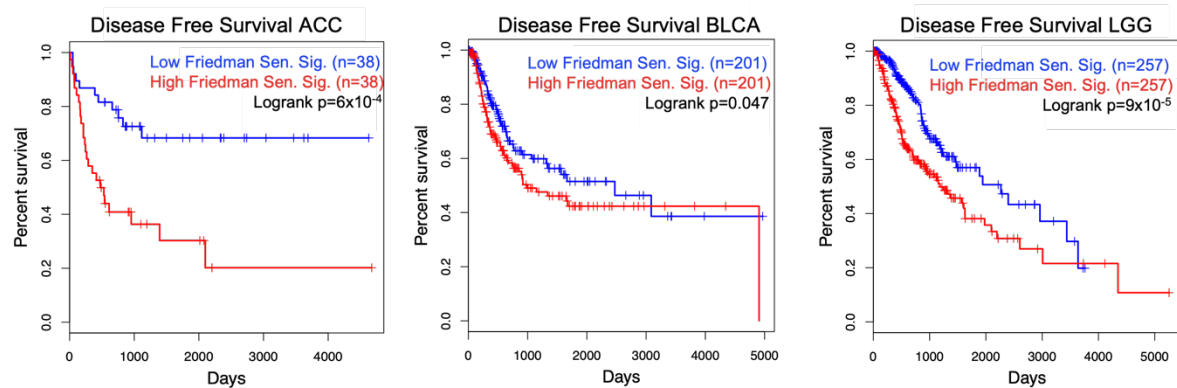
Supplementary Figure 3-7

Viable proliferating and senescent (3 days post-Dox or DNR treatment) OCI-M2 cells treated with increasing concentrations of chloroquine (CQ) or vehicle control for 48hr. The dashed red line indicated the initial number of cells seeded prior to treatment. Mean ± SEM, n = 6 independent experiments for proliferating cells, n = 3 for senescent cells obtained after Dox treatment, n = 3 for senescent cells obtained after DNR treatment; one-way ANOVA.

A.



B.

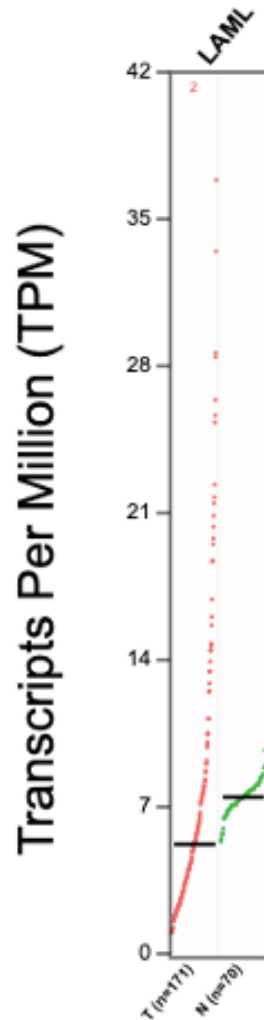


Supplementary Figure 3-8

A. Overall survival of Adrenocortical carcinoma (ACC), Bladder Urothelial Carcinoma (BLCA), Brain Lower Grade Glioma (LGG), Cervical squamous cell carcinoma and

endocervical adenocarcinoma (CESC), Liver hepatocellular carcinoma (LIHC), Lung squamous cell carcinoma (LUSC), Kidney renal papillary cell carcinoma (KIRP), Mesothelioma (MESO), and Uveal Melanoma (UVM) patients segregated based on the median high and low expression Fridman's senescent signature (Supplementary Table 1).

- B. Disease-free survival of ACC, BLCA, and LGG patients segregated based on the median high and low expression Fridman's senescent signature (Supplementary Table 1).



Supplementary Figure 3-9

RNA expression of EPOR in AML derived tumour (T) samples (N=171) compared to normal (N) bone marrow samples (N=70). GEPIA2 was used to generate expression data using tumour data from TCGA and normal tissue data from the Genotype-Tissue Expression (GTEx) project.

Supplementary table 1- 1 List of senescence-associated gene signatures

Senescent DA3/EPOR	Fridman	Casella	Pucella	Hernandez-Segura	Duy
Acox2	ALDH1A3	ARRDC4	Apol1	ACADVL	A2M
Acox1	AOPEP	BLCAP	Apol3	ADPGK	ABCA10
Acsbg1	CCN2	BMS1P9	ATF3	B4GALT7	ABCA12
Adam19	CCND1	CCND3	BATF2	BCL2L2	ABCA6
Adamdec1	CD44	CHPF2	BIRC3	CCND1	ABCC6
Adams7	CDKN1A	CLDN1	C3	CHMP5	ACKR2
Adams11	CDKN1C	CLSTN2	C9orf47	DDA1	ACSM1
Ago4	CDKN2A	DHRS7	CD163L1	DGKA	ADAMTS7
Alox15	CDKN2B	DIO2	CD82	DYNLT3	ADCY4
Atp6v0d2	CDKN2D	ELMOD1	COL17A1	FAM214B	ADGRE1
Bst1	CITED2	FILIP	CTSS	GBE1	ADGRE4P
C5ar1	CLTB	GPR155	CXCL2	GNDF	AIM2
Ccbe1	COL1A2	H2AFJ	CYP1A1	KLC1	ALOX5
Cd200r2	CREG1	JCAD	DUSP6	MT-CYB	AMPD3
Cd33	CRYAB	KIAA1467	EPSTI1	NOL3	ANKRD65
Cgn	CXCL14	KIAA1671	GBP4	P4HA2	APOBEC3H
Cgn1	CYP1B1	LRP10	GCA	PDLIM4	APOC1
Chi3l1	EIF2S2	MT-TA	GNG11	PLK3	APOD
Chst13	ESM1	MT-TC	HERC5	PLXNA3	AQP1
Clec4n	F3	MT-TN	ICAM1	POFUT2	ARHGAP6
Col5a1	FILIP1L	MT-TY	IDO1	RAI14	ASB9P1
Cped1	FN1	NCSTN	IFI27	SCOC	ATP2B2
Crtac1	GSN	PAM	IFI30	SLC10A3	AXL
Csflr	GUK1	PDLIM1	IL1A	SLC16A3	BAIAP3
Cxcl3	HBS1L	PLOD1	IL1B	SUSD6	BEST1
Dchs1	HPS5	POFUT2	IL1RN	TAF13	BTG2
Ddah1	HSPA2	PTCHD4	IL32	TMEM87B	C15orf27
Ddr2	HTATIP2	RND3	ISG20	TOLLIP	C3
Dennd2a	IFI16	SAMD9L	LCPI	TSPAN13	CALCRL
Dpys	IFNG	SARAF	LGALS9	UFM1	CASS4
Dusp13	IGFBP1	SLC16A14	MIR4632	ZBTB7A	CCDC141
Enpp2	IGFBP2	SLC9A7	MLKL	ZNHIT1	CCDC24
Fabp4	IGFBP3	SLCO2B1	MMP12		CCDC63
Ffar2	IGFBP4	SMCO3	MYD88		CCL23
Fpr2	IGFBP5	SRPX	OASL		CCL3
Gas7	IGFBP6	SRPX2	PLAU		CCR7

Gbp4	IGFBP7	STAT1	PMAIP1		CCR8
Gpr35	IGSF3	TAP1	RSPO3		CD300C
Gramd1b	ING1	TM4SF1-AS1	RTP4		cd33
Grap	IRF5	TMEM159	TNFAIP3		CD82
Gstt4	IRF7	TMEM30A	TNFAIP6		CD84
Hsd11b1	ISG15	TMEM59			CDC42BPG
Ifitm10	MAP1LC3B	TNFRSF10C			CDCP1
Il15	MAP2K3	TNFSF13B			CEL
Il1a	MDM2	WDR63			CHN2
Il1r2	MMP1	WDR78			CLEC12A
Il1rn	NDN				CLEC4M
Il7r	NME2				CLUHP3
Inhba	NRG1				CMPK2
Itgax	OPTN				CNR2
Itih1	PEA15				COCH
Jam3	RAB13				COL6A1
Lipn	RAB31				COL9A2
Lrrc3	RAB5B				CREB3L3
Ltbp2	RABGGTA				CRISPLD2
Ltf	RAC1				CSF1R
Macroh2a2	RBL2				CSGALNACT1
Mgll	RGL2				CTSF
Mmp13	RHOB				CXCL10
Mmp27	RRAS				CXCL11
Mmp7	S100A11				excl8
Ms4a7	SERPINB2				CXCR2
Mx2	SERPINE1				CXCR3
Myh14	SMPD1				CXCR5
Nlrp12	SMURF2				CYGB
Nod1	SOD1				CYP11A1
Olfm4	SPARC				CYP1A1
Patj	STAT1				CYP2A6
Pde8a	TES				CYTH4
Pdlim1	TFAP2A				CYTIP
Piwil4	TGFB1I1				DDO
Plekhg6	THBS1				DDR1
Popdc3	TNFAIP2				DHRS3
Postn	TNFAIP3				DKFZp434J0226
Ppbp	TP53				DNAH8

Prkar1b	TSPYL5				DPP4
Prune2	VIM				DUSP10
Ptger2					EBI3
Qrfpr					EFEMP2
Rsad2					EGF
Rusc2					EGR2
Sbf2					ENPP2
Scn4a					ENPP3
Serpinb2					EPAS1
Sh3bgrl2					EPS8L2
Slc15a3					ERICH4
Slc26a3					EVPL
Slc6a12					F2RL3
Slc6a8					F5
St3gal6					FAM46C
Tecta					FCAMR
Tgm2					FCER1A
Tmc7					FCGR1B
Tmem119					FCRLA
Tmtc1					FGFR3
Tnnt3					FGR
Uaca					FILIP1L
Vwf					FYB
Wnt7b					GBP1
Xdh					GBP1P1
					GHRL
					GP6
					GPR183
					GPR68
					GPRC5B
					GPRC5C
					GRID2IP
					GVINP1
					GZMA
					GZMM
					HDC
					HECW2
					HLA-DOA
					HLA-DPA1

					HLA-DPB1
					HLA-DQB1
					HLA-DRA
					HLA-DRB5
					HLA-DRB6
					HMOX1
					HTRA3
					HYDIN
					ICAM1
					ID2
					IER3
					IFI44L
					IFIT3
					IL1R1
					IL4I1
					IL7R
					IP6K3
					IPCEF1
					IQCD
					IRAK2
					IRF6
					ITGA2B
					ITGAM
					ITLN1
					KCNMB1
					KIAA1683
					KIR2DL4
					KLF2
					KRT7
					KRT79
					KRT80
					LAX1
					LDHD
					LGALS3BP
					LIF
					LMNTD2
					LPAR5
					LRRC29
					LRRC8E

					LTBP1
					MAP3K7CL
					MAP3K8
					MGAT3
					MICALL2
					MIR4420
					MLPH
					MMP1
					MMP19
					MPZL2
					MYLK3
					NCF1
					NCF1C
					NEU4
					NHLRC4
					NR5A1
					NTRK1
					OASL
					ODF3L1
					OLAH
					OPTN
					PAPSS2
					PCED1B
					PEX11G
					PGF
					PIEZO2
					PIK3IP1
					PIPOX
					PKDREJ
					PLEKHA7
					PLEKHB1
					PLIN2
					PLIN4
					PLIN5
					PLXNB3
					PMEL
					PNCK
					PRCD
					PRSS36

					PRSS8
					PTPRH
					PTPRO
					PVRL4
					RAB27B
					RAP1GAP
					RASGRP3
					RGS1
					RGS13
					RTP4
					S100A2
					S100A3
					S100B
					SAT1
					SDS
					SELENBP1
					SERPINE1
					SIGLEC9
					SLAMF7
					SLC15A3
					SLC22A20
					SLC23A1
					SLC25A18
					SLC26A11
					SLC2A14
					SLC6A13
					SMARCA1
					SPP1
					SRC
					SSC5D
					ST5
					STX11
					SUCNR1
					SYTL2
					TCN2
					THBS1
					TLR7
					TMEM198
					TMPRSS13

					TNFSF10
					TP53INP1
					TP63
					TPSB2
					TSPAN10
					TUB
					TVP23C
					TYROBP
					UBXN10
					UCA1
					VEPH1
					VNN2
					VWF
					YPEL5
					ZG16B
					ZNF469
					ZNF683
					ZNF843

Supplementary table 1- 2 List of embryonic diapause associated gene signatures.

Diapause Up	Diapause Down					
ACCSL	AACS	CSRP1	GPX2	NME1	RNPEP	TMEM150A
ADH1	AAMP	CSRP2BP	GPX3	NME2	RPL21	TMEM191C
ADHFE1	AAR2	CST3	GRB7	NMRAL1	RPL36	TMEM214
ADSS	AARS	CTSZ	GRWD1	NOL9	RPL39L	TMEM219
AHNAK2	ABCB10	CTU1	GSTO1	NPL	RPL41	TMEM248
AQP3	ABCB8	CUBN	GSTP1	NSMCE1	RPL7A	TMEM9
AQP9	ABCC1	CYB5B	GTF2F1	NSUN5	RPLP0	TMEM97
ARC	ABCF1	CYC1	GUK1	NT5C	RPLP1	TNFRSF12A
ASPG	ABCF2	CYP2S1	GUSB	NT5DC2	RPN1	TOE1
BTG1	ABHD12	DAB2	H13	NTMT1	RPS6KC1	TOMM40
CDX1	ACACA	DAD1	HDLBP	NUCB2	RPS9	TOR2A
CHD9	ACACB	DARS2	HEATR1	NXT1	RPSA	TPI1
CLDN4	ACO2	DAZAP1	HGS	OAT	RPUSD1	TPM1
COX6B2	ACTR1A	DBNL	HILPDA	OGDH	RRBP1	TRAP1
CPSF4L	ADRM1	DDOST	HMBS	OLA1	RRM2	TRAPPC5
CRYM	AGPAT4	DDR2	HMG20B	OSTC	RRP12	TRIB3
CSGALNACT1	AKT1	DDX39B	HN1	OVOL2	RRP7A	TRIM28

CTH	AKT1S1	DDX49	HNF1B	P4HA2	RXRB	TRIM44
CTNNA3	ALAD	DDX56	HNF4A	P4HB	SAC3D1	TRIP6
CUEDC1	ALDH18A1	DEDD2	HPDL	PA2G4	SAMM50	TRMT61A
DNAJC5G	ALDOA	DERA	HPN	PALM3	SARS	TTLL1
EBF1	ALG8	DERL3	HSP90AB1	PANX1	SAYSD1	TTLL4
EMP1	ALPPL2	DGKZ	HSPG2	PARP8	SCARB2	TUBA1B
ENOX1	AMN	DHX34	ICT1	PCBP4	SCYL1	TUBA1C
FEZ1	AMOT	DIEXF	IMPDH1	PDGFRA	SCYL2	TUBB4B
FGF4	AMPD2	DKK1	INO80E	PDIA6	SDF4	TUBB5
FHIT	ANAPC10	DNMT3A	INTS1	PDXK	SEC11C	TUBB6
FN1	ANGPTL4	DNMT3B	IPO4	PEBP1	SEC13	TWF2
FOS	ANKRD39	DNPEP	IPO7	PELO	SEC61A1	TXN2
FUT11	ANP32B	DOHH	ISYNA1	PFKL	SEC61B	TXNDC12
FXDYD6	AP2A1	DOK2	ITFG2	PFN1	SEMA4G	TXNDC5
GATA1	AP2A2	DOLPP1	ITFG3	PGA5	SEPW1	TXNL4A
GCH1	AP2S1	DPH3	ITGA3	PGAM1	SERPINH1	UBE2A
GIGYF1	APBA3	DPP4	ITGA5	PGD	SF3A2	UBOX5
GJC1	APOC2	DPP9	ITM2C	PGLS	SGK3	UQCRQ
GPNMB	APOM	DPPA3	JAM3	PGP	SGSM1	URGCP
GPR143	APRT	DRAM1	JMJD8	PHC2	SGTA	USP5
GRIK4	APTX	DRG2	JOSD2	PHF5A	SH3BGRL3	UTF1
GRXCR1	ARF1	DTX2	KCNK6	PHGDH	SH3PXD2B	VAMP3
GSTA3	ARFIP1	DUS1L	KDELRL1	PIGO	SHKBP1	VAMP8
GSTM1	ARFIP2	DUSP4	KDELRL2	PIK3R2	SHMT2	VAR5
HIST2H2BB	ARHGDI1A	DUSP9	KIF18B	PIN1	SHROOM3	VCP
HLX	ARHGEF16	EEF1D	KLHDC3	PITHD1	SIDT2	VIM
IDO2	ARHGEF19	EFNB2	KLRG2	PKM	SIGMAR1	VPS37C
IFI27L1	ARMC5	EFTUD2	KRT8	PLA2G16	SLC16A13	VPS51
IFI35	ARMC6	EGFL7	KRTCAP2	PLA2G6	SLC16A3	VPS52
IFITM1	ARPC1B	EHD1	LAMA1	PLAC1	SLC19A1	WARS
IGDCC3	ARPC4	EIF1AD	LAMB1	PLAC8	SLC25A10	WBSCR16
IGFBP2	ARPC5	EIF2B2	LAMC1	PLBD2	SLC25A11	WDR18
JPH3	ARVCF	EIF3B	LDHA	PLEKHO1	SLC25A22	WDR92
KBTBD2	AS3MT	EIF3G	LGALS4	PLOD2	SLC25A4	XPNPEP1
KDM3A	ATF6	EIF3I	LGMN	PLOD3	SLC25A5	XPO4
KLF2	ATOX1	EIF5A	LMAN2	PMF1	SLC29A1	YDJC
LMO3	ATP1A1	EIF6	LMF2	PMM2	SLC35B2	YIF1A
LRRC31	ATP5D	ELF3	LRP10	PMVK	SLC37A4	YIPF2
LRRC34	ATP5G1	ELOF1	LRP2	PODXL	SLC39A3	YIPF3

LTB	ATP5O	ELOVL1	LRPAP1	POFUT2	SLC43A3	YKT6
LY6G6E	AUP1	ELP5	LRRC59	POLDIP3	SLC44A2	ZCCHC17
MAL	AXIN1	EMB	LRRN4	POLE3	SLC44A3	ZDHHC12
MAP3K1	B3GNT7	EMC10	LSR	POLR2C	SLC46A1	ZFP13
MBNL1	B4GALNT1	EMC8	LY6E	POLR2H	SLC5A11	ZFPL1
MCAM	BAD	EME1	LYPLA2	POLR2I	SLC7A6	ZYX
MIB1	BCAR1	ENGASE	MANF	POLR2L	SLC7A7	
MMP2	BCKDK	ENO1	MAP1LC3A	POMP	SLC9A3R1	
MORC1	BEND5	ENTHD2	MAP2K2	PPA1	SMARCA4	
MTSS1	BOP1	EPHA2	MAP2K7	PPAP2C	SMARCD1	
MUP1	BRMS1	ERP29	MAPKAPK3	PPM1G	SMPD1	
MYLPF	BSPRY	ESRP2	MARCKSL1	PPP1CA	SMTN	
MYOF	BTBD6	ESRRA	MARS	PPP2R1A	SMYD5	
NANOG	BYSL	ETF1	MBD3	PPP2R4	SNF8	
NAV2	CAD	ETFB	MDH2	PPP2R5D	SNRPB	
NFIA	CALM1	ETHE1	MECR	PPP5C	SNRPN	
NMRK1	CAPN1	EXOSC2	MEF2D	PQLC2	SOAT2	
NPY	CAR2	EXOSC4	METTL21A	PRDX2	SOD1	
NTN1	CAR4	FABP5	MFGF8	PRDX5	SORD	
PATL2	CASP6	FAHD2A	MFSD5	PRDX6	SPARC	
PDGFB	CBR1	FAM109A	MFSD7C	PREB	SPG20	
PDGFC	CCDC124	FAM160B2	MGAT1	PRELID1	SPNS1	
PITPNC1	CCM2	FAM173A	MGST3	PRELID2	SPR	
PIWIL4	CCND3	FAM98A	MIF	PRKAG1	SRM	
PLAGL1	CCNE1	FASN	MMP15	PRKAR2A	SRPRB	
PMEL	CD164L2	FASTK	MOGAT2	PRKCSH	SSNA1	
PRICKLE2	CD320	FDPS	MON1A	PSAT1	SSR4	
PRPS1	CDC34	FECH	MPP1	PSMB10	SSSCA1	
PRRX1	CDC37	FKBP11	MRI1	PSMB3	STAG3	
PSD	CDC42EP1	FKBP2	MRPL10	PSMB4	STARD10	
PTPRD	CDCA3	FKBP9	MRPL12	PSMB6	STK40	
RAB26	CDIPT	FOLR1	MRPL17	PSMC3	STX17	
RRAGD	CDT1	FRMPD1	MRPL2	PSMC5	STXBP2	
S100A1	CFL1	FTH1	MRPL20	PSMD13	SUPT5H	
S100A13	CGNL1	FXN	MRPL28	PSMD3	SURF4	
SAT1	CHAF1A	GADD45GIP1	MRPL34	PSMD6	SUSD3	
SDC2	CHCHD3	GALK1	MRPL52	PSMD8	SYS1	
SLC28A1	CHGA	GATA4	MRPS11	PSME3	SYVN1	
SLC30A9	CIB1	GATA6	MRPS12	PSMG3	TAF1C	

SLC52A3	CITED1	GATSL3	MRPS18A	PSPH	TALDO1	
SOCS3	CITED2	GDAP2	MRPS25	PTGR1	TBC1D7	
SOD3	CKAP4	GDPD5	MRRF	PTH1R	TBCB	
SOX2	CLDN6	GEMIN7	MRT04	PTPN13	TBL2	
SPIC	CLDN7	GET4	MTHFSD	PTPN18	TBL3	
SRCAP	CLIC3	GFER	MVD	PUF60	TBRG1	
TCF4	CLPP	GGT1	MVK	PXK	TCF25	
TDGF1	CLPTM1	GLIPR2	MXD3	PYCR1	TEAD4	
TEF	CLTA	GLTSCR2	MYC	PYCR2	TELO2	
TFAP2C	CNDP2	GMPPA	MYG1	PYGO2	TEN1	
TFCP2L1	COL4A1	GMPPB	MYL6	RAB11FIP4	TFE3	
THG1L	COL4A2	GNA12	MYO1C	RAB1B	TFPT	
TRO	COMMD5	GNB2	NAA10	RAB35	THOC3	
TRPM1	COPE	GNE	NABP2	RAB3IL1	THOP1	
U1	COX16	GOT2	NACC1	RBFA	TIMM13	
U6	COX5B	GPANK1	NANS	RBM33	TIMM50	
UBD	CPN1	GPAT2	NCAPH2	RCC1	TK1	
XKR9	CPT2	GPD1L	NCKIPSD	RCE1	TMBIM6	
YPEL2	CRELD2	GPN2	NDUFA11	RCN3	TMED1	
ZBTB44	CRIP2	GPR137B	NDUFS8	REEP1	TMEM109	
ZFP423	CSK	GPS1	NLE1	REXO4	TMEM120B	

Spontaneous reversal of drug-induced senescent-like state in BJ fibroblasts

4.1 Summary

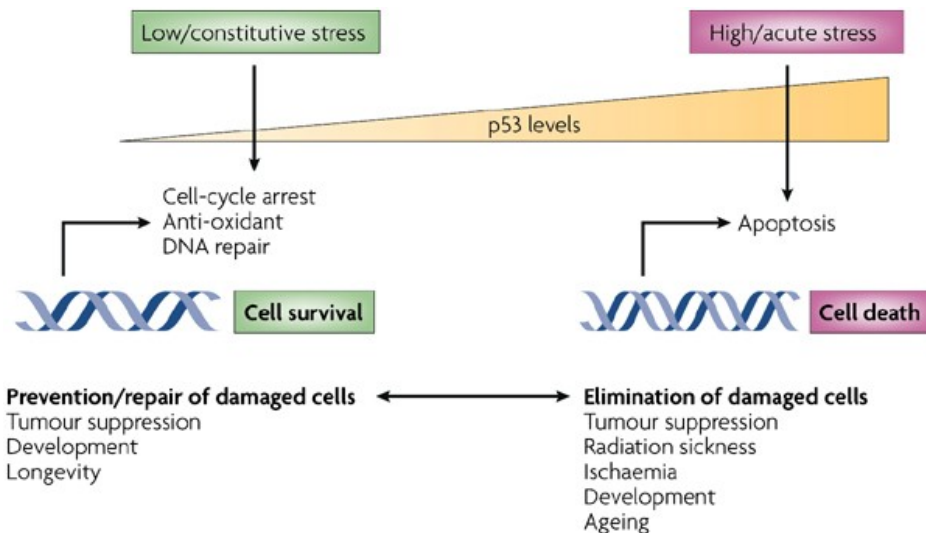
My previous work demonstrated that escape from a drug-induced senescent-like state occurs in both murine and human leukemia cells in a p53-independent manner. Here, I address whether normal human fibroblasts that enter a wtp53-driven doxorubicin-induced senescent-like state can escape. Rare clonal drug-recovered BJ fibroblast foci were observed proliferating between 2-3 weeks after doxorubicin treatment. These results were supported by performing a single cell cloning assay, where doxorubicin treated cells demonstrated the rare ability to resume proliferation after 1-3 weeks of arrest. Escaped cells demonstrated asynchronous escape and morphological differences. Despite the drug-recovered population showing resolution of drug-induced DNA damage, a subset of the drug-recovered population showed elevated p53 and p21 protein levels. Furthermore, the drug-recovered population had a more robust and heterogenous response to nutlin-3a treatment. Using nutlin-3a-induced senescence as a cellular model, I demonstrate that escape from senescence occurs as nutlin-3a is removed suggesting that continuous stabilization of p53 is required to maintain the arrested state.

4.2 Introduction

The DNA damage response is a partial determinant of cell fate; cells will retain proliferative capacity by either continuing proliferation or enter a transient cell cycle arrest to facilitate DNA repair, or enter an anti-proliferative program such as apoptosis or senescence. Although senescence has been traditionally considered to be a terminal state, my previous work in Chapter 3 has demonstrated that some leukemia cells can resume proliferation after entering a drug-induced senescent-like state. Earlier studies have shown that senescence maintenance mechanisms are required to conserve the arrested state; the ectopic expression of shRNA or viral proteins that target and inactivate p53 are required to permit cell cycle re-entry (Beausejour et al., 2003).

Tumour suppressor p53 plays a critical role in response to DNA damage. In response to DNA damage, p53 is stabilized and activated. p53 functions as a transcription factor, upregulating multiple pathways and genes that determine cell fate, such as the antiproliferative gene p21, and pro-apoptotic gene, PIDD (Lin et al., 2000). A model describing the variable outcome of p53 response is dependent on the extent of the p53 levels, where low levels permit cell proliferation or a transient cell cycle arrest and high levels promotes a terminal apoptotic response or stably arrested senescent response (**Figure 4-1**; Vousden and Lane, 2007). Regulation of p53 levels is tightly controlled to prevent aberrant activation. P53 promotes the upregulation of E3 ligases such as MDM2 and PIRH2, which bind and negatively regulate p53 levels through proteasomal degradation (Haupt et al., 1997; Leng et al., 2003). Resolution of stress leads to destabilization of p53 and p53-mediated transcription. However, it remains unclear whether spontaneous reversal

of a drug-induced senescent state can occur in normal cells that have entered p53-mediated senescence.



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Figure 4-1 Stress-induced p53 protein levels determine cell fate.

Adapted from Vousden and Lane (2007). High levels of cell stress promote high levels of p53 protein, whereas low levels of stress promote a low levels of p53 protein expression. High levels of p53 result in terminal cell fate such as apoptosis; low levels of p53 promote cell survival by upregulating various pathways such as temporary cell cycle arrest, anti-oxidant response, and DNA repair.

In this chapter I address whether BJ fibroblasts can spontaneously escape a drug-induced senescent-like state. Cell cycle re-entry is a rare event and is associated with phenotypic heterogeneity, with respect to the duration of arrest and morphology. The doxorubicin-recovered population has an intact early DNA damage response and retains nutlin-3a responsive p53. Unexpectedly, the drug-recovered population demonstrates a more heterogenous yet robust p53 response to nutlin-3a treatment. I further show that nutlin-3a induced senescence is reversible in BJ fibroblasts upon removal of nutlin-3a. My findings reveal that 1) doxorubicin-induced

senescent-like state is reversible in a subpopulation of rare cells, and 2) the drug-recovered population displays phenotypic heterogeneity.

4.3 Results

4.3.1 Doxorubicin-induced senescence in BJ fibroblasts

My previous work showed that rare leukemia cells resume proliferation after entering a drug-induced senescent-like state. I questioned whether escape from senescence was unique to cancer cells or whether normal cells could also escape. To determine whether normal cells could escape a drug-induced senescent-like state, I utilized the normal human BJ fibroblast model. BJ fibroblasts enter a p53-dependent senescent state, and do not express p16. I reasoned that escape may be more likely to occur in this normal cell model since senescence is maintained by p53, and not p53 and p16 together (Beauséjour et al., 2003). To examine whether doxorubicin (Dox) treatment promoted senescence in BJ fibroblasts, cells were treated with dox for 24 hours, and rates of proliferation were assessed by counting viable cells 3-, 5-, and 7-days post-treatment and an SA- β -gal was performed. Vehicle control treated BJ fibroblasts demonstrated steady proliferation, whereas dox-treated BJ fibroblasts remained arrested over the 7-day period; cell viability was not impacted by dox treatment (**Figure 4-2A**). 7 days post-Dox treatment, BJ fibroblasts demonstrated an enlarged flattened morphology, where 75% stained positive for SA- β -gal (**Figure 4-2B-C**). To assess p53 activity, a westernblot was performed on senescent and proliferating BJ fibroblasts. Westernblot analysis demonstrated that p53, and p21, a canonical p53 target gene, were upregulated in Dox-treated senescent BJ fibroblasts compared with control treated proliferating BJ fibroblasts (**Figure 4-2D**). In addition, westernblot analysis showed that

lamin B1, a biomarker of senescence, was decreased in Dox-treated fibroblasts compared with untreated control cells (**Figure 4-2D**).

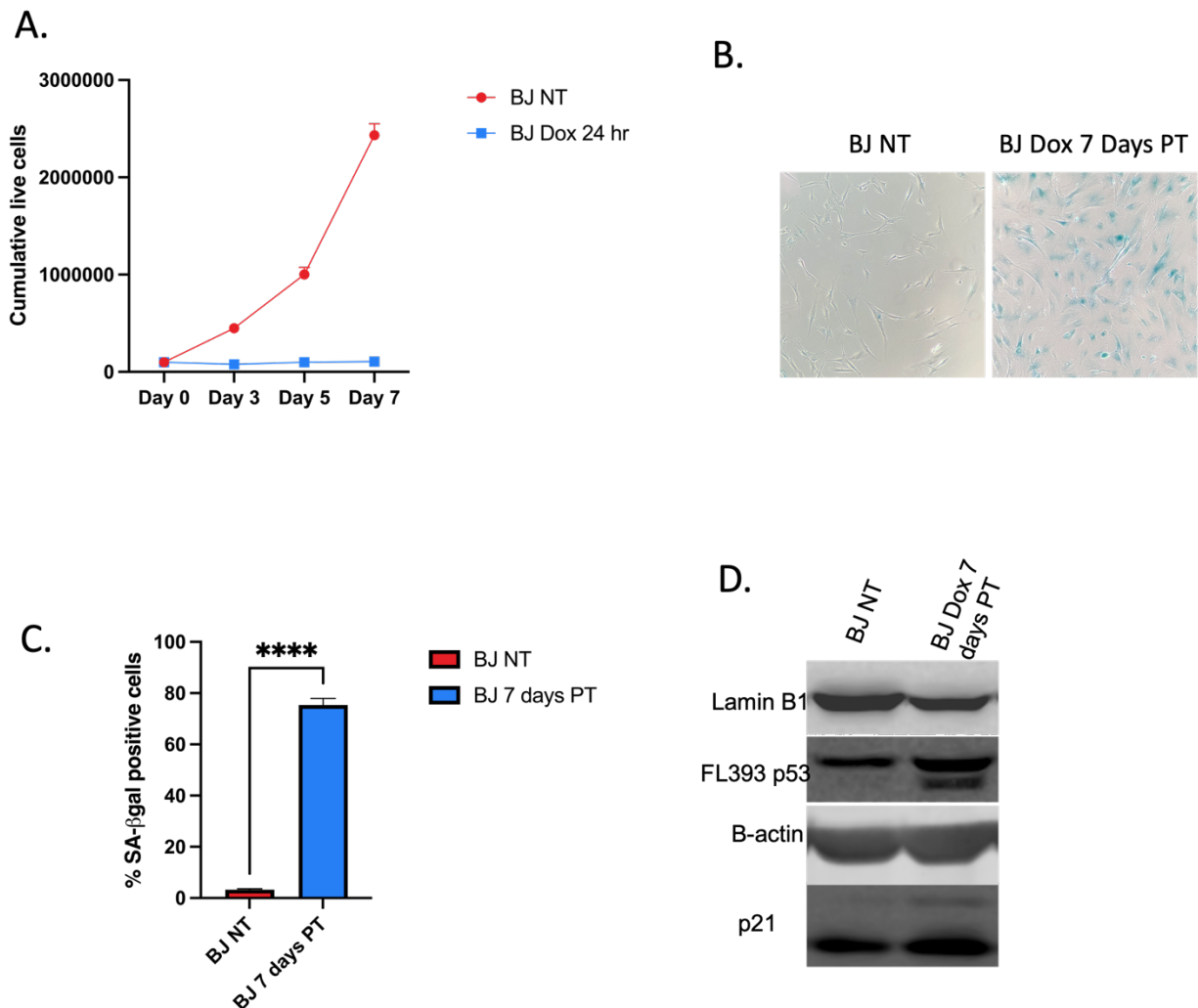


Figure 4-2 Doxorubicin-induced senescence in BJ fibroblasts.

Normal human BJ fibroblasts at a low population doubling (<20) were used.

- Proliferation curves of doxorubicin (Dox) treated and NT BJ fibroblast. Viable cells were counted using trypan blue staining. BJ fibroblasts were treated with or without 200 ng/ml Dox for 24 hr. After drug treatment, all cells were maintained in drug-free media. (n=3 biological replicates).
- Representative images and quantification of SA-β-gal staining of BJ fibroblasts. Related to Figure 5C.
- Quantification of SA-β-gal. SA-β-gal staining of BJ fibroblasts were performed 7 days post-treatment. BJ fibroblasts were treated with or without 200 ng/ml Dox for 24 hr. After drug treatment, all cells were maintained in drug-free media. (n=3 biological replicates).

replicates; a minimum of 100 cells were counted for each biological replicate). A two-tailed student t-test was performed.

- D. Representative western blot images of NT and Dox-treated BJ fibroblasts. Western blot of BJ fibroblasts were performed 7 days post-treatment. BJ fibroblasts were treated with or without 200 ng/ml Dox for 24 hr. After drug treatment, all cells were maintained in drug-free media. (n=3 biological replicates).

Statistical significance is shown by ****P<0.0001.

4.3.2 Doxorubicin-induced senescence is associated with cytosolic DNA

Immunofluorescent microscopy confirmed that lamin B1 decreased in BJ fibroblasts 3- and 7-days post Dox treatment compared with proliferating control BJ fibroblasts and BJ fibroblasts harvested immediately after 24 hr Dox treatment (**Figure 4-3A-B**). Dox-treated BJ fibroblasts showed evidence of DNA damage when treated with Dox. Cells displayed a robust DNA damage response, demonstrating a uniform increase in γ H2AX immediately after drug-treatment that persisted in roughly 60% of the population 3- and 7-days post-treatment, as is typically seen during senescence (**Figure 4-3A-B**; Gorgoulis et al., 2019).

DAPI staining revealed that micronuclei and chromatin bridges were observed in BJ fibroblasts 3 and 7 days post-dox treatment (**Figure 4-3A, C-D**). Consistent with the observations made in the elderly and progeria fibroblast cells, the majority of the micronuclei observed 3- and 7-days post-Dox treatment stained positive for γ H2AX and decreased levels of lamin B1 compared with primary nuclei of control proliferating BJ fibroblasts (**Figure 4-3A**). Rare chromatin bridges that were devoid of lamin B1 stained positively for γ H2AX; surprisingly, some ultra rare chromatin bridges appeared to continue beyond the cytoplasmic membrane and be located in the extracellular space, while retaining sporadic γ H2AX staining (**Figure 4-4**).

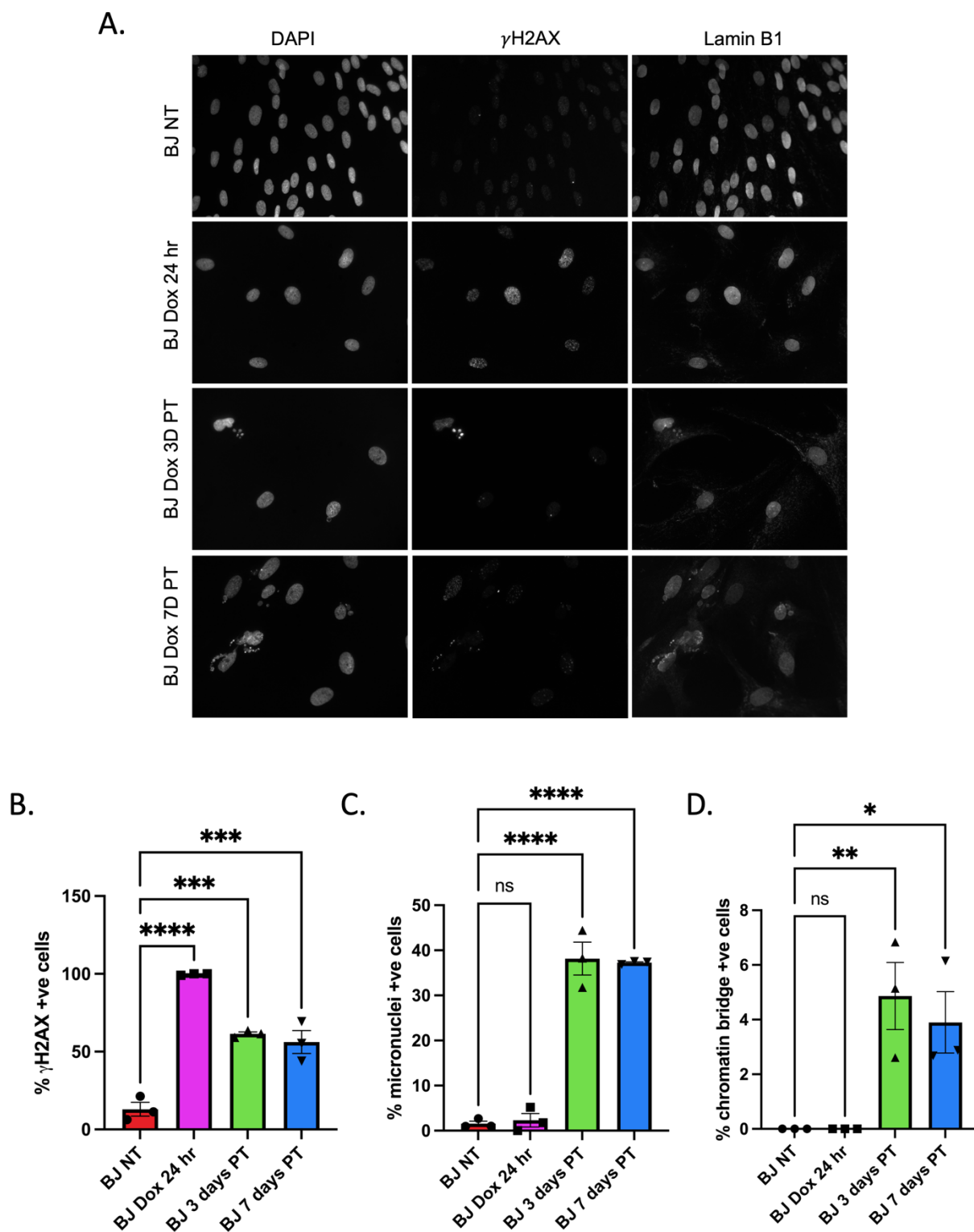


Figure 4-3 Persistent DNA damage in doxorubicin-induced senescent BJ fibroblasts.

A-D. Normal human BJ fibroblasts at a low population doubling (<20) were used. Immunofluorescence was performed on BJ fibroblast immediately after 24 hr Dox treatment, 3-

and 7-days post-Dox treatment and under no treatment. Cells were stained using DAPI, lamin B1 antibody and γ H2AX antibody.

- A. Representative immunofluorescent images of BJ fibroblasts.
- B. Quantification of γ H2AX positive BJ fibroblasts, as defined by ≥ 3 γ H2AX foci. (n=3 biological replicates; a minimum of 100 cells were counted for each biological replicate). ANOVA was performed to significance.
- C. Quantification of micronuclei positive BJ fibroblasts. (n=3 biological replicates; a minimum of 100 cells were counted for each biological replicate). ANOVA was performed to significance.
- D. Quantification of chromatin bridge positive BJ fibroblasts. (n=3 biological replicates; a minimum of 100 cells were counted for each biological replicate). ANOVA was performed to significance.

Statistical significance is shown by not significant (ns), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

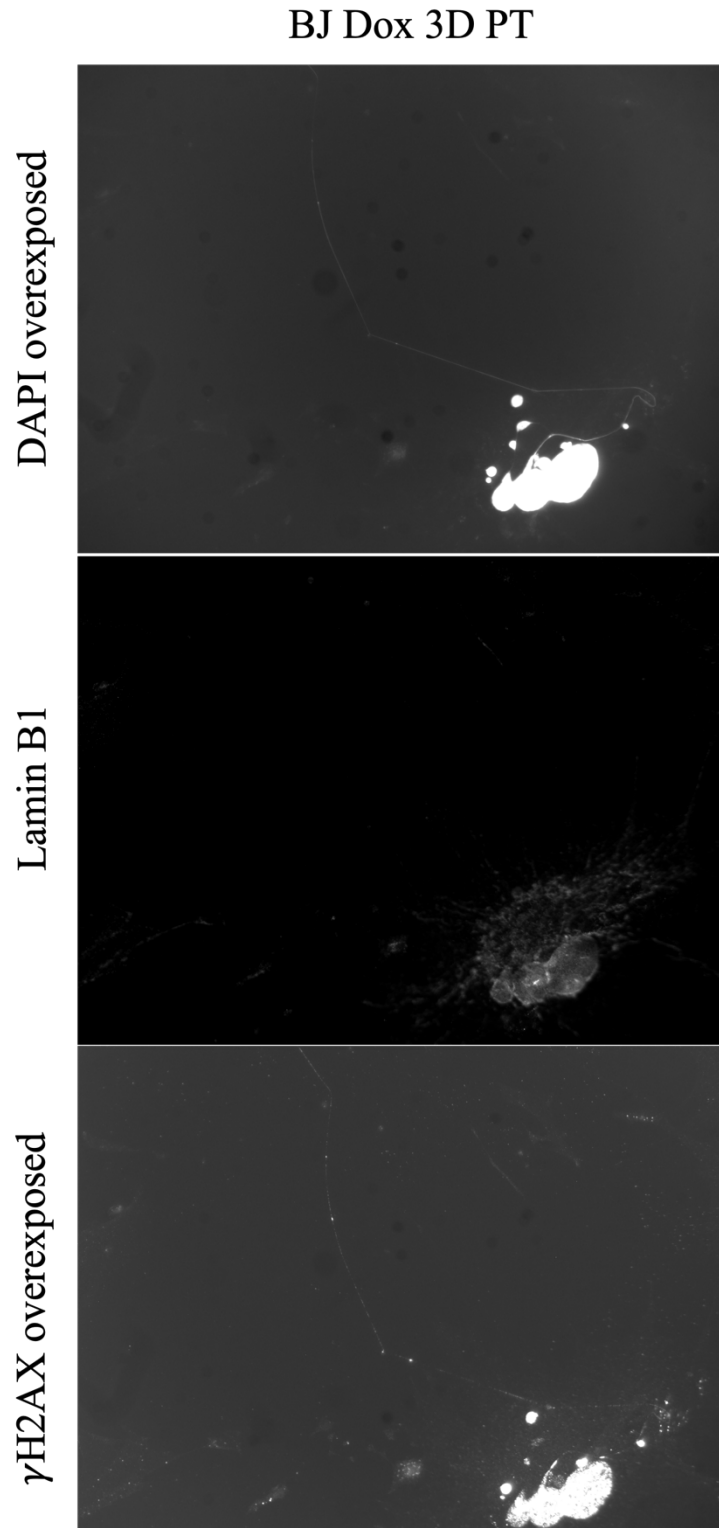


Figure 4-4 Doxorubicin-induced extracellular chromatin bridge undergoing DNA
 Representative immunofluorescent image of a chromatin bridge forming 3 days post-Dox treatment. Related to Figure 4-3.

To determine whether micronuclei and chromatin bridges formed after Dox treatment were exposed to the cytosol, immunofluorescence was performed using a cGAS antibody. cGAS demonstrated colocalization with micronuclei and chromatin bridges in BJ fibroblasts that formed 3- and 7 days post-treatment (**Figure 4-5; Figure 4-6; data not shown**). Noteworthy, cGAS favoured recruitment to micronuclei that had weaker lamin A/C staining (**Figure 4-5; Figure 4-6**). Moreover, portions of chromatin bridges that retained lamin A/C were largely cGAS negative; cGAS was heavily recruited to areas of chromatin bridges that were devoid of lamin A/C (**Figure 4-6**).

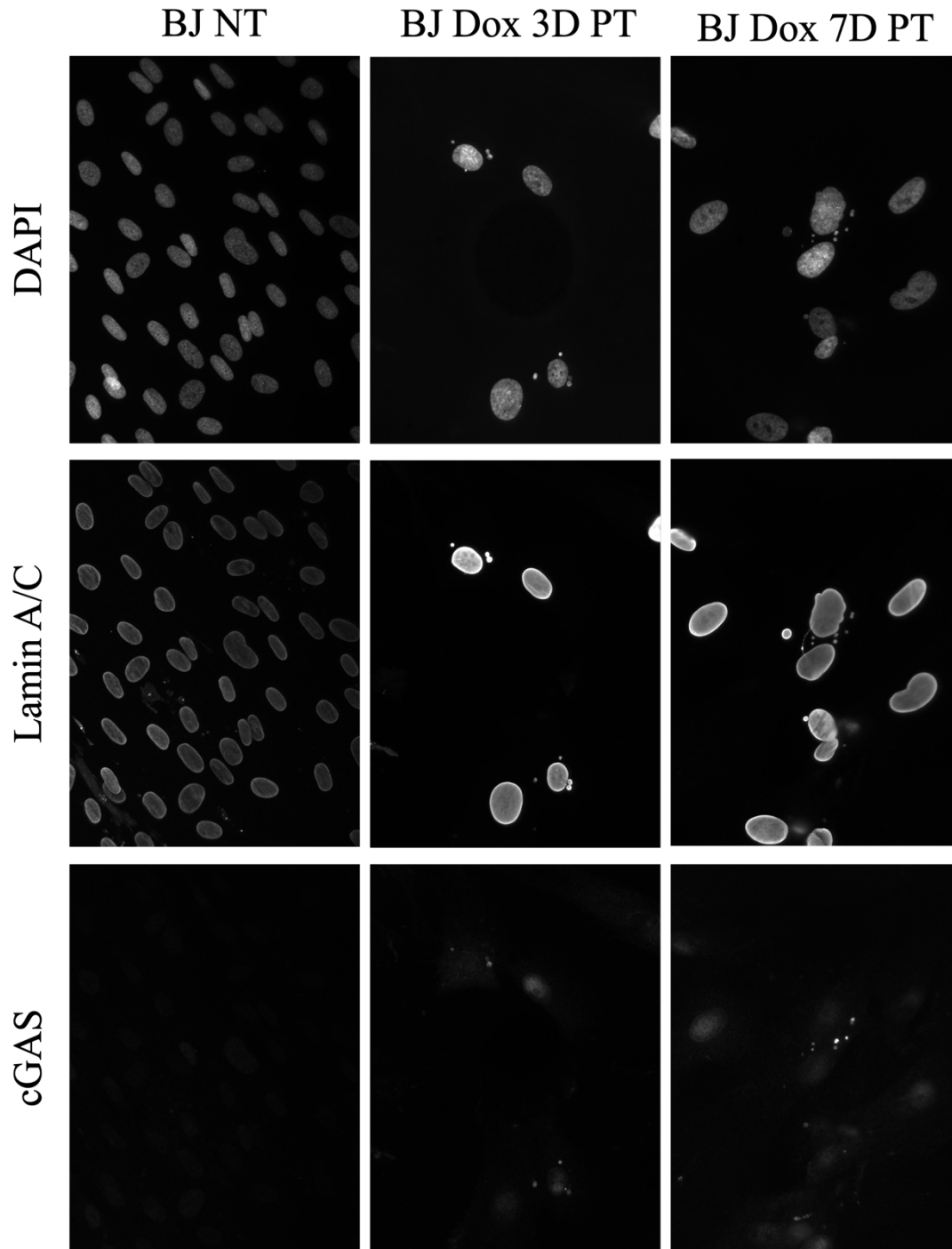


Figure 4-5 cGAS is recruited to cytosolic DNA in senescent BJ fibroblasts.
Representative immunofluorescence images of BJ fibroblast 3- and 7-days post-Dox treatment. Cells were stained using DAPI, lamin A/C antibody and cGAS antibody.

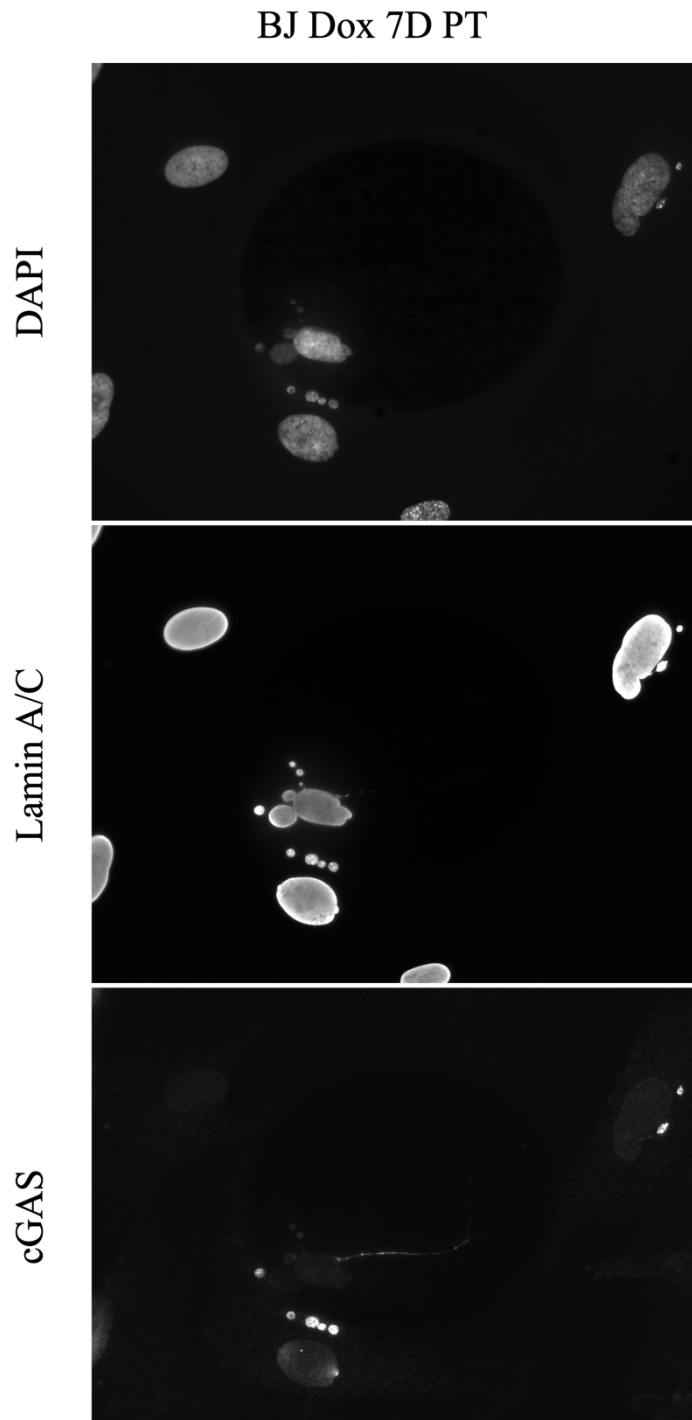


Figure 4-6 Co-localization of cGAS to exposed cytosolic chromatin in senescent BJ fibroblasts.

Representative immunofluorescent image of a chromatin bridge 7 days post-Dox treatment. Related to Figure 4-5.

4.3.3 Doxorubicin-induced senescence of normal fibroblasts is reversible

To determine whether senescence was spontaneously reversible in Dox-treated BJ fibroblasts, cells were allowed to recover in drug-free media until proliferation was observed. Despite cells having entered senescence, small clonal patches of BJ fibroblasts were observed proliferating in Dox-treated BJ fibroblast flasks 3-4 weeks post-treatment. After the initial emergence of the initial clonal patches, new patches were observed emerging in a sporadic nature. Proliferating clonal patches appeared to have normal fibroblasts morphology, and were SA- β -gal negative, surrounded by senescent, SA- β -gal positive BJ fibroblasts, suggesting escaped cells are unresponsive to anti-proliferative signaling from paracrine SASP factors that promote senescence (**Figure 4-7A**). To further demonstrate that Dox-treated BJ fibroblasts resume proliferation after an extended recovery period, a long-term proliferation assay was performed, where total viable cells were counted every 7 days. BJ fibroblasts treated with Dox for 24 hr remained stably arrested for 14 days post-treatment, a minimal increase in cell number was observed on 21 days post-treatment, suggesting that some clones began to expand; after the initial small increase in cell number on day 21, recovering BJ fibroblasts continued to expand at a comparable rate as the non-treated BJ fibroblasts, which remained proliferating for the duration of the experiment (**Figure 4-7B**).

The evidence demonstrating that doxorubicin induces senescence in BJ fibroblasts is limited to the phenotype of the population; hence, it is possible that rare non-senescent cells are present in the population that are not initially detected. Therefore, in order to convincingly demonstrate that Dox-treated fibroblasts escape senescence, a single cell-based assay was performed. Untreated or Dox-treated single cells were seeded into wells of a 96-well plate, and monitored for

proliferation on a weekly basis (**Figure 4-7C**). 26.7% of wells seeded with untreated control BJ fibroblasts cells contained proliferating cells, whereas only 0.6% of wells seeded with Dox-treated BJ fibroblasts contained proliferating clones (**Figure 4-7D**). Notably, of the wells that contained proliferating untreated BJ fibroblasts, 88.3% of the wells contained proliferating cells within 7 days of seeding, whereas Dox-treated BJ fibroblast clones were observed proliferating after 7 days post-treatment (**Figure 4-7E**).

Clones demonstrated differences in rates of proliferation (data not shown), and morphology. Two morphologies were observed in the escaped clones: i) a normal fibroblast morphology, ii) a flattened cobblestone-like morphology (**Figure 4-7F**). These results indicate that single cell senescent BJ fibroblasts can escape and may undergo a senescence-associated reprogramming event. The majority of wells seeded with single proliferating cells that were not treated with doxorubicin showed colony formation within the first two weeks. and retained normal fibroblast morphology. However, it remains unclear whether the difference in morphology is due to heterogeneity among cells that enter senescence, heterogeneity gained during senescence, or heterogeneity during escape.

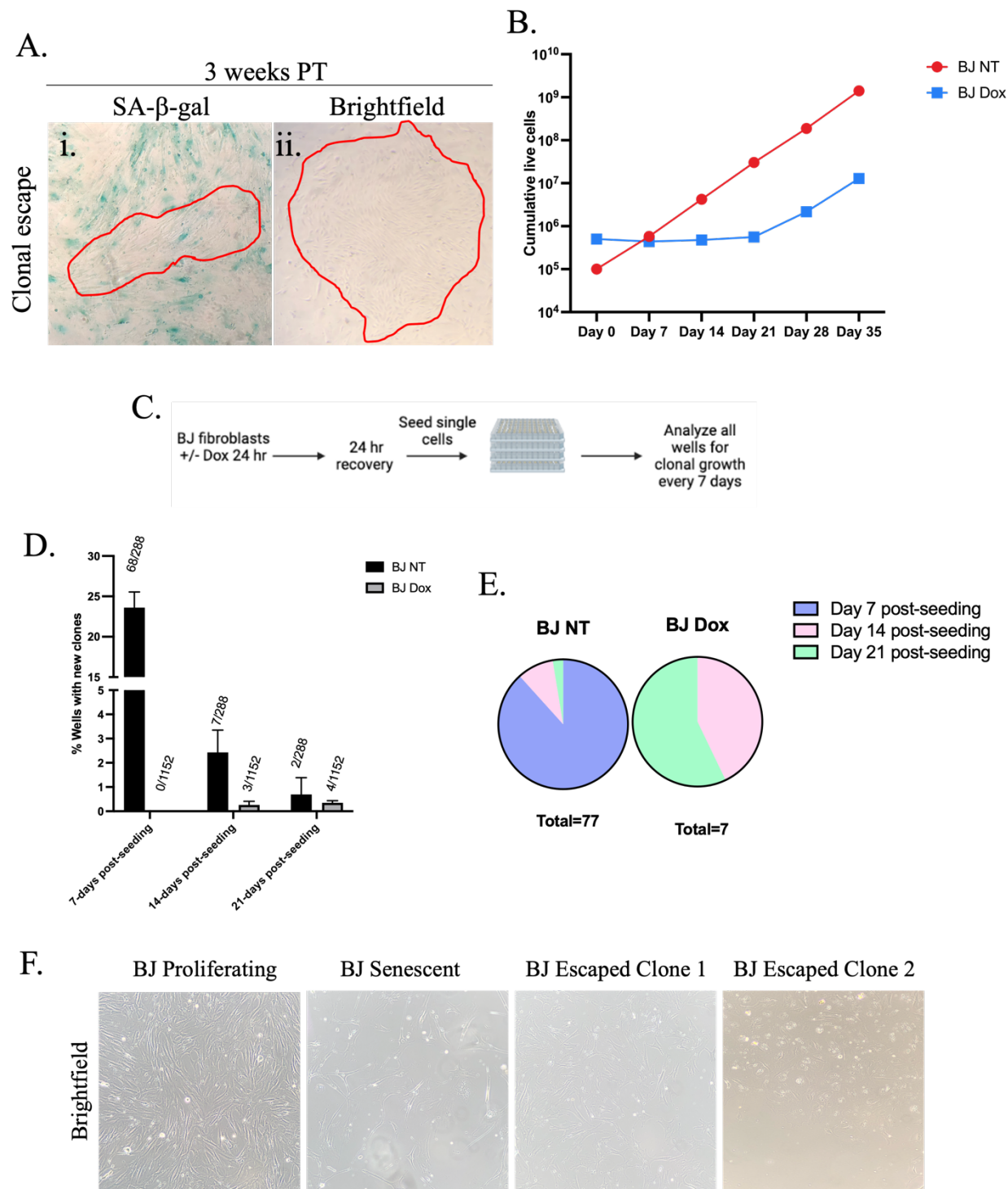


Figure 4-7 Escape from doxorubicin-induced senescence in BJ fibroblasts.

- A. i. Representative image of SA- β -gal negative BJ fibroblast colony forming 3 weeks post-Dox treatment. ii. Brightfield microscopy image of a proliferating BJ fibroblast colony forming 3 weeks post-Dox treatment.

- B. Proliferation curves of doxorubicin (Dox) treated and NT BJ fibroblast. Viable cells were counted every 7 days for a total of 35 days using trypan blue staining. BJ fibroblasts were treated with or without 200 ng/ml Dox for 24 hr. After drug treatment, all cells were maintained in drug-free media. (n=3 biological replicates).
- C. Experimental scheme. Dox-treated and untreated single cells were seeded into 96 well plates and monitored every 7 days for new expanding clones. Expanding clones were defined by ≥ 5 cells/ well.
- D. Percentage of wells containing new colonies. Mean $\pm$ SEM, n = 3 independent experiments.
- E. Pie chart showing proportions of when new expanding clones were detected.
- F. Representative brightfield images of BJ fibroblasts two clones that have escaping senescence, senescent BJ fibroblasts and proliferating parental BJ fibroblasts.

To determine whether escaped cells gained a mutation that directly or indirectly affected the ability of p53 to maintain senescence, the expanding population of emerging BJ fibroblasts (XBJ fibroblasts) were treated with nutlin-3a and compared to naïve, never senescent fibroblasts treated with Nutlin-3a. Immunofluorescence analysis demonstrated with single cell resolution that nutlin-3a treatment promoted an increase in p53 protein in both the XBJ population and the naïve BJ population relative to their respective DMSO controls. The XBJ fibroblasts appeared to have a more heterogenous and robust increase in p53 expression compared to naïve BJ fibroblasts (**Figure 4-8A**). Furthermore, a larger proportion of DMSO treated XBJ fibroblast had a higher basal p53 levels than the naïve DMSO treated controls (**Figure 4-8A**). To determine whether p53 target gene expression was altered in the XBJ fibroblasts, I repeated the nutlin-3a treated single cells immunofluorescence experiment, and co-stained for p53 and p21. As expected, XBJ fibroblasts showed a more robust and heterogenous expression of p53 in response to nutlin-3a than naïve BJ fibroblasts, and the basal level of p53 was higher in a proportion of XBJ fibroblasts than naïve BJ fibroblasts (**Figure 4-8B**). In keeping with a more robust p53 response in the drug-recovered population, p21 expression was more robust in nutlin-3a treated XBJ fibroblasts than nutlin-3a treated naïve BJ fibroblasts. Moreover, a fraction of XBJ

fibroblasts has higher basal p21 levels than naïve fibroblasts (**Figure 4-8C**). Both naïve BJ fibroblasts and XBJ fibroblast demonstrate a correlation between p53 expression and p21 expression upon nutlin-3a treatment (**Figure 4-8D**). Taken together, these results suggest that the drug-recovered population did not undergo a loss-of-function p53 mutation during treatment or that a pre-existing p53 mutant clone bypassed Dox-induced senescence.

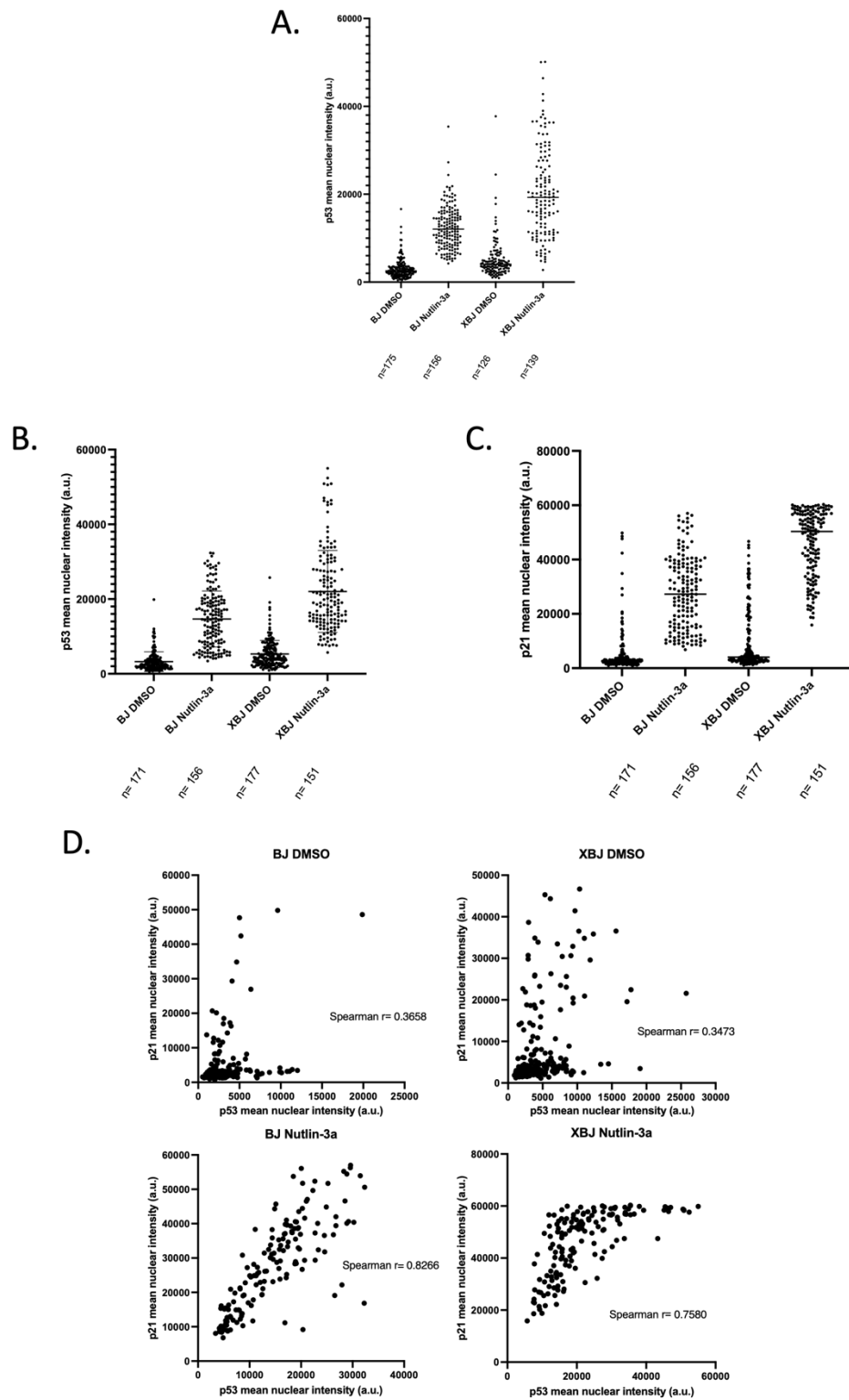


Figure 4-8 Drug-recovered escaped BJ fibroblasts have a functional p53 response.

- A. Immunofluorescence staining with FL393, detecting p53 nuclear intensity of naïve BJ fibroblasts and drug-recovered escaped (XBJ) fibroblasts treated with DMSO or 10 μ M nutlin-3a for 24 hr. n=3 independent replicates.
- B-D. Immunofluorescence co-staining with FL393 and F5, detecting p53 and p21 nuclear intensity of naïve BJ fibroblasts and XBJ fibroblasts treated with DMSO or 10 μ M nutlin-3a for 24 hr. (n= 3 independent replicates).
- B. Immunofluorescence staining with FL393, detecting p53 nuclear intensity of naïve BJ fibroblasts and drug-recovered escaped (XBJ) fibroblasts treated with DMSO or 10 μ M nutlin-3a for 24 hr.
- C. Immunofluorescence staining with F5, detecting p21 nuclear intensity of naïve BJ fibroblasts and XBJ fibroblasts treated with DMSO or 10 μ M nutlin-3a for 24 hr. n=3 independent replicates.
- D. Correlation of p53 and p21 nuclear intensity in single cells across DMSO and nutlin-3-a treatment groups for naïve BJ fibroblasts and XBJ fibroblasts. Spearman correlation was performed.

Since p53 appeared to remain functionally intact in response to DNA damage induced by Dox, we questioned whether the DNA damage was resolved in some senescent BJ fibroblasts that permitted cell cycle re-entry. To determine whether the XBJ fibroblasts had resolved the persistent DNA damage that remained during senescence, and to address whether the DDR was still functional in the senescent and escaped population, naïve, senescent, and escaped BJ fibroblasts were treated with Dox for 2 hr and subjected to immunofluorescence. As expected, non-treated naïve BJ fibroblasts showed low levels of γ H2AX, while some senescent BJ fibroblasts showed persistent γ H2AX signaling; the non-treated escaped BJ fibroblast population show low levels of γ H2AX, comparable to the naïve, never treated BJ fibroblasts (**Figure 4-9**). Dox-treatment resulted in a uniform increase of γ H2AX in both naïve and escaped BJ fibroblasts, indicating that the initial DDR pathways remained intact in escaped BJ fibroblast (**Figure 4-9**). Interestingly, only some senescent BJ fibroblasts demonstrated an increase in γ H2AX in response to Dox treatment, suggesting that some senescent BJ fibroblasts have lost a functional early DDR (**Figure 4-9**). Furthermore, co-staining with lamin B1 demonstrate that escaped BJ

fibroblasts increased lamin B1 levels compared with senescent BJ fibroblast that resembled lamin B1 levels seen in proliferating naïve BJ fibroblast (**Figure 4-9**).

Taken together these results suggest that BJ fibroblasts can escape drug-induced senescence.

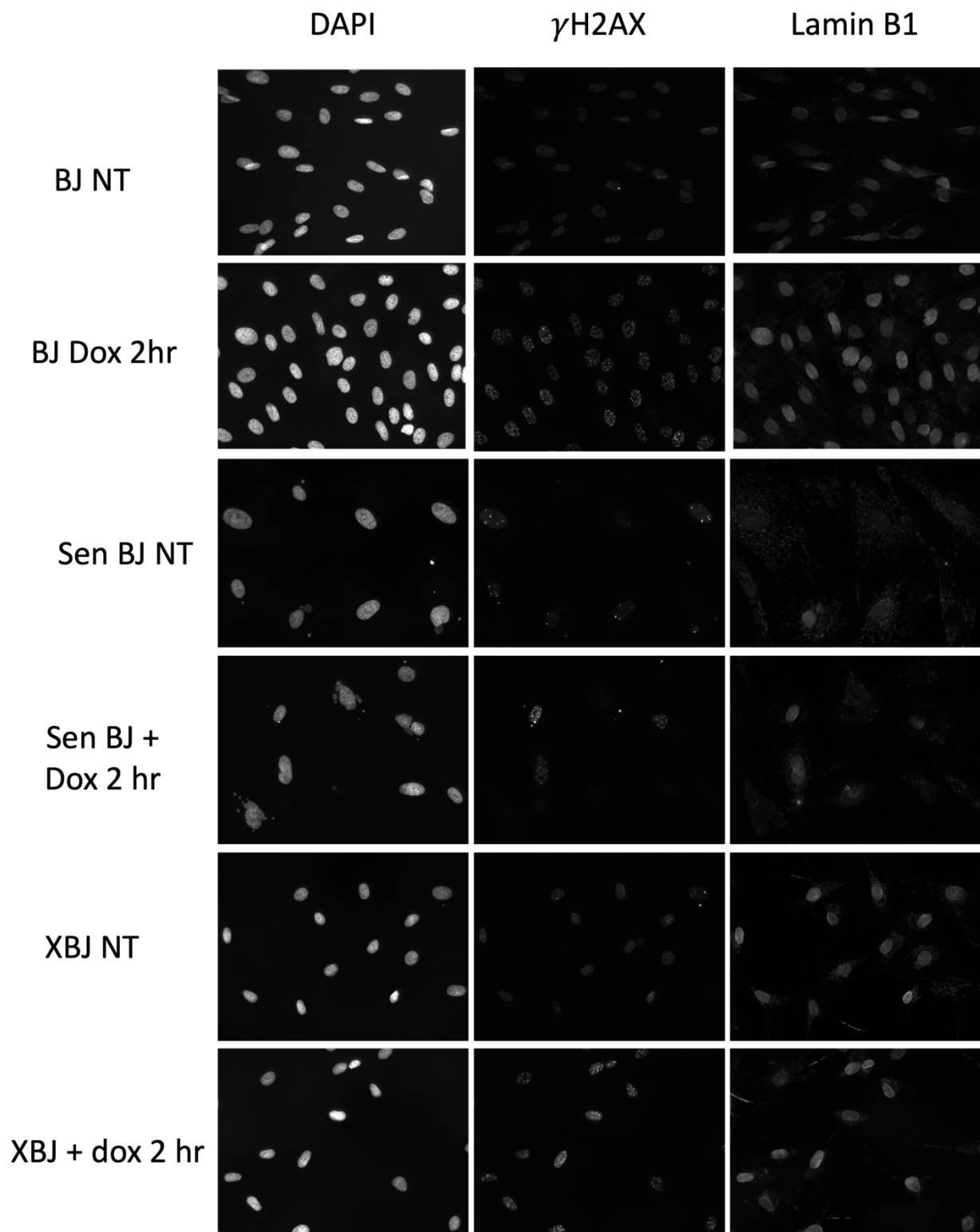


Figure 4-9 BJ fibroblasts that escape doxorubicin-induced senescence resolved persist senescence-associated DNA damage foci and respond to doxorubicin.

Immunofluorescence was performed on naïve BJ fibroblasts, doxorubicin-induced senescent BJ fibroblasts, and drug-recovered escaped BJ fibroblasts immediately after 2 hr Dox treatment, or no treatment. Cells were stained using DAPI, lamin B1 antibody and γ H2AX antibody.

4.3.4 Nutlin-3a induced senescence is reversible

To provide further evidence that drug-induced senescence is a reversible state when stress is resolved, I employed BJ fibroblasts treated with nutlin-3a, a MDM2 inhibitor that promotes p53 stability independent of genotoxicity. BJ fibroblasts do not express p16 and undergo p53-mediated senescence (Beauséjour et al., 2003). BJ fibroblasts were continuously treated with Nutlin-3a or DMSO for 7 days. To monitor levels of proliferation, BJ fibroblasts were counted on days 3, 5, and 7 of continuous nutlin-3a treatment. No proliferation was observed over the 7 day period of nutlin-3a treatment, whereas DMSO treated cells retained proliferative activity (**Figure 4-10A**). To confirm that nutlin-3a was promoting senescence, a SA- β -gal stain was performed. Nutlin-3a treated fibroblast showed a nearly homogenous level of increased SA- β -gal positivity (88%) compared with DMSO treated fibroblasts; nutlin-3a-treated BJ fibroblasts demonstrated needle-like morphology, consistent with previous reports (Wiley et al., 2018) (**Figure 4-10B-C**). To further characterize the nutlin3a-induced senescent state, a western blot was performed that demonstrated an increase in p53, p21 and decrease lamin B1 protein during nutlin-3a treatment compared with DMSO-treated control cells (**Figure 4-10D**) Performing immunofluorescence microscopy confirmed that lamin B1 was decreased in nutlin-3a treated fibroblasts compared with DMSO controls (**Figure 4-10E**). In keeping with our understanding of the mechanism of nutlin-3a-induced senescence, BJ fibroblasts treated with nutlin-3a did not display an increase in double-strand break marker, γ H2AX, nor an increase in micronuclei and chromatin bridges (**Figure 4-10E-H**).

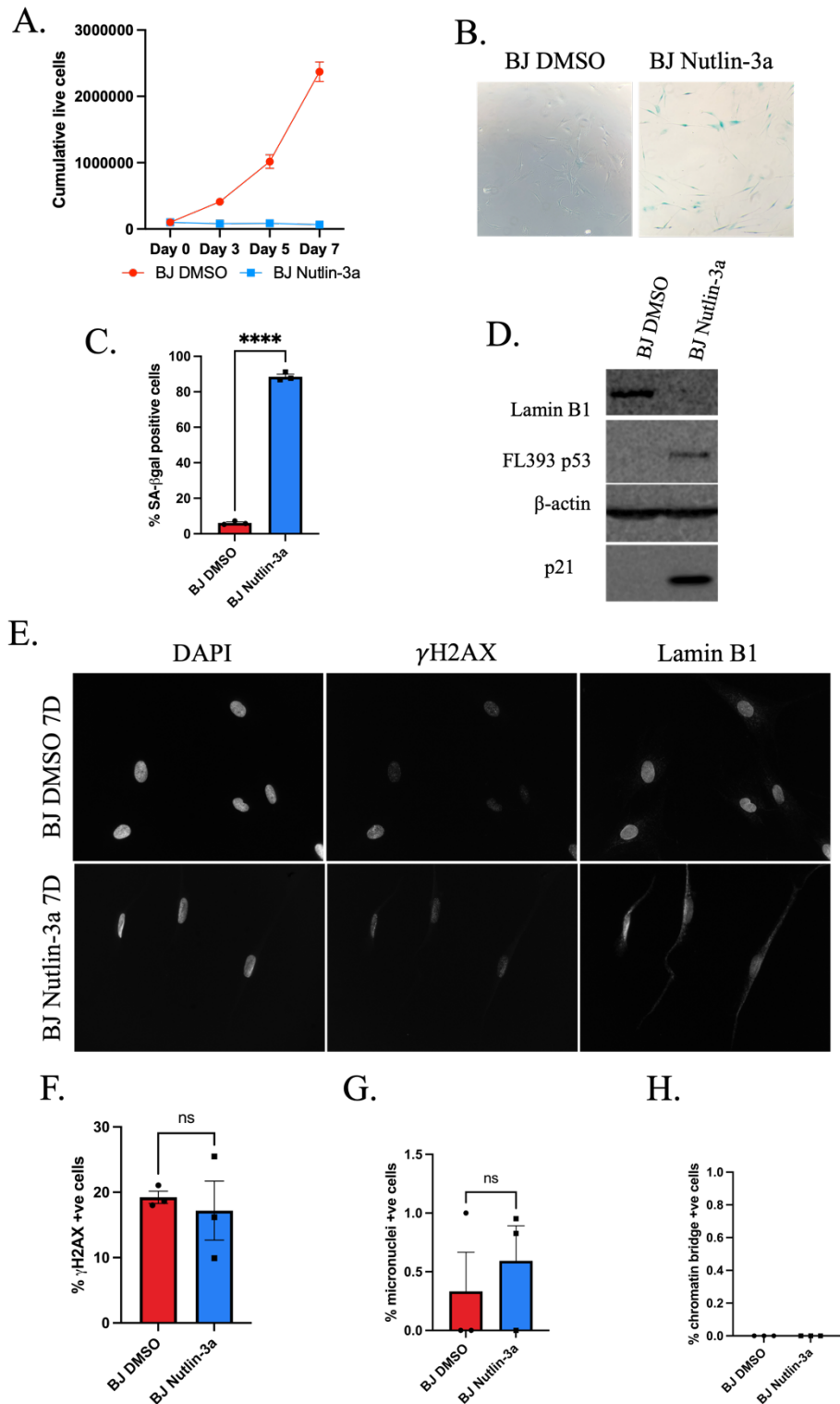


Figure 4-10 Nutlin-3a-induced senescence in BJ fibroblasts.
Normal human BJ fibroblasts at a low population doubling (<20) were used.

- A. Proliferation curves of Nutlin-3a and DMSO treated BJ fibroblast. Viable cells were counted on days 3, 5 and 7 of the experiment, using trypan blue staining. BJ fibroblasts were treated with DMSO or 10 μ M Nutlin-3a for 7 days. (n=3 biological replicates).
- B. Representative images and quantification of SA- β -gal staining of BJ fibroblasts. SA- β -gal staining of BJ fibroblasts were performed after 7 days of continuous 10 μ M Nutlin-3a or DMSO.
- C. Quantification of SA- β -gal staining related to figure 10B. Two-way student t-test was performed. (n=3 biological replicates; a minimum of 100 cells were counted for each biological replicate).
- D. Representative western blot images of DMSO or 10 μ M Nutlin-3a BJ fibroblasts. Western blot of BJ fibroblasts were performed on the 7th day of treatment. (n=3 biological replicates).
- E. Representative immunofluorescent images of DMSO and Nutlin-3a treated BJ fibroblasts immuno-stained with lamin B1 and γ H2AX antibodies. Related to Figure 10E-G.
- F. Quantification of γ H2AX positive DMSO and Nutlin-3a treated BJ fibroblasts, as defined by ≥ 3 γ H2AX foci. Two-way student t-test was performed. (n=3 biological replicates; a minimum of 100 cells were counted for each biological replicate).
- G. Quantification of micronuclei positive DMSO and Nutlin-3a treated BJ fibroblasts. Two-way student t-test was performed. (n=3 biological replicates; a minimum of 100 cells were counted for each biological replicate).
- H. Quantification of chromatin bridge positive DMSO and Nutlin-3a treated BJ fibroblasts. (n=3 biological replicates; a minimum of 100 cells were counted for each biological replicate).

Statistical significance is shown by not significant (ns), ****P<0.0001.

Initial removal of nutlin-3a after a 1 week treatment resulted in a heterogenous population. The majority of cells acquired a classical senescent morphology that were SA- β -gal positive; sporadic clonal patches were observed proliferating with normal BJ fibroblast morphology and were SA- β -gal negative (**Figure 4-11A**). In order to demonstrate that nutlin-3a treated cells resumed proliferation after nutlin-3a withdrawal, a long-term proliferation assay was performed, in which cells were counted every 7 days over a course of 35 days. BJ fibroblast treated with nutlin-3a for 7 days resulted in sustained growth arrest, in keeping with our previous findings (**Figure 4-10A; Figure 4-11B**). Following nutlin-3a removal, BJ fibroblasts gradually began to resume proliferation (**Figure 4-11B**). To provide support that the resumption of proliferation was due to the withdrawal of nutlin-3a, and not a decrease in the long-term potency of nutlin-3a,

proliferation of BJ fibroblasts treated with nutlin-3a were monitored for 14 days. BJ fibroblasts treated with nutlin-3a for 7 days were able to resume proliferation, whereas continuous nutlin-3a treatment resulted in cell cycle arrest for the entirety of the 14-day experiment (**Figure 4-11C**). Notably, no proliferation was observed in BJ fibroblasts treated with continuous nutlin-3a for an entire 2-month period (**data not shown**). Together these results support the idea that continuous p53 activation is necessary to maintain senescence in BJ fibroblasts, and that cells that no longer retain nutlin-3a-induced p53 activity during senescence may be able to resume proliferation.

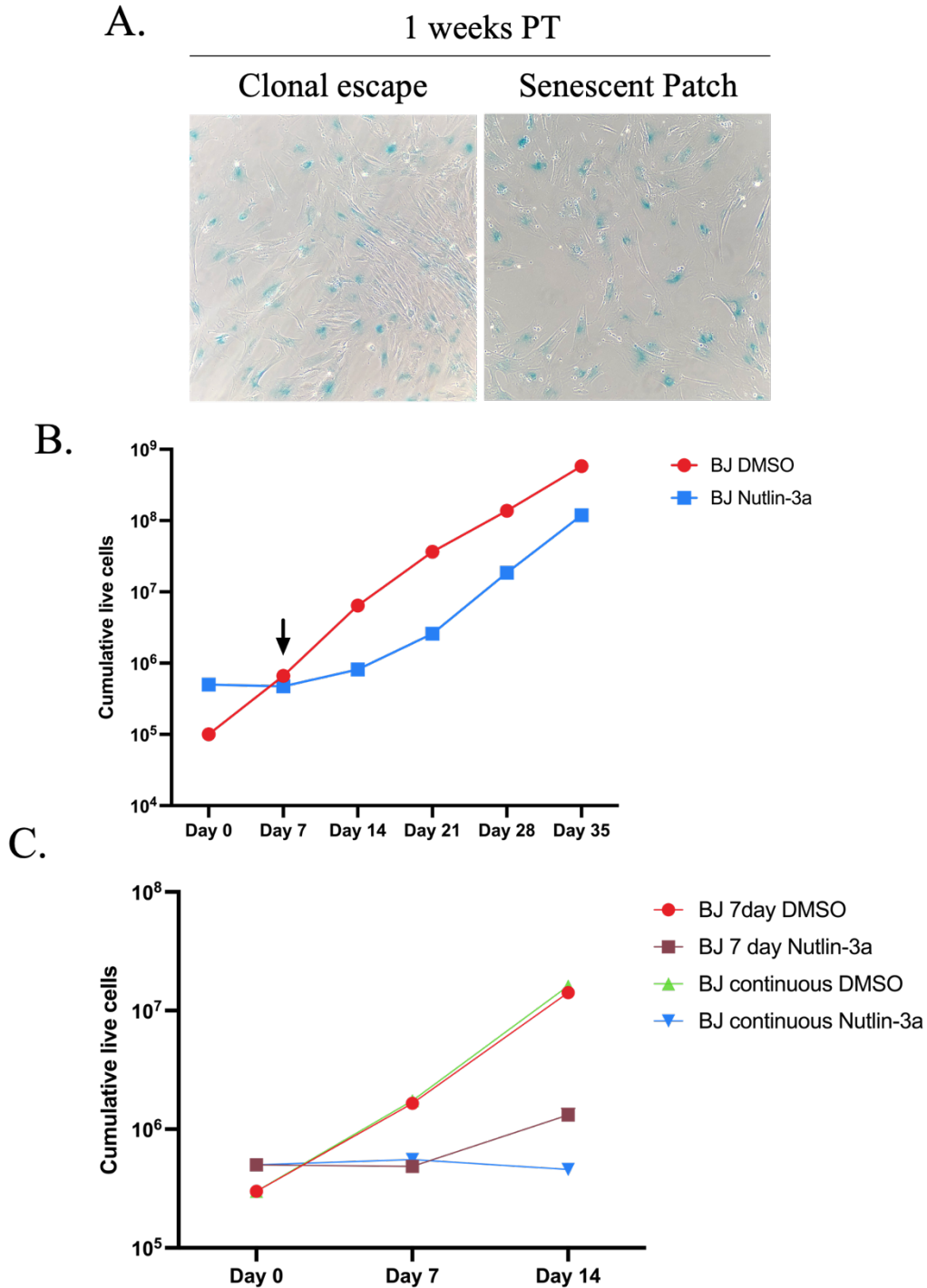


Figure 4-11 Nutlin-3a-induced senescence is reversible once nutlin-3a is removed.

Normal human BJ fibroblasts at a low population doubling (<20) were used.

A. Representative images of SA-β-gal staining of BJ fibroblasts. SA-β-gal staining of BJ fibroblasts were performed 7 after the removal of 10 μM Nutlin-3a or DMSO treatment.

- B. Proliferation curves of Nutlin-3a and DMSO treated BJ fibroblast. Viable cells were counted every 7 days for a total of 35 days using trypan blue staining. BJ fibroblasts were treated with DMSO or 10 μ M Nutlin-3a for 7 days. (n=3 biological replicates).
- C. Proliferation curves of BJ fibroblast treated with Nutlin-3a or DMSO for 7 days and 14 days. Viable cells were counted every 7 days for a total of 14 days using trypan blue staining. BJ fibroblasts were treated with DMSO or 10 μ M Nutlin-3a. (n=3 biological replicates).

4.4 Discussion

In keeping with my previous findings in leukemia models, I demonstrate that normal BJ fibroblasts are capable of spontaneously escaping doxorubicin- and nutlin-3a- induced senescence. Escape from nutlin-3a-induced senescence appears to occur in a sporadic manner where rare clones resume proliferation, potentially followed by a larger portion of the population escaping. In contrast, I demonstrate with single cell resolution, that escape from doxorubicin-induced senescence is a rare asynchronous clonal event, associated with resolution of genomic instability and p53 activation. I propose a model in which rare cells that repair DNA damage and resolve persistent DNA damaging signalling during senescence, may undergo a loss of p53 stabilization that allows cell cycle re-entry and progression.

A fraction of the drug-recovered population displays a more robust p53 and p21 expression, indicating that there is heterogeneity in the basal p53 response within the drug-recovered population and some cells may be undergoing stress. Furthermore, when challenging naïve and drug-recovered cells with nutlin-3a treatment, the drug-recovered population showed an overall higher level of p53 and p21 expression, and more variability within p53 levels, further supporting a more heterogenous escaped population. It remains unclear what biological

significance a more robust p53 response would have in an escaped population, or what the underlying mechanism may be.

Despite having unresolved DNA damage, single division escape from irradiation-induced cell cycle arrest has recently been shown to occur in normal epithelial cells (Reyes et al., 2018). Irradiation promotes oscillation of p53 protein levels (Lahav et al., 2004). Lower amplitude oscillation of p53 protein levels after irradiations resulted in transient decrease of p21, which permits rare cells to escape arrest (Reyes et al., 2018). Cells that undergo a single division were subsequently arrested and display high non-oscillating state of p53. The high levels of p53 were associated with increased micronuclei suggesting that inappropriate escape prior to resolving DNA damage lead to mitotic errors and a more robust p53 response (Tsabar et al., 2020). The switch in p53 response was dependent on PIDD1 expression. PIDD1 association with caspase-2 and RAIDD, known as the caspase-2-PIDDosome, resulted in MDM2 degradation in cells that went through mitotic errors, thus stabilizing p53 and prompting the switch (Tsabar et al., 2020).

My initial interest in determining whether senescent cells could escape, was driven by observations made in chapter 2 and by others that there is an age- and senescence-associated increase in micronuclei and chromatin bridges (Dou et al., 2015; Dou et al., 2017), which I speculate could lead to the loss of genes maintaining the senescent state. This remains an unaddressed question, and a theoretical mechanism that could facilitate escape. Elucidating the mechanism underlying senescence maintenance and the phenotypes of escaped cells is critical for our understanding of tumour progression and human disease. Although genetic loss of p53 and p16 have been shown to permit escape, alternative mechanisms may exist. Furthermore,

addressing how p53 maintains senescence will give insight into the complexity of p53 mediated tumour suppression and cell cycle arrest. The ability of cells to escape a drug-induced senescent-like state raises the possibility that escaped damaged cells may contribute secondary therapy-related cancers or age-associated disease.

4.5 Materials and methods

4.5.1 Cell lines and cell culture

BJ fibroblasts were obtained from the American Type Culture Collection. All cells were maintained in Alpha Minimum Essential Medium supplemented with 10% fetal bovine serum at 37°C at 5% CO₂. Cells were split by rinsing cells with 1xPBS, pH 7.4, and incubating cells with 0.05% trypsin until all cells lifted from the tissue culture flask. Trypsinization was then stopped by adding complete media, followed by pelleting cells by centrifugation.

4.5.2 Drug treatment

BJ fibroblasts were treated with 200 ng/ml doxorubicin (Dox) for 24 hr or the specified amount of time, washed with 1xPBS, pH 7.4, and allowed to recover in drug-free media. BJ fibroblasts were treated with 10 µM Nutin-3a for 7 days or as specified. Nutin-3a was replenished in new media every 3 days for the duration for the experiment.

4.5.3 Cell proliferation and viability

Cell number and cell viability was determined by trypan blue staining, as previously mentioned in Chapter 3.

4.5.4 Single cell cloning

The single cell cloning assay was performed as stated in Chapter 3, however, cells were assessed for colony formation every 7 days.

4.5.5 Immunofluorescence

Immunofluorescence was performed as previously stated in Chapter 2, with the following exception. Immunofluorescence performed on drug-treated cells seeded on coverslips 24 hr prior to drug-treatment. Nuclear intensity was measured in image J. The following additional primary antibodies were used: FL393 (sc-6243, Santa Cruz Biotechnology) and F5 (sc-6246, Santa Cruz Biotechnology).

4.5.6 Scoring γ H2AX positivity

Random fields of view were observed by immunofluorescent microscopy and γ H2AX was scored for each individual primary nucleus. Nuclei containing ≥ 3 γ H2AX foci were considered γ H2AX positive. γ H2AX positive cells were summed and divided by total number of nuclei.

4.5.7 Measuring micronuclei and chromatin bridges

Micronuclei and chromatin bridges were scored as indicated in Chapter 2.

4.5.8 Western blotting

Western blotting was performed as indicated in Chapter 2. The following primary antibodies were used:

4.5.9 Senescence-associated- β -galactosidase staining

SA- β -gal staining was performed as stated in Chapter 2 and 3.

4.5.10 Statistical analysis

Graphpad Prism 9 was used to determine statistical significance. Two-way Student's *t*-test (to compare means of two samples different samples), by one-way ANOVA (to compare means of more than two different samples) was performed to determine P values. Spearman' Rank-Order Correlation analysis was performed to determine

Discussion, limitations, and future directions

5.1 Discussion

The focus of my thesis is on aging, senescence, and their relationship with cancer. Initially I focus on describing the accumulation of cytosolic DNA with aging and in premature aging diseases, which underwent DNA damage and cGAS recognition. The increase of micronuclei and chromatin bridges during aging and senescence led to the hypothesis that the resolution of genomic instability or mutations that occur during senescence may lead to rare cells escaping. This led me to challenge the pre-existing concept that senescence is an irreversible state of cell cycle arrest. I utilized normal human BJ fibroblasts, murine DA3/EPOR leukemia cells, and human OCI-M2 leukemia cells to address whether senescence is reversible. In these studies, I provide *in vitro* evidence that rare cells can resume proliferation after a prolonged period of drug-induced cell cycle arrest, where the drug-treated arrested population display markers of senescence. Here I will discuss the significance of the accumulation of micronuclei and chromatin bridges with age, the broader biological significance and the clinical implications of escaping senescence. I will also discuss the experimental limitations in demonstrating escape from senescence, and the future directions of my project and the field.

5.1.1 Altered secretion during senescence, and aging

A hallmark of both senescence and aging is increased genomic instability, and the altered secretion of proteins and metabolites (López-Otín et al., 2013; Hernandez-Segura et al., 2018).

Telomere attrition, mitotic errors, unrepaired DNA breaks and inappropriate DNA repair can all contribute to the formation of micronuclei and chromatin bridges. My results suggest that the cGAS-STING pathway is activated in response to the elevated levels of micronuclei and chromatin bridges that increase with age. In keeping with these findings, Lan et al. (2019), demonstrated that depletion of STING reduced the innate immune inflammatory response in cells derived from elderly donors. STING-mediated inflammation may be playing a critical role in age-related disease and human health. Supporting this view, individuals over 65 years of age carrying the STING R293Q variant, known to impair STING function, showed a lower susceptibility to age-associated chronic lung disease (Hamann et al., 2020). The influence of cytosolic DNA on other phenotypes of senescence needs to be explored. Premature segmental aging diseases have proved to be a powerful tool to investigate age-associated biological processes and disease. These diseases may be useful models to explore the influence of spontaneously occurring micronuclei and chromatin bridges with respect to their own disease state and normal aging (McClintock et al., 2007; Olive et al., 2010; Scaffidi and Misteli 2006).

Chronic inflammation is a complex process driven by multiple factors. The accumulation of cytosolic DNA represents one element that drives the inflammatory response and one aspect of SASP in aging and senescent cells. Chemotherapeutics have systemic harmful effects throughout the body, promoting DNA damage, senescence, and cell death in non-diseased organs. The short- and long-term effects of therapy have negative consequences on healthspan (Hudson et al., 2013). Chemotherapy increases the risk factor of thrombotic events in cancer patients (Ashrani et al., 2016; Heit et al., 2000; Huang et al., 2011). The rapid accumulation of drug-induced senescence promotes a sudden flux in SASP genes. SILAC driven analysis of senescent cell

supernatant demonstrated an increased secretion of coagulation-related proteins and the removal of drug-induced senescent cells *in vivo* increased tail bleeding time (Wiley et al., 2019). Additionally, normal cells that undergo therapy-induced senescent have been shown to contribute to cancer relapse and metastasis through SASP (Demaria et al., 2017).

Seno-therapy, therapy aimed at killing or modulating the SASP of senescent cells, is beginning to show promise as a therapy for both age-related diseases and as an adjuvant therapy for cancers (Wang et al., 2022). The first senolytic clinical trial showed improved physical function in idiopathic pulmonary fibrosis (Justice et al., 2019). Several other senolytic clinical studies are underway for diseases such as Alzheimer's, chronic kidney disease, COVID-19, and others (Gerdes et al., 2021). The removal of senescent cancer cells and senescent normal cells with a senolytic after chemotherapy may improve certain side effects, improve quality of life, and alter the rate of tumour progression.

I have presented evidence that non-transformed cells are capable of escaping a drug-induced senescent-like state. The escaped population has an altered, more robust albeit heterogenous p53 response to nutlin-3a. Although my work is limited to an *in vitro* setting, *in vivo* escape may be possible. It will be interesting to determine whether *in vivo* escape can occur and if *in vivo* escaped cells are altered from their naïve state. Escaped cells may retain aspects of senescence, as demonstrated by the XC2 DA3/EPOR clone. Senolytics are unlikely to eliminate escaped cells and may contribute to age-related disease.

5.1.2 Escape from senescence

The reversibility of a drug-induced senescent-like state raises the possibility that senescent states may also be reversible. Cdc6-mediated oncogene-induced senescence has recently been shown to be reversible (Zampetidis et al., 2021). Whether normal cells can escape from RAS or BRAF-induced senescence has remained unknown. Ectopic expression of mutant RAS or BRAF in normal cells *in vitro* promotes oncogene-induced senescence, halting proliferation.

Paradoxically, a frequent early event in the evolution of pancreatic ductal adenocarcinoma (PDAC) and melanoma are mutations in K-RAS and BRAF, respectively (Shain et al., 2015; Yachida and Iacobuzio-Donahue, 2013). Both K-RAS and BRAF mutations are often succeeded by the loss of CDKN2A (p16) and TP53 (p53), two master regulators of senescence. How can a mutation in RAS/BRAF be beneficial for tumour development if proliferation is halted in the presence of wtp53 and p16? Nevertheless, RAS/BRAF mutations must endow an early selective advantage in PDAC and melanoma. Despite senescence being a tumour suppressive barrier, I have shown that senescent gene sets are associated with poor overall survival, suggesting that senescence may be playing a role in supporting tumour progression. Senescence is associated with transcriptional reprogramming where senescent cells gain stem cell-like properties and undergo epithelial-to-mesenchymal transition (Milanovic et al., 2017; Zampetidis et al., 2021). I speculate that senescence may be an important stage in the evolution of some cancers. I think that the role of oncogene-induced senescent cells in melanoma and pancreatic cancer ought to be re-evaluated in light of the ease with which cells can escape senescence.

5.1.3 Drug-induced senescence may be a form of non-genetic resistance

My findings highlight that escape from a drug-induced senescent-like state can occur in both normal and transformed cancer cells. Cells that escape the senescent-like state appears to not

always be resistant to second round of drug-treatment or incapable of re-enter senescence, suggesting a plastic mechanism of escape. The senescent-like state may be a non-genetic mechanism that cells exploit to survive drug treatment and perhaps other stresses. Senescent cells upregulate the anti-apoptotic response, enabling survival through drug-treatment. The rare ability of some senescent cells to escape and resume proliferation suggests that cancer cells may exploit the non-proliferative senescent state to persist through therapy only to emerge after the removal of drug. Such a survival mechanism would be independent of pre-existing mutations that enable resistance to drug. Alternative non-genetic mechanisms of survival through drug treatment have been described and are under the umbrella term ‘persister cancer cells.’

Cancer relapse has often been viewed as a form of Darwinian selection. Where therapy imposes a selective pressure eliminating tumour cells but rare clones that harbour genetic mutations or stable epigenetic changes in genes that confer resistance are able to survive therapy. Whether these rare cells are capable of persisting through drug treatment pre-exist within the population or adapt to drug during treatment has recently begun to be elucidated. The residual resistant clones are able to proliferate, and the progeny are resistant to therapy. Cancers that relapse and stem from a resistant clone will be resistant to the previous therapy. However, a retreatment response, where relapsed tumours are responsive to the same primary treatment, in relapsed ovarian, colorectal, and small-cell lung carcinoma have been reported (Kuczynski et al., 2015). The retreatment response suggests that in some cases of relapse, tumours gain transient non-genetic ‘resistance’ during therapy termed drug-persistence.

Multiple, non-mutually exclusive, mechanisms have been shown to facilitate survival of drug-persister cells, including epigenetic remodelling, altering metabolism, decreasing proliferation and changing cell state (**Figure 5-1**). Sharma and colleagues (2010) were the first to demonstrate that PC9 non-small cell lung cancer cells were able to enter a reversible drug-tolerant persistent state. A sub-population of cells survived without de novo mutations in known resistance genes. Persister cells temporarily decreased their rate of proliferation and expressed stem cell markers during drug-treatment. Drug-tolerant persister (DTP) cells retained drug sensitivity after a short recovery period. DTP PC9 cells undergo a KMD5A dependent chromatin remodelling, which is necessary for survival through drug-treatment (Sharma et al., 2010).

Rehman and colleagues showed that all cancer cells have an equal likelihood of entering a DTP state, and that there was not a sub-population of DTP cancer cells that was selected for. DTP colorectal cancer cells survive treatment by reducing their rate of proliferation and entering diapause-like state (Rehman et al., 2021). In keeping with this work, my work and Duy et al. (2021) have also demonstrated that drug-treated leukemia cells enter a senescent- diapause-like state. Together these findings suggest that the some DTP stress states are conserved across tumour type and species.

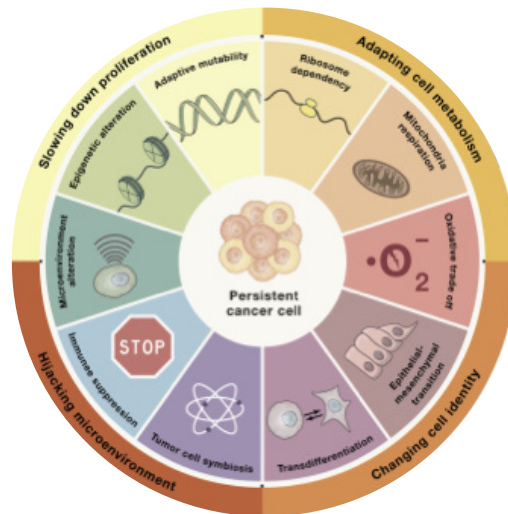


Figure 5-1 Multiple pathways that facilitate drug-tolerant persistent cancer cells.

Adapted from Shen et al., 2020. The various pathways that facilitate drug-persistence fall within four major categories: 1) Slowing down proliferation, 2) Adapting cell metabolism, 3) Changing cell identity, 4) Hijacking the microenvironment. Importantly, these pathways are likely interconnected.

Development of the watermelon system, a single cell immunofluorescent based assay that monitors proliferation status of cells that is compatible with barcoding and single cell RNA-seq, revealed heterogeneity in PC9 persister cells during drug treatment. During drug treatment PC9 persister cells demonstrated proliferative heterogeneity; some clones were able to retain proliferative capacity at a fast rate, some proliferated at a medium rate, and others arrested. Single cell RNA-seq demonstrated metabolic distinctions between dividing and non-dividing persister cells; increased glutathione and NRF2 expression in cycling persisters linked the maintenance of reactive oxygen species (ROS) was responsible for the two different persister states. Clearance of ROS by N-acetylcysteine, knockout of KEAP1, a negative regulator of NRF2, or overexpression of glutathione peroxidase 2 (GPX2) resulted in an increased proportion of cycling persisters (Oren et al., 2021). Similarly, GPX4 expression was previously shown to be necessary for persister cell survival, by preventing ferroptosis-mediated death (Hangauern et al., 2017).

Although senescence is a tumour suppressive barrier and a largely beneficial outcome of cancer therapy with respect to preventing tumour growth, my results and other recent studies have begun to suggest that senescence may not an optimal outcome of therapy and warrants reservation. Drug-induced senescence, like drug-tolerant persister cells, may be a mechanism exploited by cancer cells to survive therapy, and re-emerge at a later time. The presence of senescent cancer cells presents an opportunity to utilize senolytics as an adjunctive therapy, and fully eliminate residual cancer cells.

5.2 Conclusion

In this thesis I have provided evidence of two important aspects of drug-induced senescence in normal BJ fibroblasts and leukemia cells. First, escape from drug-induced senescence is rare but importantly can occur. Secondly, the escaped population is diverse. My findings are important to the fields of senescence, cell fate, and cancer biology. However, my PhD work has been largely observational and has only begun to shed light on the fate of senescent cells. Future work should focus on further validating escape from drug-induced senescence using a more rigorously defined senescent population and determine the mechanism or mechanisms of escape. It will be interesting to determine whether escape from senescence can occur *in vivo*; identifying biomarkers of escape will aid in this goal and may be useful in clinical settings. Finally, studying the extent of the heterogeneity and the mechanism of heterogeneity in escape cells would be interesting; are escaped cells heterogenous due to pre-existing differences in the population or arises de novo during senescence and escape.

5.3 Future directions and limitations

The strength and novelty of my work relies on the use of a single cells assay that monitors clonal expansion from single senescent cells. This assay demonstrates that rare cells do escape senescence and resume proliferation. Previous work has relied on cell population studies and on cell sorting of senescent cells by flow cytometry. Population studies do not differentiate between senescent cells that escape senescence, transiently arrested or slow dividing cells that never arrested. Flow cytometry does not yield entirely pure populations of senescent cells that are necessary to measure escape.

An inherent issue in describing escape from senescence is limited by the lack of a universal and specific senescence biomarker. Senescent cells show extensive phenotypic heterogeneity making it difficult to find a conserved senescent biomarker (Cohn et al., 2022). Although there are many biological markers of senescence, each is limited SA- β -gal positivity, along with the absence of proliferation has been the gold standard in demonstrating senescence. However, SA- β -gal positivity alone cannot accurately define a senescent cell, as SA- β -gal can increase in other stress conditions, is largely limited to in vitro assays that require cell fixation, and subjective classification of positivity. Therefore, additional biomarkers are used to define senescent cells such as the accumulation of cytosolic DNA and SASP, nuclear changes such as decrease of lamin B1, telomere loss, and senescence-associate heterochromatin foci. To address this issue, Gorgoulis and colleagues recommend using multiple markers associated with senescence to increase the confidence of identifying a senescent cell. The proposed algorithm to detect a senescent cell would be to first screen for senescence using SA- β -gal or the accumulation of lipofuscin, followed by further validation of senescence using additional markers such as

decreased lamin B1 expression, the increase of markers of cell cycle arrest, such as increased levels of p21 and p16. Following the identification of some of these markers, senescent cells can be further examined by looking for markers of SASP and the DDR (Gorgoulis et al., 2019).

5.3.1 Confirm escape from drug-induced senescence

Further evidence that cells escape senescence would help support my claim that senescence is a reversible process in some rare cells. In order to confidently show escape from senescence, cells will be followed with live cell imaging utilizing multiple fluorescent markers of senescence. One allele of CDKN1A (p21), LMNB1 (lamin B1), and SERPINB2 (SerpB2) will be tagged with different constitutively fluorescent protein (i.e. mCherry, mVenus, and mTurquoise), from here on termed senescent-associated tagged (SAT) cells. SAT cells will be treated with drug to promote senescence and be sorted based on SA- β -gal positivity. Using all four markers of senescence, cells will be monitored using live cell imaging for several days or weeks after drug exposure to determine whether p21 and Serpin B2 levels decrease, lamin B1 levels increase and whether proliferation resumes. Clones that resumed proliferation and initially displayed the markers of senescence will clearly demonstrate that senescence is a reversible program.

5.3.2 Determine whether cancer cells that escape from drug-induced senescence has clinical relevance

Escape from drug-induced senescence may be a process that contributes to tumour relapse. The question whether escape from senescence may contribute to tumour relapse remains largely unexplored due to the preconceived notion that senescence is permanent state of cell cycle arrest. To address whether escape from drug-induced senescence has clinically significant, two main

questions need to be addressed: 1) Can cells escape drug-induced senescence *in vivo*? 2) Is there a biomarker of escape and, if so, is it associated with tumour relapse?

5.3.2.1 In vivo assessment of escape from drug-induced senescence

The difficulty in assessing escape from senescence *in vivo* stems from the lack of senescence biomarkers that can be used in an *in vivo* environment while preserving cell viability. To determine whether escape from senescence can occur *in vivo* and is clinically relevant, two similar experimental approaches will be used. The first approach will be a proof-of-concept experiment, in which SAT AML cells will be treated with doxorubicin or daunorubicin in an *in vitro* setting, sorted based on the fluorescent tagged markers of senescence, and cells will be injected intrafemorally into NOD/SCID mice. Tumour progression and the xenograft cell population will be observed by femoral sampling, and overall survival will be assessed.

A drug-induced senescence xenograft tumour model will be informative however, lacks clinical relevance. To compliment the findings in a more clinically relevant manner, naïve, proliferating SAT AML cells will be injected intrafemorally into NOD/SCID mice. Once mice show more than 50% engraftment, mice will be treated with daunorubicin and vehicle control for 3 days. One week after treatment, drug- and vehicle control-recovering SAT AML cells will be isolated and sorted into two sub-populations, SAT positive AML cells and SAT negative AML cells. SAT positive and negative cell sub-population will be xenografted into new NOD/SCID mice. Survival and tumour growth will be monitored to determine whether tumour cells that escape from drug-induced senescence contribute to tumour relapse and influence the overall

aggressiveness of the relapsed tumour. Control group will consist of mice injected with cells derived from vehicle control treated mice.

5.3.2.2 Identifying a biomarker of escape from drug-induced senescence

To provide further insight as to whether escape from drug-induced senescence contributes to tumour relapse in patients, identification of a biomarker of escape is of key importance.

Identifying a marker of escape will be useful for basic research and may also serve as a diagnostic or prognostic tool for tumour relapse. To identify a biomarker of escape, I will screen multiple SAT expressing AML cell lines that entered senescence and escaped, based on the aforementioned SAT criteria (5.3.1). Escaped, senescent and naïve proliferating cells will be subjected to bulk RNA-sequencing to determine conserved differentially expressed genes in the escaped populations compared to both senescent and naïve populations. Candidate genes will be validated by qRT-PCR and, if applicable, western blotting. Once a biomarker of escape is established, publicly available gene expression data can be analyzed to determine whether AML relapse is associated with the expression of the candidate genes.

5.3.3 Determine which genes are responsible for maintaining senescence

A key outstanding question from the work I presented is determining the mechanism of escape from drug-induced senescence, for both DA3/EPOR cells and BJ fibroblasts. In order to determine which genes maintain the senescent phenotype, I will promote drug-induced senescence DA3/EPOR cells and BJ fibroblasts, and perform Perturb-seq. Perturb-seq is a novel technique developed by the Weissman laboratory, that utilizes single cell RNA-seq to analyze a

pooled genome-wide CRISPR-mediated gene inactivation (CRISPRi) screen (Replogle et al., 2022). Cells will be treated with drug and sorted based on SA- β -gal positive cells using the fluorescent C12-FDG dye. C12-FDG positive cells will be transduced with a genome-wide CRISPRi lentiviral library of an MOI of <0.3 and undergo selection. Two unique gRNAs will be used for each gene. Multiple scramble guide RNAs (gRNAs) will be utilized as negative control. Cells will be harvested over multiple days after infection. Single cell RNA-seq will provide insight into the transcriptional states of each individual cell and determine the effect of each gene perturbation on the transcriptional state. Cells will be assessed based on cellular transcriptional states; Fridman's senescent gene signature and proliferating gene signature will be used to differentiate senescent and proliferating cells, respectively. gRNA expression will be correlated with depletion of target RNA, to ensure efficient knockdown, and the associated transcriptional state. Genes that display both gRNA are associated with a loss of the Fridman's senescent gene signature and increase of the proliferation gene signature will be considered candidate genes that promote escape from senescence. Furthermore, harvesting samples over multiple timepoints and determining the number of cells with unique gRNA will suggest which gRNA promote cell proliferation. To validate that candidate genes maintain senescence, shRNA that target candidate genes will be cloned into doxycycline-inducible vectors and expressed in BJ fibroblasts and DA3/EPOR cells; cells will be treated with drug to promote senescence, and subsequently treated with doxycycline to determine whether knockdown of candidate genes promotes escape.

5.3.4 Identify whether chloroquine has *in vivo* senolytic potential

From my work in chapter 4, I have identified that chloroquine specifically eliminates some drug-induced senescent leukemia cells. Whether chloroquine has similar senolytic potential *in vivo*

remains unknown. AML cells will be inject intrafemorally into NOD/SCID mice. Once mice show more than 50% engraftment, mice will be treated with daunorubicin or vehicle control for 3 days. Blood and femoral samples will be obtained to ensure that AML cells entered drug-induced senescence. One-week post-drug treatment, daunorubicin and vehicle control treated mice will be treated with chloroquine or vehicle control. Overall survival will be documented for all four groups; i) vehicle control, vehicle control, ii) vehicle control, chloroquine, iii) daunorubicin, vehicle control, iv) daunorubicin, chloroquine.

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