

Single-cell transcriptomics identifies Mcl-1 as a target for senolytic therapy in cancer

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sant'Agostino

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List of abbreviations

PCa	Prostate cancer
BHP	Benign prostatic hyperplasia
PIN	Prostatic intraepithelial neoplasia
LGPIN	Low-grade intraepithelial neoplasia
HGPIN	High-grade intraepithelial neoplasia
PSA	Prostate-specific antigen
ADT	Androgen deprivation therapy
CSPC	Castration sensitive prostate cancer
CRPC	Castration resistant prostate cancer
LHRH	Luteinizing hormone-releasing hormone
AR	Androgen receptor
PTEN	Phosphatase and Tensin-Homolog-Gene
PI3K	phosphoinositide 3-kinase
PI (3,4,5)P3 or PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PI (4,5)P2 or PIP2	Phosphatidylinositol 4,5-bisphosphate
LOH	Loss of heterozygosity
GEMMs	Genetically Engineered Mouse Models
SA- β -Gal	Senescence-associated β -galactosidase
BrdU	5-bromodeoxyuridine
RB	Retinoblastoma
miRNAs	microRNAs
SASP	Senescence-associated secretory phenotype
ROS	Reactive oxygen species
MMPs	Matrix metalloproteinases
MSC	Mesenchymal stem cell
OIS	Oncogene-induced senescence
HCC	Hepatocellular carcinoma
PICS	PTEN induced cellular senescence
DDR	DNA damage response
CDKs	Cyclin-dependent kinases

TIS	Therapy-induced senescence
AURKs	Aurora kinases
PLKs	Polo-like kinases
NF-kB	Nuclear factor-kB
C/EBP β	CCAAT/enhancer-binding protein- β
TME	Tumor microenvironment
MDSCs	Myeloid derived suppressor cells
NK	Natural killer
Nav-Gal	Galacto-conjugated navitoclax drug
PROTAC	Proteolysis-targeting chimaera
BET	Bromodomain and extraterminal domain
uPAR	Urokinase-type plasminogen activator receptor
CTCs	Circulating tumor cells
DCCs	Disseminated cancer cells
EMT	Epithelial-to-mesenchymal
CXCL1	C-X-C-motif ligand
OPN	Osteopontin
CTGF	Connective tissue growth factor
ECM	Extracellular matrix
DAMPs	Damage-associated molecular patterns
GPX4	G glutathione (GSH)-dependent enzyme glutathione peroxidase
PUFAs	Phosphatidylethanolamine-containing polyunsaturated fatty acids
PARP1	poly (ADP-ribose) polymerase 1
MIF	Macrophages migration inhibitory factor
AIF	Apoptosis inducing factor mitochondria associated 1
DISCs	Death-inducing signaling complexes
BCL2	BCL2, apoptosis regulator
BH	BCL2 homology domain
MOMP	Mitochondria outer membrane permeabilization
BAX	BCL2 associated X, apoptosis regulator
BAK	BCL2 antagonist/killer 1
BCL2L1 or BCL-XL	BCL2 like 1

MCL1	Myeloid Cell Leukemia Sequence 1
BCL2L2 or BCL-W	BCL2 like 2
scRNA-seq	Single-cell RNA sequencing
GEMMs	Genetically engineered mouse models
Pten ^{pc/-}	Pten-null prostate conditional
FACS	Fluorescence-activated cell sorting
UMAP	Uniform Manifold Approximation and Projection
SIT	Senescence Index Tool
GSEA	Gene Set Enrichment Analysis
NES	Normalized enrichment score
TF	Transcription factor
c.m.	Conditioned media
IHC	Immunohistochemistry
TAMs	Tumor-associated macrophages

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Abstract

Prostate cancer (PCa) is one of the most diagnosed cancers in men worldwide, prompting extensive research into effective treatment strategies. This type of cancer is an androgen dependent tumor. The initial therapeutic options involve prostatectomy and androgen deprivation therapy (ADT), which in most of the cases is sufficient to treat the cancer. However, in some cases the phenotype of PCa becomes more aggressive, developing resistance to ADT. In this context, chemotherapies and androgen target therapies are needed to control tumor progression. All these treatments aim to trigger apoptosis and programmed cell death. However, new research indicates that most of the anticancer therapies in clinics can induce senescence; consequently, cancer cells use cellular senescence as an alternative survival strategy to avoid apoptosis.

Cellular senescence is a state in which cells don't divide and undergo a stable irreversible growth arrest. Various internal and external factors, including normal aging processes, DNA damage, stress, cellular insults, and therapies can trigger this phenomenon.

Since senescent cancer cells stop proliferating, cellular senescence initially acts as a protective mechanism against tumor progression and initiation of potential malignancies. However, senescent tumor cells, despite being non-proliferative, remain metabolically active and exhibit a distinct secretory phenotype, called senescence-associated secretory phenotype (SASP), which contribute to tumor progression, proliferation, and metastasis over time.

For these reasons, the elimination of detrimental senescent cells using specific drugs named senolytic is needed.

In this PhD project, we have used single-cell RNA sequencing as a powerful technique to investigate in depth the survival mechanisms of senescent tumor cells. I have developed a bioinformatic algorithm named Senescence Index Tool (SIT), to identify senescent cells in two prostate cancer mouse models, the $Pten^{PC-/-}$ and the $Pten^{PC-/-}; Timp1^{-/-}$. The SIT is based on two main features of senescence: the upregulation of canonical senescence markers and the cell cycle arrest.

In this context, the antiapoptotic gene Mcl-1 has been identified as a key player in the survival of senescent cells. Remarkably, Mcl-1 shows elevated expression levels in senescent tumor cells, including the senescent cells where Bcl-2, a well-known senolytic target, is expressed at low levels.

We have investigated both in vitro and in vivo the potential of targeting Mcl-1 as an innovative senolytic therapy in comparison with Navitoclax, the gold standard used in the clinics. While the Bcl-

2 inhibitor Navitoclax demonstrated efficacy in reducing metastases, the Mcl-1 inhibitor S63845 resulted in the complete elimination of senescent tumor cells and the associated metastases.

MCL-1 also emerged as a pro-survival factor of senescent cells in the application of SIT on datasets of human single-cell RNA sequencing. Moreover, the SASP analysis in the human senescent cells highlighted the presence of factors involved in the promotion of migration of tumor cells, enhancing the role of senescence in promoting metastases.

In conclusion, in this project, we have discovered that, although senescent tumor cells are a heterogenous population, they rely on Mcl-1 for survival. Indeed, a potent senolytic therapy targeting Mcl-1 impacts metastases outcomes. By expanding our comprehension of prostate cancer possible treatment, this research offers a promising opportunity to enhance therapeutic effects and counteract aggressive phenotypes and therapy-induced senescence.

1 Introduction

1.1 Prostate cancer

Prostate cancer (PCa) is the second most common diagnosed solid tumor in men, with one out of 8 men being diagnosed with PCa during their lifespan, representing a major cause of cancer-related mortality in men¹. The prostate is a gland of the male reproductive system, which has the function of producing and secreting fluids, part of semen, to maintain sperm viability.

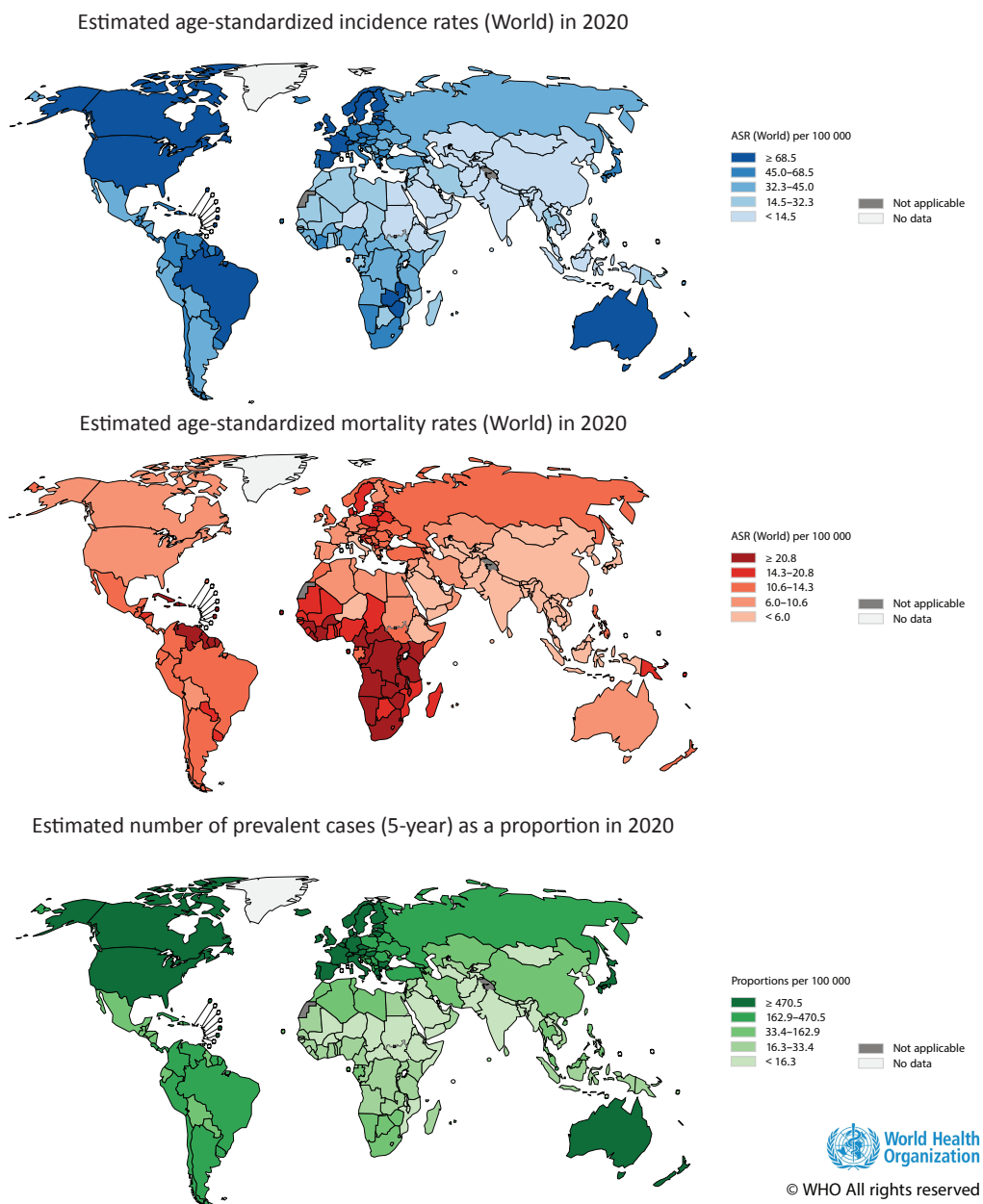


Figure 1: Global geographical incidence, mortality and prevalent cases of prostate cancer

(Top) Global incidence of prostate cancer in 2020; (Middle) Global mortality from prostate cancer in 2020; (Bottom) Global incidence of prevalent cases in 2020. Figures are adapted from the Global Cancer Observatory (<https://gco.iarc.fr/today/home>)

Different risk factors influence prostate cancer onset and outcome, including age, race, nationality, diet/obesity and familiarity. Diet and obesity are the only modifiable risk factors and it has been demonstrated that the adoption of a low-fat diet is associated with a lower risk of developing prostate cancer². Furthermore, the risk of developing prostate cancer increases with age, with 70% of men diagnosed with the disease being over 65 years old. The race is considered another important risk factor for PCa. African Americans have the highest incidence rate globally. Interestingly, the disease is common in North America and Europe, while it is rare in Asia, Africa, and South America (Fig. 1)^{3,4}.

In addition, PCa risk is strongly associated with familiar background. In fact, individuals with a familial history of PCa have the highest incidence of the disease (20.2% of men up to age 72), followed by breast cancer (13.6%) and colorectal cancer (12.8%)⁵.

Benign prostatic hyperplasia (BPH) can occur during aging, causing an increase in the size of the prostatic gland. However, this condition is not directly linked to PCa onset.

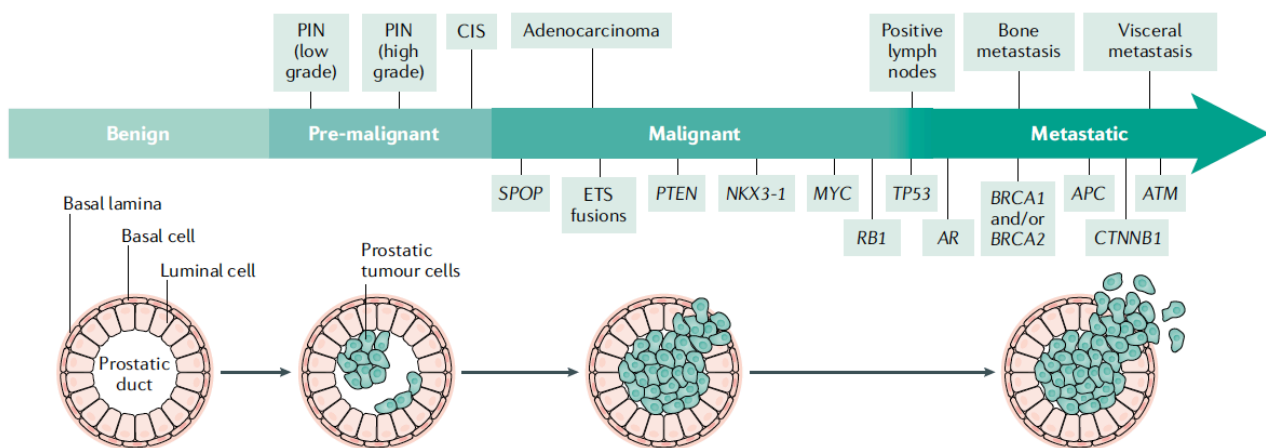


Figure 2: Prostate cancer development and genetic alteration during progression.

Prostate hyperplasia PIN develops into malignant adenocarcinoma, which can acquire metastatic phenotype and spread outside to the lymph nodes, bones, or viscera. Figure adapted from³.

Oxidative stress, DNA damage, genetic and epigenetic alteration, inflammation, genetic factors and telomere shortening are processes known to initiate prostate cancer tumorigenesis, which is a multi-step process. In fact, in pathological conditions, the prostate gland starts to proliferate abnormally rising to prostate hyperplasia (PIN), without affecting the gland structure. Subsequently, when tumoral cells invade the stroma surrounding the glands, PCa is classified as low-grade intraepithelial neoplasia (LGPIN). If the stromal infiltration is massive, high-grade PIN (HGPIN) of the tumor is reached. Subsequently, when the migratory and invasive capability of tumor cells is enhanced, PCa develops as adenocarcinoma and then progresses as metastatic prostate cancer when tumor cells

reach distant organs and form new niches called metastases (Fig. 2)^{6,7}. The most common sites of metastases formation are pelvic lymph nodes (99%) and bones (84%), however distant lymph nodes (10.6%), liver (10.2%), lungs (9.1%) and brain (<2%) can be uncommon sites of metastatization. Of note, metastases in soft tissues are associated with poorer survival⁸.

Since prostatic tumor cells express high levels of androgen receptor (AR), PCa is considered a hormone dependent-tumor. Typically, prostate cancer is detected through a blood test to measure prostate-specific antigen (PSA) level, a serine protease activated by AR and elevated in patients with prostate cancer. When a high level of PSA is detected, appropriate examinations like echography, multiparametric magnetic resonance imaging or micro-ultrasound technology on the prostate with subsequent prostate biopsy are needed to diagnose prostate cancer⁹.

PCa treatment depends on the stage of the disease at diagnosis and involves a combination of surgical removal, hormonal therapy, androgen deprivation therapy and chemotherapy³ (Fig.3).

When prostate cancer is diagnosed at the localized stage, standard treatments include surgical excision (prostatectomy) and irradiation using radiotherapy (beam therapy) or brachytherapy (implantation of radioactive sources)¹⁰.

To target the androgen sensitivity of prostate tumor cells, patients undergo androgen deprivation therapy (ADT), also known as chemical castration. ADT aims to lower androgen levels to control tumor progression. Several strategies have been developed for ADT. Luteinizing hormone-releasing hormone (LHRH) using agonists or antagonists, AR blockers such as Enzalutamide, Apalutamide or Darolutamide, and Abiraterone, an inhibitor of CYP17, an enzyme involved in androgen synthesis, are the gold standard therapy in the castration-sensitive phase³ (Fig.3).

Response to the therapy is achieved through regular monitoring of PSA levels and/or radiological examinations. Of note, a decrease in PSA levels indicates a positive response to the therapy. However, in case of biochemical relapse, when PSA levels increase, prostate cancer transforms from castration sensitive prostate cancer (CSPC) phase to a castration resistance prostate cancer phase (CRPC).

CRPCs are the most lethal form of PCa, characterized by the metastasis's onset. In CRPC phase, chemotherapy with the administration of Docetaxel, Cabazitaxel and Radium-223 is employed as palliative treatment (Fig.3).

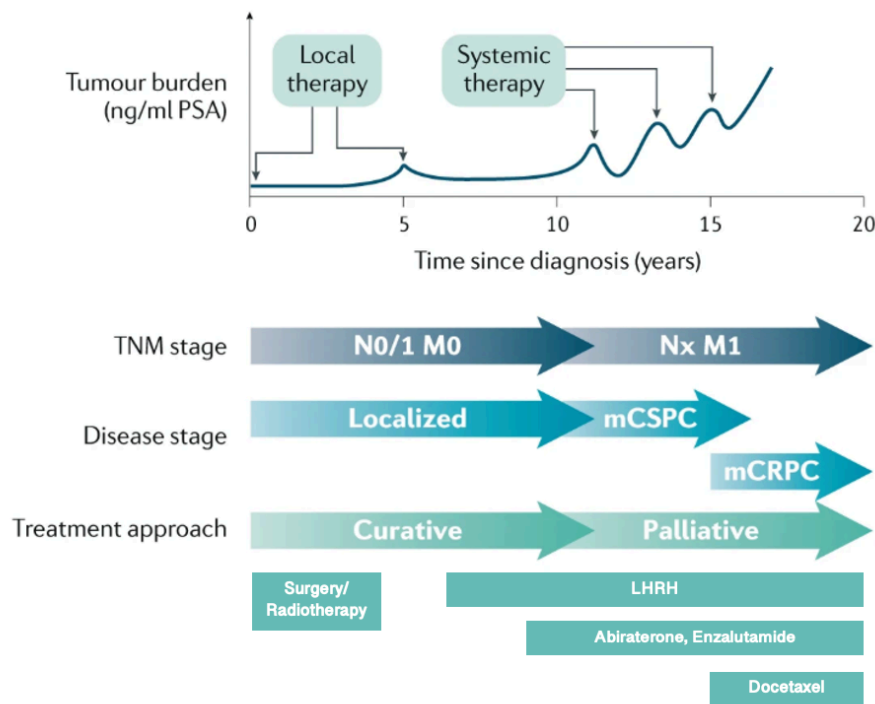


Figure 3 : Prostate cancer treatments across the progression

Schematic scheme of disease stage and the treatment approaches starting from the diagnosis. Figure adapted from ³.

PCa is the most heritable cancer since its onset is related to genetic factors. Germline mutations in DNA repair genes such as BRCA1, BRCA2, ATM, ATR, PALB2, CHECK2, and mismatch repair genes (MSH2, MSH6 and PMS2) increase the PCa susceptibility¹¹. Interestingly, men harboring these mutations represent a large proportion of those developing metastatic prostate cancer¹².

Moreover, the accumulation of somatic mutations in the prostatic epithelium is associated with PCa development. Aberrations can occur in either oncogenes or tumor suppressor genes, resulting in alteration of transcription and translation of tumor cells. These changes affect the functional homeostasis of the prostate gland.

The most common genetic aberrations in prostate cancer patients consist of TP53 and SPOP mutations, RB1 and PTEN deletion, MYC amplification and TMPRSS2–ERG fusion¹³. Of note, the androgen receptor (AR), one of the key modulators of PCa, undergoes amplification in the late stage of the disease, in the CRPC phase. More than one genetic alteration can coexist in a patient. Mutations can drive tumor development and/or can be acquired during tumor progression and treatment, resulting in therapy resistance and enhanced aggressiveness. For instance, although AR amplification is completely absent in primary tumors, genetic alterations in AR emerge during treatment and progression of prostate cancer to CRPC, indicating a role in ADT therapy resistance¹⁴.

1.1.1 Phosphatase and Tensin-Homolog-Gene (PTEN)

The loss of Phosphatase and Tensin-Homolog-Gene (PTEN) is one of the most frequent mutations in advanced and metastatic prostate cancer patients¹⁵. PTEN is a tumor suppressor gene and its inactivation or deletion has been associated with different types of cancer, including glioma, kidney disease, breast, lung, and prostate cancers¹⁶. The human PTEN gene locus is located on chromosome 10 and has a critical role in regulating cell survival mediated by phosphoinositide 3-kinase (PI3K)-AKT signaling^{13,17,18}. In particular, PTEN functions as a phosphatase with dual specificity, meaning it can convert phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3 or PIP3) into phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2 or PIP2). This mechanism makes PTEN the direct antagonist of the PI3K-AKT pathway. When PTEN is lost or mutated, AKT and the downstream mTOR pathway are activated, eventually promoting uncontrolled cell proliferation, increased angiogenesis, and resistance to apoptosis^{19,20}. The most diffused mutation in PTEN happens within exon 5, which encodes the catalytic domain, resulting in PTEN loss-of-function²¹. However, PTEN can also show point- or epigenetic mutations at lower rates of loss of heterozygosity (LOH).

Progression from localized to metastatic disease and from CSPC to CRPC has been thought to involve the deregulation of key genes in growth control. PTEN loss-of-function mutations can be found in over 12-17% of localized and mCSPC tumors. However, PTEN deletions are more prevalent in mCRPC, being present in over 40% of tumors¹³. This suggests that these mutations play a significant role both in the transformation of normal cells into cancerous ones and in the progression of the disease. In conclusion, approximately 70% of PCa exhibit mutation or loss of PTEN, being absent in the benign prostatic epithelium or in PIN. However, PTEN alterations are enriched in HGPIN, prostate adenocarcinoma, and metastatic prostate cancer patients. Therefore, PTEN alterations confer malignant capabilities to prostate cancer²².

The use of prostate cancer genetically engineered mouse models (GEMMs) has significantly enhanced knowledge of different stages of the disease, including the molecular mechanisms of metastasis formation^{23,24}. For example, loss of PTEN alone can lead to the development of adenocarcinoma²⁵. However, when combined with other genomic alterations like mutation in Kras (Kras^{G12D})²⁶, loss of Smad4²⁷, and loss of TIMP1²⁸, it can activate the metastatic potential of prostate cancer (PCa).

1.2 Senescence

Cellular senescence is a stable cell cycle arrest that occurs in diploid cells in physiological and/or pathological conditions, limiting the proliferative capability of the cells. Since the cell growth arrest in senescence is irreversible, it differs from the reversible cellular quiescence, in which cells remain capable of starting division upon stimulation²⁹.

The term “cellular senescence” by Hayflick and Moorhead dates back to the 1960s. They observed that human fibroblasts had a limited proliferative capability and can reach a maximum number of cell divisions³⁰. This observation was defined as replicative senescence and it arises from a progressive reduction in telomere length with each cell division, serving as a natural response to control genomic instability and the subsequent accumulation of DNA damage³⁰. In addition, the accumulation of senescent cells has been reported during aging and age-related disorders. Of note, cellular senescence can occur in cells subjected to different insults, including treatment with chemo-radiotherapy or targeted therapies, loss of tumor suppressor genes and overexpression of oncogenes, as well as genotoxic stress and metabolic shock^{29,31-33}.

1.2.1 Hallmarks of senescence

Senescent cells can be identified through a variety of biomarkers. However, it is essential to note that none of these individual markers can be universally applied to all types of senescence²⁹. The primary characteristic that identifies all senescent cell types is the cell cycle arrest, specifically in the G1 or G2 phase, which differs from the growth arrest during quiescence that occurs in the G0 phase of the cell cycle³⁴.

Additionally, senescent cells are characterized by several other hallmarks. These include increased senescence-associated β -galactosidase (SA- β -gal) activity at pH 6, which can be revealed using proper kits as blue color staining followed by microscopic examination.

Senescent cells lack proliferative markers such as Ki-67 protein or 5-bromodeoxyuridine (BrdU). Senescent cells also express cell cycle inhibitors, such as p16^{INK4a}, p21^{Waf1/Cip1}, p15^{INK4b} and p27^{Kip1} as well as exhibit the hypophosphorylation of Retinoblastoma (RB)^{29,35}. Morphologically, senescent cells commonly exhibit altered cell size, with a smoother shape than proliferating cells. They also

display the formation of senescence-associated heterochromatin foci³⁶, loss of lamin B1³⁷, DNA damage foci³⁸, and senescence-associated distension of satellites³⁹.

Additionally, it is known that senescent cells upregulate certain microRNAs (miRNAs) and secrete many factors, including growth factors, cytokines, chemokines, and proteases. This secretory phenotype is commonly referred to as the senescence-associated secretory phenotype (SASP) or senescence-messaging secretome.

Further, senescent cells are metabolically active. To maximize energy production, arrested cells engage in abnormal fatty acid oxidation, oxidative phosphorylation, and glycolysis processes, eventually increasing their oxygen consumption^{40,41}. Indeed, senescent cells often exhibit impaired respiratory chain function and an overabundance of reactive oxygen species (ROS) in their mitochondria⁴². It is known that cellular senescence is linked to an increased quantity of mitochondria, but these mitochondria have a reduced membrane potential, resulting in amplified ROS generation. Intriguingly, mitochondrial dysfunction can directly trigger senescence^{43,44}.

In conclusion, there are four main hallmarks of senescence that can be used to identify senescent cells (Fig. 4).

- 1- Cell cycle arrest: Inhibition of the cyclin activity occurs in cellular senescence by upregulation of p21^{Waf1/Cip1} and p16^{INK4a}. Mainly, p21^{Waf1/Cip1} plays a role in the early events of senescence, while p16^{INK4a} is involved in the later phases of senescence^{45,46}.
- 2- DNA damage: A DNA damage response of cells induces cellular senescence by activation of canonical p53/p21 pathway⁴⁷.
- 3- Secretory phenotype.
- 4- Altered metabolism.

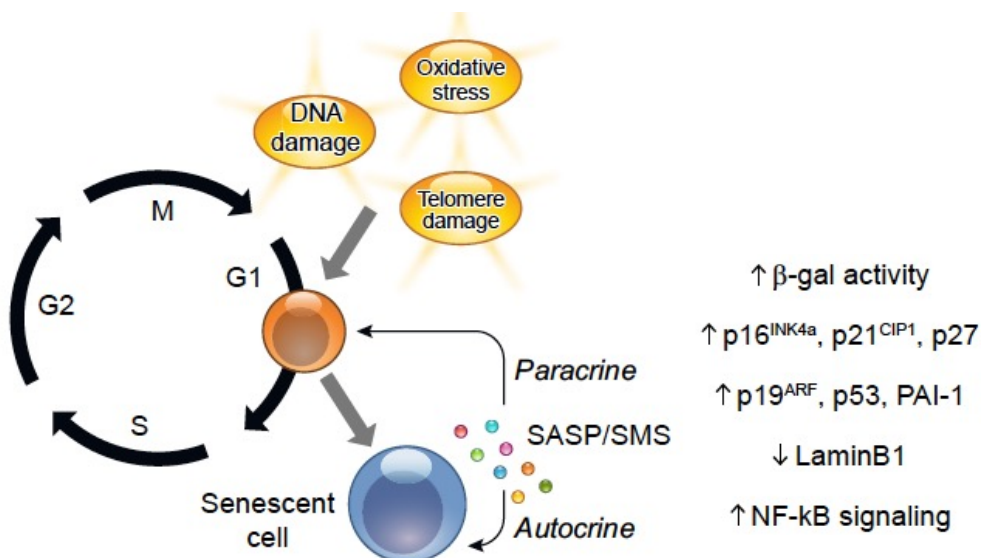


Figure 4 : Hallmarks of senescence

Cells can become senescent after different stimuli (yellow circles) and are characterized by a variety of features, such as SASP, increased SA- β -gal activity, increased expression of specific markers and many others. Figure adapted from ³².

1.2.2 *Types of senescence*

Over the years, numerous studies have identified different subtypes of senescence. Developmental and replicative senescence naturally occur during embryogenesis and the aging process, respectively, under normal physiological conditions. On the other hand, various forms of senescence can be triggered by pathological stressors like tumorigenesis, chemotherapy, and radiotherapy.

Senescence in physiology

During embryogenesis and development, senescence is essential to maintain fetal integrity. In mammalian embryos, multiple tissues are affected by senescence, including limbs, nervous system, gut endoderm and mesonephros, where senescent cells attract macrophages for their phagocytosis^{48,49}. In some cases, the occurrence of cellular senescence in embryogenesis is essential to avoid morphological defects in adult. Indeed, a canal that connects mesonephric tubules, kidney and cloaca, called the Wolffian duct, is subjected to cellular senescence impacting vagina formation. If senescence is avoided, an abnormal function of the duct occurs in the vagina morphogenesis, causing defects in the adults⁵⁰.

Apart from embryonic development, cellular senescence occurs in many physiological progresses in adults. Indeed, placental syncytiotrophoblasts and megakaryocytes undergo senescence during their maturation^{51,52}. If senescence is avoided in megakaryocytes, myeloproliferative disorders, like primary myelofibrosis, can occur, indicating the physiological relevance of senescence in this context⁵¹.

Wound healing is another physiological reaction to tissue injury in which cellular senescence is implicated. Of note, senescence can be either beneficial or detrimental in the tissue repair process, depending on the injury site. In cutaneous and corneal wound healing, the occurrence of senescence positively reduces fibrosis, thanks to the secretion of matricellular protein CCN1, reduction in fibroblast growth factor2 (FGF2) and increased expression of metalloproteases (MMPs)⁵³. On the other hand, wound healing activity is negatively impaired when senescence occurs in lung fibroblast, mesenchymal stem cells (MSC) and diabetes-induced cell⁵³.

Another mechanism of physiological senescence is the replicative senescence. This mechanism is caused by telomere shortening, which is perceived as a DNA-damage response. This activates the DNA damage kinases ATM, ATR, CHK1 and CHK2 activity, which enhances cell cycle protein production and the p53-p21^{Waf1/Cip1} pathway.

Oncogene-induced senescence

Induction of senescence can occur by either oncogene activation or tumor suppression gene inactivation and it is termed oncogene-induced senescence (OIS). Oncogene activation results in proliferative stress and senescence induction that act as a protective mechanism limiting tumor growth. Indeed, senescence is a physiological tumor-suppressive mechanism that prevents benign lesions from developing into malignant tumors. Particularly, cell cycle arrest prevents malignant cells from proliferating, passing carcinogenic mutations to the child cells, and improves the immune clearance of damaged cells.

OIS was first observed with HRAS^{G12V} oncogenic activation, which involves Raf/MEK/MAPK pathways, and activation of p53 and p16^{INK4a} cascades⁵⁴. From this study, a variety of interconnections between senescence onset and oncogene activation were discovered, such as the involvement of KRAS^{G12V} in lung adenoma and pancreatic intraductal neoplasia⁵⁵, the presence of KRAS^{G12D} mutation in lung adenoma⁵⁶, the role of NRAS^{G12D} in T-cell lymphoma⁵⁷, the involvement of BRAF^{V600E} in skin melanocytes and lung adenoma and melanoma, as well as aberrant activation of Rheb⁵⁸ and Akt1⁵⁹ in prostate intraepithelial neoplasia⁶⁰.

On the other hand, OIS can be driven by the inactivation of oncogenes. MYC-driven OIS is an example in which inactivation of the oncogene MYC induces cellular senescence and regression in different tumors, including lymphoma, osteosarcoma, and hepatocellular carcinoma (HCC)⁶¹.

In addition, the depletion or inactivation of tumor suppressor genes is a different mechanism for triggering senescence. The first discovery that the inactivation of a tumor suppressor gene can induce senescence was demonstrated in the prostate epithelium lacking Pten expression. This senescence response occurring upon PTEN loss is known as PTEN Induced cellular senescence (PICS)⁶².

PICS, contrary to OIS, occurs even without the presence of DNA damage response (DDR). In the case of PICS, the loss of PTEN results in the activation of p53 via the mTOR signaling pathway and the inhibition of p19^{Arf}-MDM2 signaling. Furthermore, PTEN depletion mediates the increase of p16^{INK4a}

gene, a key player in senescence response. The induction of PICS has been documented in precancerous lesions, indicating its potential involvement in inhibiting the advancement of these lesions into invasive cancer⁶³. However, in murine models of prostate cancer, the deletion of PTEN leads to the development of a benign prostatic intraepithelial neoplasia, characterized by the presence of numerous senescent tumor cells⁶³. Additionally, PICS induction has been observed as a response to different therapies, such as chemotherapy and radiation, suggesting its potential role in the treatment response^{64,65}.

Another tumor suppressor gene that plays a crucial role in the regulation of cellular senescence is TP53. TP53 induces senescence through multiple mechanisms by acting as a transcription factor and by regulating the expression of genes involved in the cell cycle. One of the primary pathways through which p53 induces senescence is by upregulating the expression of p21^{Waf1/Cip1}, which inhibits the activity of cyclin-dependent kinases (CDKs) and prevents cell cycle progression⁶². Additionally, TP53 promotes senescence by activating the expression of pro-senescent factors such as p16^{INK4a}⁶².

In the context of cancer, loss of TP53 function or alterations in its pathway, like in MDM2 gene that interacts with TP53, favor the proliferation of potentially cancerous cells, thus contributing to tumor development and progression^{28,66}. Indeed, when both PTEN loss and p53 inactivation are present, the cells are able to bypass senescence, evading PICS⁶². As a result, the damaged cells can continue to proliferate and acquire additional genetic alterations, leading to the development of invasive prostate cancer.

Therapy induced senescence

Cellular senescence occurs in the initial stages of tumorigenesis and in more advanced tumors as a response to treatment. Conventional anticancer therapy, chemotherapy and radiotherapy are known to trigger senescence in cancer cells through different mechanisms (Fig. 5).

Therapy-induced senescence, known as TIS or pro-senescence therapies, are used as therapeutic strategies with favorable outcomes in the treatment of cancer, as they impair the proliferation of cancer cells and promote a strong antitumor immune response⁶⁷.

Among the anticancer therapies tested in the clinic, a variety of chemotherapies induce senescence, either as main targeted effect or as a side effect. The chemotherapeutic dosage and posology are crucial in determining whether a cell will undergo apoptosis or senescence. Generally, lower doses of chemotherapy induce cellular senescence while higher doses trigger apoptosis of most cancer cells⁶⁸⁻⁷⁰.

At a molecular level, some chemotherapy treatments have the capacity to impact DNA and the DDR machinery. This leads to the accumulation of DNA damage and triggers the activation of TP53, as well as downstream molecules like p21^{Waf1/Cip1} and PAI1, resulting in a senescence response. Among chemotherapies, Doxorubicin, Etoposide and Camptothecin are topoisomerase inhibitors that inhibit cell replication, and they are commonly used for the treatment of colon, breast and lung cancer⁷¹⁻⁷⁴. Alkylating agents are another group of chemotherapy agents used as pro-senescence. Cisplatin, Carboplatin, Oxaliplatin and Temozolomide, which induce massive DNA damage in cancer cells by cross-linking the DNA, are the commonly used alkylating agents in cancer therapy⁷⁵. In addition, microtubule inhibitors such as Paclitaxel and Docetaxel arrest the proliferation of cancer cells by interfering with microtubule dynamics^{76,77}. The dose is essential with this pro-senescence therapy to avoid side effects on normal cells. Moreover, by preventing DNA synthesis, Methotrexate and Gemcitabine cause genotoxic stress, which induces senescence.

Other pro-senescence treatments aimed to block the cell cycle are Palbociclib, Ribociclib and Abemaciclib, which impede CDK4/6 complex formation preventing cell cycle progression. CDK4/6, over-expressed in many cancers⁷⁸, are cyclin-dependent kinases important for the cell cycle progression from G1 phase to S phase, whereas the inhibition of CDKs induce senescence. Another important cyclin-dependent kinase in pro-senescence therapy is CDK2, a cyclin-dependent kinase that becomes a target when resistance to CDK4/6 inhibition occurs.

Although clinical resistance to CDK4/6 inhibitors is associated with RB1 loss, the amplification of CDK6 or CCNE1, encoding for cyclin E1, the inhibition of CDK2 offers a promising approach to overcome this problem and become a potential therapy for promoting senescence⁷⁹.

In addition, a way to induce senescence is by targeting serine/threonine kinases that control the mitotic process. Particularly, aurora kinases (AURKs), such as Alisertib and Barasertib, and polo-like kinases (PLKs), including BI-2535 and Bi-6727, are studied as pro-senescence therapy in preclinical and clinical models⁸⁰⁻⁸³.

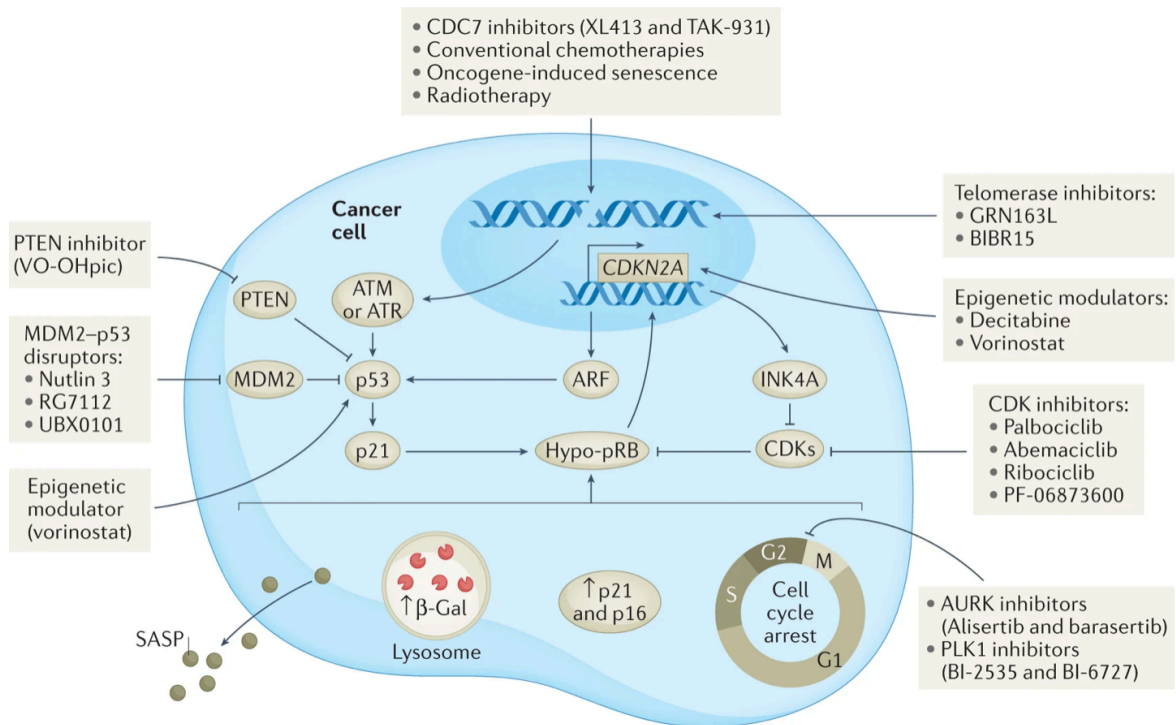


Figure 5: Therapy-induced senescence treatments in cancer.

Schematic representation of different therapies which induce senescence in cancer cells and their area of action inside the cells. Figure adapted from ⁸⁴.

Similar to chemotherapy, radiation therapy is one of the main treatments for multiple cancer types. This anticancer therapy has the ability to induce through localized radiation an acute DNA damage response that activates ATM, ATR and p53-p21^{Waf1/Cip1} signaling causing apoptosis and cellular senescence^{85,86}.

Additionally, some drugs can cause senescence in specific cancers based on the phenotypes of the malignancy. For example, Vorinostat, a histone deacetylation inhibitor, induces senescence in urothelial carcinoma and leukemia^{87,88}. Drugs inhibiting the VEGF pathway can promote senescence in preclinical models of colorectal and renal cancer^{89,90}. Finally, some therapeutic antibodies, such as the anti-CD20 antibody Rituximab are able to drive senescence in lymphoma⁹¹.

1.2.1 SASP

Senescent cells are characterized by a peculiar secretome called senescence-associated secretory phenotype (SASP). SASP is a mixture of many growth factors, proinflammatory cytokines, chemokines, pro-angiogenesis factors, proteases, and reactive oxygen species (ROS)⁹². The nuclear factor-kB (NF-kB) and the transcription factor CCAAT/enhancer-binding protein-beta (C/EBPβ) are the main responsible for SASP transcription⁹³. The SASP can trigger senescence in a paracrine way,

affecting the neighborhood cells, and in an autocrine manner, establishing a pro-inflammatory tumor microenvironment (TME).

This secretome has both beneficial and deleterious functions, depending on the timing and the condition in which is produced (Fig. 6). At the beginning of senescence, SASP factors suppress tumor proliferation by causing cell cycle arrest and promoting immune surveillance⁹⁴. IL-1 α , secreted by senescent tumor cells, activates the NF- κ B pathway and the transcription of IL-6 and IL-8⁹⁵, enhancing the recruitment of anti-tumor immune cells, M1-like macrophages, T helper cells and natural killer (NK) cells. These infiltrating immune system aims to eliminate senescent cells. However, IL-6 and IL-8 are also known as driver factors of cancer proliferation⁹⁶. Particularly, IL-6 factor promotes both angiogenesis⁹⁷ and the recruitment of myeloid-derived suppressor cells (MDSCs), an immune cell subset that antagonize CD8⁺ T cells and NK cells, favoring a pro-tumoral TME^{98,99}.

Additionally, several different SASP factors have the ability to promote angiogenesis, migration and metastasis formation by remodeling the TME. CXCL5 and VEGF are implicated in blood vessel density^{100,101}. Again, metalloproteases family, such as MMP1 and MMP3, favor tumorigenesis and migration of cancer cells^{28,102}. The balance of advantages and disadvantages of senescence depends on whether senescent cells' presence is transient or slowly accumulates over time, eventually causing an inflammatory TME.

In conclusion, senescent cells communicate and impact the surrounding tissues through various mechanisms mediated by the SASP. Altogether, they can promote senescence, inflammation, recruitment of immune cells, and alter the TME metabolism¹⁰³.

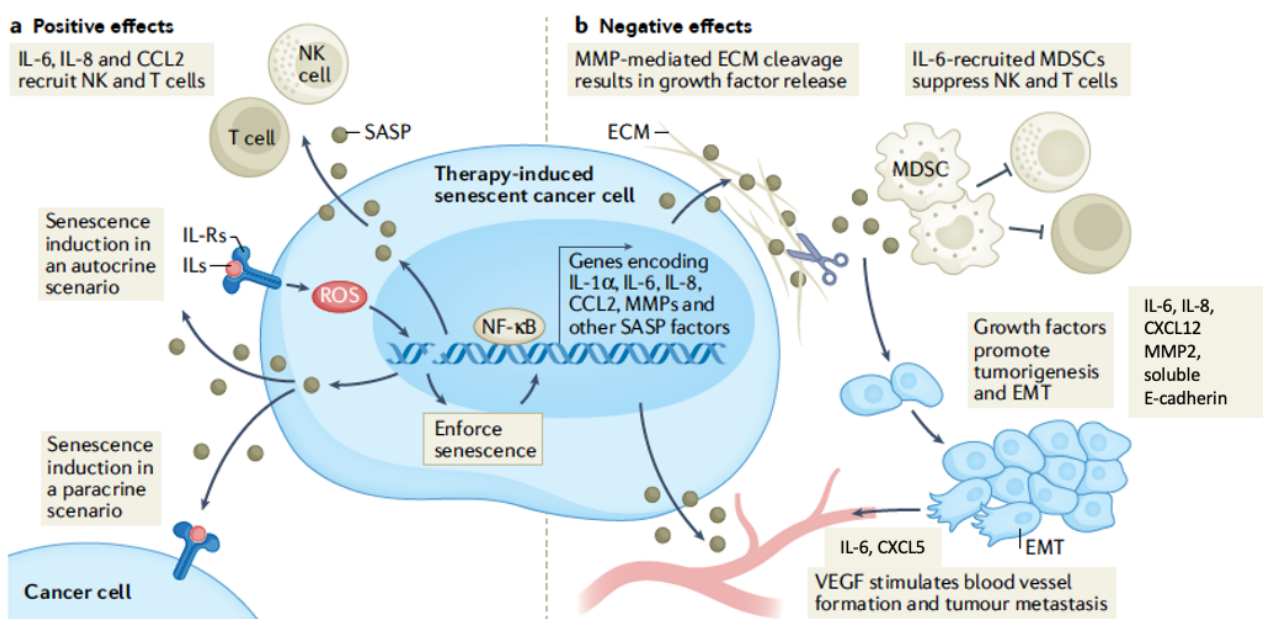


Figure 6 : Positive and negative effects of SASP

Based on the context, SASP factors can have both beneficial and detrimental effects on the surrounding cells. Moreover, the same factors can either trigger senescence or promote tumor aggressiveness. Figure adapted from ⁸⁴.

1.2.2 *Senolytic therapy*

Although cellular senescence has beneficial effects in the beginning, the persistence of senescent cells in tumors can be detrimental. This scenario underlines the need for developing therapies to remove or reshape senescent cells selectively. Indeed, drugs that eliminate senescent cells are known as senolytic, while drugs that modulate the SASP are named senomorphic (Fig. 7).

Since many cancer therapies unintentionally trigger senescence as a side effect, many studies are focused on the discovery of vulnerabilities to eliminate these detrimental cells. The main objective is to enhance the positive aspects of senescence while restricting the deleterious effects, thereby optimizing the overall therapeutic outcome^{53,84,104}.

Cells becoming senescent start to increase the expression of many anti-apoptotic factors of BCL-2 family¹⁰⁵. Hence, different senolytic drugs aim to inhibit these factors, thereby inducing apoptosis. Among them, Navitoclax (ABT-263) and ABT737, selective inhibitors of BCL-2, BCL-XL and BCL-W, reactivate the apoptosis pathway in senescent cells¹⁰⁶⁻¹⁰⁸. In many preclinical models of different cancer types, Navitoclax in combination with pro-senescence therapy, such as Etoposide, Doxorubicin, and Olaparib, was able to regress tumor growth by eliminating senescent cells¹⁰⁹⁻¹¹². However, administering Navitoclax to patients has shown many side effects related to the high toxicity or the lack of efficacy, depending on the drug dose, inducing thrombocytopenia and neutropenia^{113,114}. Due to the side effects of Navitoclax, Venetoclax (ABT199) and galacto-conjugated navitoclax drug (Nav-Gal) have been developed. Venetoclax, a BCL2-selective inhibitor, aims to reduce the side effects on hematological cells but is inactive in eliminating senescent cells^{105,115}. However, Nav-Gal is a nanoparticle specifically activated by high SA- β -gal activity, one hallmark of senescent cells, which significantly reduces side toxicity¹¹¹. Moreover, inhibitors of BCL-XL, such as A1155463 and A1331853, have been developed for senolytic therapy and tested in lung and colon cancers¹¹⁶.

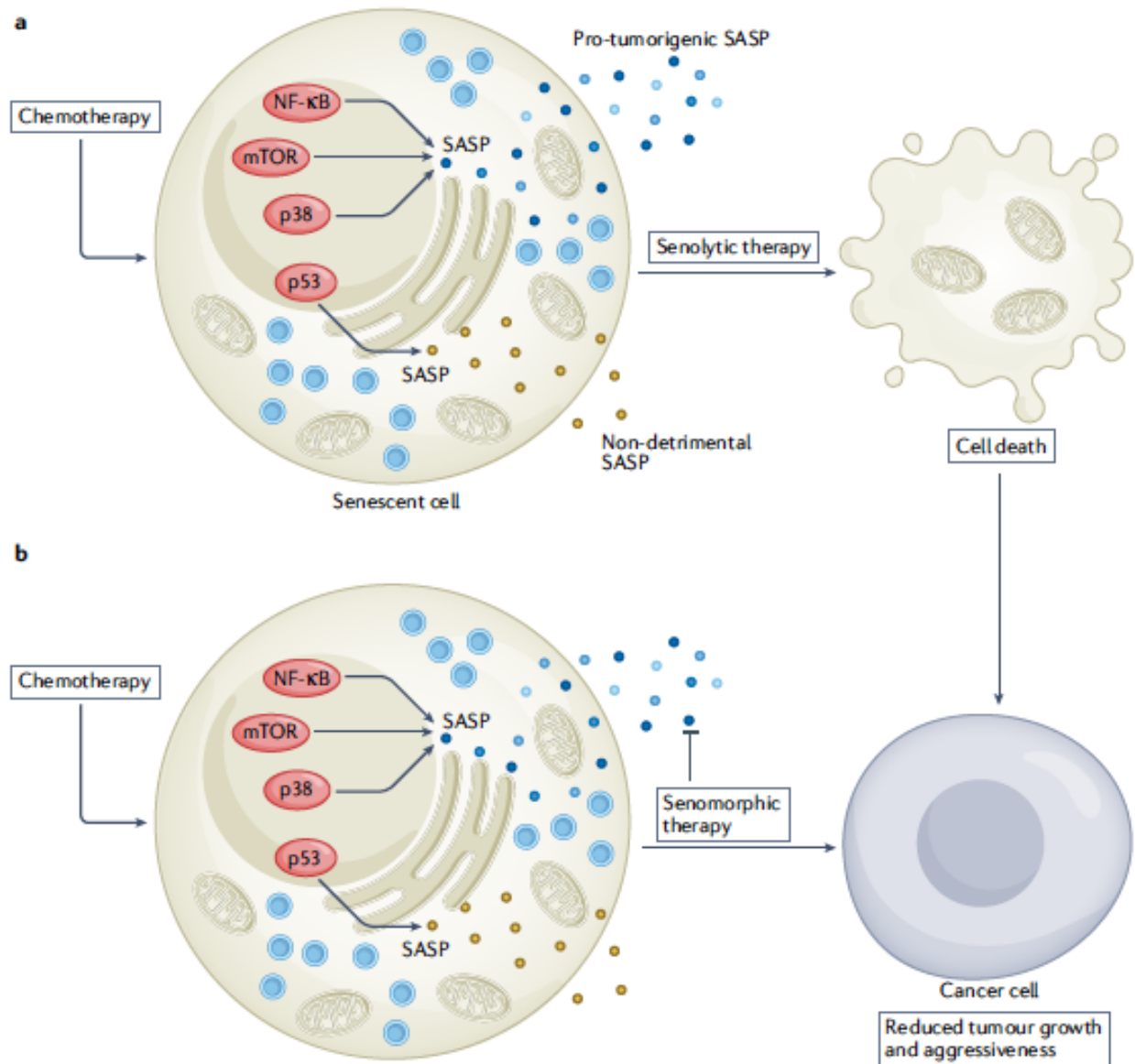


Figure 7 : Schematic representation of senolytic and senomorphic therapies.

a) Senolytic therapies aim to kill senescent cells by inducing cell death; b) Senomorphic therapies block the pro-tumorigenic SASP. Figure adapted from ¹⁰⁴.

Other senolytics include proteolysis-targeting chimera (PROTAC) drugs. PZ15227 is a BCL-XL specific PROTAC that confiscates the cereblon E3 ubiquitin ligase to degrade BCL-XL¹¹⁷. ARV-825 is a PROTAC drug containing bromodomain and extraterminal domain (BET) BRD2, BRD3 and BRD4 inhibitors and E3 ligase binder pomadomide^{118,119}. ARV-825 downregulates XRCC4 inhibiting the p53 pathway, interfering with the DDR response and activating the apoptotic response¹¹⁸.

Given the crucial role of p53 in senescence, there is a growing interest in targeting p53 activity as a potential approach for senolytic therapy. Researchers have developed specific interventions to interfere with p53-related processes and induce apoptosis in senescent cells. One approach involves

the use of the FOXO4-DRI peptide. This peptide is designed to disrupt the interaction between p53 and FOXO4, a protein that can inhibit p53-mediated apoptosis in senescent cells. By interfering with this interaction, FOXO4-DRI promotes the apoptotic elimination of senescent cells¹²⁰.

Another strategy to modulate p53 activity is through the use of compounds such as UBX0101, which prevents the interaction between p53 and MDM2, a negative regulator of p53. Therefore, UBX0101 helps to maintain p53 activity, allowing it to induce apoptosis in senescent cells¹²¹.

Considering that SASP is primarily responsible for the adverse effects of senescence, the use of SASP inhibitors with senomorphic properties may be a remarkable alternative for senolytic therapy.

Since senescent cells are also characterized by activation of peculiar pathways, including SRC, PI3K and mTOR, p38 MAPK, NF- κ B signaling, inhibition of those will disrupt senescent cell homeostasis. For example, Metformin acts on NF- κ B pathway preventing the release of SASP¹²². Rapamycin is a mTOR inhibitor that suppresses SASP by diminishing NF- κ B pathway¹²³. Ruxolitinib is a JAK inhibitor that reduces inflammation in TME limiting the action of SASP^{124,125}. SB203580 is a p38 MAPK inhibitor that represses SASP activity in breast cancer¹²⁶.

Unconventional approaches to kill senescent cells are based on immune cell modulation, such as the development of CAR T cells targeting urokinase-type plasminogen activator receptor (uPAR), a membrane protein expressed in senescent cancer cells¹²⁷.

In addition, hormones like androgen, estrogens, melatonin, and glucocorticoid can have senomorphic effects by controlling cytokine and SASP release¹²⁸⁻¹³¹.

The combination of pro-senescence therapy and senolytic/senomorphic therapies represents a promising approach in cancer treatment as it can promote, on one hand, the arrest of tumor growth and, on the other hand, the elimination of a pro-inflammatory TME caused by the SASP.

1.2.3 Senescence and metastasis formation

Metastasis, the primary cause of cancer recurrence and mortality in patients¹³², has been the subject of many studies over the past few decades, significantly increasing our understanding of its connection with cellular senescence¹³³.

The metastasization process consists of the release of cancer cells, called circulating tumor cells (CTCs) or disseminated cancer cells (DCCs) inside the bloodstream until they stop in a secondary site, promoting tumors in distant organs.

Many studies have described the relationship between senescent tumor cells and metastasis, particularly but not limited to prostate cancer, thyroid cancer, and melanoma^{28,134,135}.

In fact, SASP factors could promote the epithelial-to-mesenchymal transition (EMT) by increasing the migratory phenotype of cells and impacting the angiogenesis and vascularization¹³⁶. Among secreted factors, IL-6 and IL-8 are the drivers of SASP-mediated EMT in many cancer, including colorectal¹³⁷⁻¹³⁹; C-X-C-motif ligand CXCL1 is a crucial element in the development of the premetastatic niche in the liver in colorectal cancer models¹⁴⁰; CCL5 drives the spread of tumor cells in the lung and lymph nodes; Osteopontin (OPN) induces the expression of SNAIL, SLUG and TWIST, key mediators of EMT and metastasis¹⁴¹; Connective tissue growth factor (CTGF) enhances vessel formation in prostate cancer¹⁴².

In addition, SASP induces cytoskeleton and actin reorganization by impairing focal adhesions and boosting cell migratory phenotype¹⁴³. Senescent cells can release factors involved in the remodeling of extracellular matrix (ECM) such as TGF- β and MMPs which reduce matrix stiffness favoring the invasion^{28,144,145}. In PCa model, it has been demonstrated that TIMP1 loss in PTEN^{pc/-} mouse is characterized by senescence and that the reprogramming of SASP factor and up-regulation of GDF-15, FGF1 and MMPs support for metastasis formation in prostate cancer²⁸.

SASP-mediated EMT involves not only neighboring tumor cells but also the senescent cells themselves. Senescent cells have higher migratory capability as compared to proliferating cancer cells¹³⁴. CXCL12, a SASP factor, has been shown to promote in vivo an environment favoring the invasion of senescent cells but also non senescent cancer cells¹³⁴.

In breast cancer, it has been proved that the pro-senescence doxorubicin treatment causes the spread of tumor cells and metastasis formation. In fact, by removing p16^{INK4a} positive cells after doxorubicin treatment, tumor re-growth and metastases loci were reduced. This result suggests that chemotherapy promotes metastasis¹⁴⁶.

Another SASP gene involved in the extravasation of tumor cells is IL-1 α . The endothelial surface adhesion molecule regulates the mechanical attachment of CTCs to vascular vessels and the penetration of CTCs in the bloodstream.

The interconnection between metastasis and senescence is also given by the receptor CD36¹⁴⁷. This protein is upregulated in response to different types of senescence¹⁴⁷, and its activation in senescent cells stimulates NF-kB-dependent chemokines and cytokines production.

Since cells that form metastasis express high levels of CD36, the formation of metastasis is reduced by blocking the receptor in human oral carcinoma¹⁴⁸. Moreover, due to the frequent amplification

of CD36 in metastatic cancers and its correlation with a poor survival prognosis¹⁴⁹, nowadays, CD36 is used as a diagnostic biomarker for metastatic tumors¹⁴⁸.

Collectively, these studies provide evidence that the SASP plays a role in facilitating tumor metastasis. Senescence may contribute to tumor cell dissemination by enhancing the migratory capabilities of tumor cells within the local environment and by preparing the distant organ microenvironment to support tumor seeding. This highlights the interconnection between senescence and metastasis in cancer progression¹³³.

1.3 Cell death modalities

Cells subjected to different types of stress can also undergo cell death. Cell death is a mechanism by which dangerous and useless cells are eliminated. Like senescence, cell death plays a crucial role in many physiological functions, such as development, homeostasis and the immune system. Moreover, dying cells can signal with the surrounding cells by releasing molecules known as alarmins or damage-associated molecular patterns (DAMPs)^{150,151}.

Cells can die in multiple ways, which differ from the signals involved in the initiation, execution, and propagation. The different cell death modalities are described mainly in the work of Galluzzi et al.¹⁵² and can be classified as:

Necroptosis: it is triggered by extracellular and intracellular perturbations which are detected by specific receptors such as FAS, TNRF1, TLR3 and TLR4^{153,154}. Necroptosis activation depends on RIPK3 and MLKL sequential activation and on RIPK1 catalytic activity^{155,156}.

Ferroptosis: it is controlled by G glutathione (GSH)-dependent enzyme glutathione peroxidase (GPX4) and takes place after lipid peroxidation, which depends on ROS production and iron availability^{157,158}. Particularly, GPX4 is the main inhibitor of ferroptosis by reducing lipid peroxidation, specifically of phosphatidylethanolamine-containing polyunsaturated fatty acids (PUFAs)¹⁵⁹.

Pyroptosis: it is the result of the caspases activation, particularly of CASP1, CASP3, CASP4 and CASP5, and critically depends on the formation of plasma membrane pores by members of the gasdermin protein family¹⁵².

Parthanatos: it is driven by the hyperactivity of poly (ADP-ribose) polymerase 1 (PARP1), a component of DDR response, and the degradation of DNA mediated by Macrophage migration inhibitory factor (MIF) and apoptosis-inducing factor mitochondria associated 1 (AIF)¹⁶⁰⁻¹⁶².

Apoptosis: it can be initiated by two pathways: the intrinsic pathway (mitochondrial pathway) and the extrinsic pathway (death receptor pathway). The intrinsic pathway allows the outer membrane of the mitochondria to permeate, leading to the release of cytochrome c and activation of the enzyme caspases. In contrast, the external pathway is triggered by external signals such as cytokines or death ligands, which bind to death receptors on the cells' surface. This binding results in the formation of death-inducing signaling complexes (DISCs), which activate caspase and induce apoptosis.

The balance between cells' homeostasis and apoptosis is mediated by two opposite signaling: the pro-apoptotic one, aiming at cell death, and the anti-apoptotic one, which prevents cell death. Specifically, apoptosis is regulated by a group of proteins belonging to the BCL2, apoptosis regulator (BCL2) protein family, which is characterized by BCL2 homology (BH) domain, fundamental in the cascade.

The anti and the pro-apoptotic signaling control the mitochondria outer membrane permeabilization (MOMP), which is the crucial step in the apoptosis leading to the release of cytochrome C. More specifically, BCL2-associated X, apoptosis regulator (BAX), and BCL2 antagonist/killer 1 (BAK) form hetero/homodimers to release the BH3-only proteins, leading to the formation of lipid pores and alteration of mitochondrial permeability, eventually leading to cell death. This phenomenon is blocked by the anti-apoptotic BCL2 family members, which antagonize BAX and BAK by either sequestering them or impeding the pore formation. The anti-apoptotic members include BCL2 itself, BCL2 like 1 (BCL2L1, BCLXL), Myeloid Cell Leukemia Sequence 1 (MCL1), BCL2 like 2 (BCL2L2, BCL-W), and BCL2 related protein A1 (BCL2A1).

Of note, senescent cells are known to be resistant to apoptosis¹⁶³. When an irreparable damage occurs, a cell has two possible outcomes: either undergo apoptosis or senescence. The decision between apoptosis and senescence is based on the severity and duration of the DNA damage signaling. If the DNA damage is intense but short-term, it triggers apoptosis. On the other hand, if the damage is mild but prolonged, it leads to cellular senescence⁶⁸.

These findings imply that both senescence and apoptosis pathways might be activated simultaneously during certain processes or stress responses and that the specific outcome, whether apoptosis, senescence or a combination of the two, depends on each cell type's unique characteristics and signaling mechanisms.

In summary, the cell's internal state determines which response (apoptosis, senescence, or both) will occur first.

2 Aim

The studies about cellular senescence in prostate cancer and its influence on tumor progression and metastasis formation gained more attention in recent years. In particular, the dual faces of cellular senescence in cancer therapy open more interest in the field of research focused on the elimination of senescent cells (senolysis) combined with pro-senescence therapy.

In this work, by using different prostate cancer mouse models and single-cell RNA sequencing (sc-RNA seq) technologies, we aimed to identify senescent cells in prostate cancer at single-cell resolution and find new vulnerabilities for their elimination.

Therefore, the specific aims of this project were:

1. The finalization of the 10x single-cell RNA-seq analysis in different genetically engineered mouse models (GEMMs) and the identification and characterization of senescent cells at single-cell resolution.
2. Identification of senescence vulnerabilities for senolytic therapy through 10x single-cell RNA seq data.
3. In vitro and in vivo validation of the vulnerability with therapy-induced senescence (TIS).
4. Clarification of the interconnection between metastases onset, senescence and senolytic therapy in vitro and in vivo at single-cell level.

3 Methods

The methods section is partially reported in the publication related to this PhD thesis where I'm first author^{164,165}.

Mouse Models. All mice were maintained under specific pathogen-free conditions in the animal facilities of the IRB institute. Experiments were performed according to the state guidelines and approved by the local ethical committee ("Dipartimento della Sanità e Socialità, Esperimenti su animali" Canton Ticino), authorization number TI-51/2018. Prostate-specific Pten^{pc/-} transgenic mice¹⁵² were crossed with Timp1^{-/-} mice (Jackson Laboratory, 6243) to generate Timp1 knock out in Pten^{pc/-}²⁸. NRG male mice, at 12 weeks of age, were used for subcutaneous cell injection of PC3 shCtrl and shTIMP1.

In vivo Treatments. Docetaxel (TEVA Pharma AG 6984894) intraperitoneally at 10mg/kg once a week. ABT263 (MedChemExpress HY-10087) by oral gavage 50mg/kg, daily. S63845 (MedchemExpress HY-100741) at the dose of 25 mg/kg by oral gavage. Mice were monitored for any suffering of distress or weight loss by measuring total body weight of mice weekly and monitoring the behavioral changes every day for a total of 4 weeks of treatment.

Prostate Cancer Cell Culture. PC3 and LNCaP human prostate cancer cells were purchased from ATCC and were cultured according to the manufacturer's instructions. Cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% P/S. HEK-293T (human embryonic kidneys) and TrampC1, cells were obtained from ATCC. RapidCap were obtained from Trotman laboratory¹⁶⁶. Cells were cultured in DMEM supplemented with 10% FBS and 1% P/S. All cell lines were kept under controlled temperature (37C) and CO2 (5%) and used for experiments at early passages. All the cell lines were tested negative for Mycoplasma (MycoAlertTM mycoplasma detection kit, LT07-418, LONZA).

In vitro treatments. Palbociclib was used at the concentration of 10 µM; Docetaxel was used at 1 nM; ABT263 was used at the of 2.5 µM; S63845 was used at 10 µM, UMI-77 was used at 10 µM ; AZD5991 was used at 10 µM.

Generation of shTIMP1 Human Prostate Cancer Cell Line. PC3 cell line was transfected with shRNA using the human TIMP1-directed shRNA RHS4430-200284918-V3LHS_317110. To prepare lentiviral particles, HEK-293T cells were transfected using JetPRIME transfection reagents (JetPRIME, Polyplus

transfection, 114-07/712-60) as per the manufacturer's instructions. PC3 cells were infected with the filtered lentiviral supernatant obtained from transfected HEK-293T cells. Infected human prostate cancer cells were subsequently selected using Puromycin (3mg/ml).

Generation of Pten-/- TrampC1 murine prostate cancer cell line. TrampC1 cells were purchased from ATCC and cultured according to manufacturer's instructions (DMEM, 10% heat-inactivated FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin). The transfection of the PTEN CRISPR/Cas9 KO plasmid (Santa Cruz Biotechnology) was performed using jetPRIME® transfection reagent according the manufactory protocol at the 1:2 DNA / jetPRIME® ratio. 24h after transfection, the GFP transduced cells were sorted to purity 99% and plated as single cell on 96-well plates. At day 7 after cell sorting the grown cell colonies were moved into 24-well plates for further expansion.

Generation of shMCL-1 human and murine Prostate Cancer Cell Line. LNCaP and RapidCap cell line was transfected with shRNA using the human and mouse MCL-1-directed shRNA RHS4696-200751526 and RMM4431-200332978, respectively. To prepare lentiviral particles, HEK-293T cells were transfected using JetPRIME transfection reagents (JetPRIME, Polyplus transfection, 114-07/712-60) as per the manufacturer's instructions. Both LNCaP and RapidCap cells were infected with the filtered lentiviral supernatant obtained from transfected HEK-293T cells. Infected prostate cancer cells were subsequently selected using Puromycin (3mg/ml). The shRNA was activated in both the cell lines adding Doxycycline in the cell culture media (1mg/ml).

Immunohistochemistry (IHC). IHC tissue sections were processed as follows: deparaffinized, unmasked, pre-staining, blockings and secondary stainings. Deparaffinization was performed using three-step procedure, first and second step the slides were immersed in OTTIX plus solution (Diapath, Cat No. X0076) for 5 mins each followed by third and last step of OTTIX shaper solution (Diapath, Cat No. X0096) for 5 mins. The slides were drained off the excess solution and were then immersed in ionized water for 5 mins. Further, un- masking or antigen retrieval procedure was followed which involved immersing the section slides in pH solutions (depending upon the antibodies) either pH 6 (Citrate, Company: Diapath, Cat No. T0050) or pH 9 (DAKO, Cat No. K800421-2) in water bath at 98°C for 20-25 mins. The slides were allowed to cool at room temperature for 20-25 mins. The section slides were washed with 1xPBST (0.5% Tween20), 2 times for 3 mins each, followed by staining procedure. Blocking procedure began by incubating the slides with 3% H2O2 (VWR chemicals, Cat no: 23615.248) for 10 mins followed by 1xPBST washes as before and performing protein block. Protein blocking was performed using Protein-Block solution (DAKO

Agilent technologies, Cat No. X0909) for 10 mins at room temperature. Depending upon antibodies, if they were developed in mouse host, the tissues were blocked for mouse cross-reactivity using biotinylated Anti-Mouse antibody (Vector Laboratories, Cat No. BP-9200). Sections were stained with respective primary antibodies at room temperature for 1 hour followed by 3 washes with 1xPBST as before. These slides were further incubated with respective secondary antibodies, Anti-Mouse (Vector Laboratories, Cat No. BP-9200), Anti-Rabbit (Vector Laboratories, Cat No. BP-9100). During secondary antibody incubation, Vectastain ABC solution was prepared (Company: Vector laboratories, Cat No. PK-6100) at the dilution of 1:150 of both Solution A and Solution B in 1xPBS solution followed by 30 mins incubation at room temperature. Upon completion of secondary antibody stainings, slides were washed for 3 times with 1xPBST followed by ABC solution staining for 30 mins at room temperature. After ABC, slides were washed 3 times with 1xPBST and final steps of IHC stainings were performed. DAB staining was performed using DAB solution (Company: Vector laboratories, Cat No. SK-4105. One drop of Chromogen in 1ml of Diluent solution) and allowed to stain for no more than 3-4 mins at room temperature. Immediately slides were washed 3 times with 1xPBST and counter staining was performed using hematoxylin solution (Diapath, C0303). At the end of IHC staining, sections were dehydrated using deparaffinization procedure after which slides were mounted with coverslip using aqueous mounting media (Diapath, 060200). Tumor tissue samples were fixed in 10% neutral-buffered formalin (Thermo Scientific, Cat No. 5701) overnight. Tissues were washed thoroughly under running tap water followed by processing using ethanol and embedded in paraffin according to standard protocols. Sections (5 mm) were prepared for antibody detection and hematoxylin and eosin staining.

Senescence associated β -galactosidase (SA- β -gal) Assay. For tissue-specific SA- β -gal assay, tumor samples were immediately frozen in OCT solution at -80°C and sections of 4 mm were prepared. Senescence-associated SA- β -gal staining was performed using Senescence β -Galactosidase Staining Kit (Cell Signaling Cat. No 9860) according to the manufacturer's instructions. Counter staining was performed using Eosin staining (Alcohol-based Diapath, C0352). For in vitro experiment, SA- β -gal staining was performed using Senescence β -Galactosidase Staining Kit (Cell Signaling Technology, Cat. No 9860) according to the manufacturer's instructions.

Proliferation and cell death Assay. Proliferation assay PC3, TrampC1, Pten -/- TrampC1 and Rapidcap cell lines was performed by plating 1×10^4 cells per well of 96-well plate in at least sextuplicate. Proliferation was monitored and analyzed by using Incucyte S3 in vitro system (Essenbioscience).

Migration Assay. Migration assay were performed in PC3 by plating 20x10⁴ cells per well of 96-well plate in at least sextuplicate. Wound was performed using Incucyte Wound Maker and cell migration was monitored and analyzed by using Incucyte S3 in vitro system (Essenbioscience).

Condition Media Assay. Cell supernatants were harvested and spun down at 1500 rpm for 10 mins and the supernatant was filtered using 0.22 µm filters. Conditioned media was administered to parental cells at time zero or 48 hours prior the assay for proliferation and migration assay, respectively. The conditioned media in all the experiments was normalized based on the number of cells present in the well at the moment of the harvesting.

Western Blot. Prostate tissues, tumor samples or cells were lysed using 1x RIPA buffer (Cell signaling, 9806) supplemented with Phenylmethane- sulfonyl fluoride (PMSF; Millipore Sigma, catalog 329-98-6) and incubated on ice for 30 mins. Samples were centrifuged at 14'000 rpm for 15 mins. Protein concentration was determined by the BCA kit (Thermo Fisher 23227). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), 10% and transferred on to 0.45 mm nitrocellulose membrane (Thermo Scientific, 88018). After protein transfer, membranes were blocked in 5% milk solution and membranes were probed with the indicated antibodies overnight at 4°C. The membranes were incubated with horseradish peroxidase-conjugated (HRP-linked) secondary antibodies anti-rabbit IgG (Promega, W4011, 1:5000) or anti-mouse IgG (Cell signaling, W4021, 1:5000) and developed using enhanced chemoluminescence (ECL) substrate (Thermo Scientific, 32106). Membranes were exposed to Fusion Solo S imaging system (Vilber). Blots were semi-quantitatively analyzed by densitometry using ImageJ 1.52 v (National Institutes of Health).

Antibodies. For IHC anti-Cleaved Caspase 3 (Cell signalling #9661), anti-Ki67 (RTU-Lab Vision #RM-9106-R7 Dilution Ready to use), anti-Luciferase (Abcam #ab181640), Mcl-1 (Cell signaling #5453) were used. For Western blot anti-Cleaved Caspase 3 (Cell signalling #9664), anti-BCL-2 (Cell signalling #3498S), anti-Mcl-1 (Cell signalling #5453) anti-HSP90 (Cell signalling #4874), p27 kip1 (Cell signalling #3698S), p15 ink4b (Abcam #53034), p16 ink4a (Abcam #211542), p21 (Abcam #107099), p19 ARF (5-C3-1) (Santa Cruz Biotechnology #SC-32748), PAI-1 (Abcam #66705), , Mcl-1 (Cell signaling #5453), secondary Anti-Rabbit (Promega #W4011), secondary Anti-Rat (Thermo Fisher #31470).

Quantitative Real-Time PCR (RT-qPCR). RNA extraction from cells or tissues samples was performed using Trizol (Ambion, life technologies, 15596026), according to the manufacturer's instructions.

cDNA was obtained using ImPROM II kit (Promega, A3800) according to the manufacturer's instructions. RT-qPCR was performed using Gotaq[®] qPCR Master Mix, Promega[®] (A6002) on Step One Real-Time PCR systems (Applied Biosystems). Primers used for RT-qPCR are listed in Supplementary Table 1. Expression levels were calculated using the ddCT method¹⁶⁷.

Single Cell Sequencing Analysis. Prostate tumors were resected from *Pten*^{pc-/-} and *Pten*^{pc-/-}; *Timp1*^{-/-} mice (all three lobes, AP, DLP and VP) and from NRG mice injected subcutaneously with 2.5 *10⁶ PC3 ShTIMP1. Sample were processed for single cell suspension followed by RNA sequencing and analysis using the following procedure:

Single Cell Suspension. Prostate tumors or xenografts tumors were isolated, minced and processed for single cell suspension. Tissues were digested in 2 ml of Digestion Buffer composed by RPMI 10% FBS + 1% P/S, 500 mL of Collagenase D (1 mg/mL), 50mL of DNase (100 U/mL) and 125mL of HEPES (25 mM). The cell suspension was incubated for 50 minutes at 37°C on a rocker. Then, the digestion was stopped adding 1 mL of RPMI 10% FBS + 1% P/S. The cells suspension was filtered through a 100 μ m cell strainer and kept on ice for 4 minutes. Then cells suspension was filtered again through a 40 μ m cell strainer and spun down at 1500 rpm for 5 min at 4°C. FACS staining was performed using EPCAM-FITC (anti-Mo CD326, eBioscience, clone G8.8 #11-5791-82) for the murine prostate tumors, while PC3 shTIMP1 were sorted thanks to GFP positivity.

Single-cell transcriptomes was performed using 10X Chromium single cell platform (10X Genomics, Pleasanton, CA. USA). FACS-sorted Epcam+ prostate cells were used as the input source for the scRNA-seq. Cells were suspended in a phosphate buffer solution containing 0.04% weight/volume bovine serum albumin (BSA). The recommended volume of single cell suspension was loaded on a Chromium Single Cell Controller (10x Genomics) targeting ~10000 cells per murine prostate samples while for xenografts tumors we used a target cell recovery between 5000 and 10000 cells per sample. Bar- coded single-cell gel beads in emulsion (GEMs) were created by 10x Genomics! Chromium TM and then reverse transcribed to generate single-cell RNA-seq libraries using Chromium Single Cell 3' Library and Gel Bead Kit v2 (10X Genomics) according to manufacturer's instructions. Resulting short fragment libraries were checked for quality and quantity using an Agilent 2100 Bioanalyzer and Invitrogen Qubit Fluorometer. Unique molecular identifiers (UMIs), which were incorporated into the 5' end of cDNA during reverse transcription, were used to quantify the exact number of transcripts in a cell.

Human single-cell RNA sequencing data were downloaded from GEO under the code GSE137829.

Single-Cell Sequencing data preprocessing and quality control. Sequencing data were processed by Cell Ranger¹⁶⁸ (version 3.1.0 for murine alignment and version 6.0.0 for xenograft derived-samples) and reads were aligned to mouse genome (mm10 v3.0.0) or human genome (GRCh38) with STAR (v.2.5.1b)¹⁶⁹. To reduce the 'dropout' phenomenon, R Magic package was used on gene-counts¹⁷⁰. Single cell sequencing analysis was performed in epithelial cells purified from both *Pten*^{pc-/-} and *Pten*^{pc-/-}; *Timp1*^{-/-} tumors or on GFP+ cells in PC3shTimp1 xenograft. For each cell, we calculated different quality measures: percent of mitochondrial genes, number of genes and gene biotype. We removed cells that had more than 25% expression on mitochondrial genes, fewer than 100 total genes expressed and we considered only protein coding genes defined using getBM (biomaRt package). All the samples were integrated using Seurat¹⁷¹⁻¹⁷⁴ package (FindIntegrationAnchors and IntegrateData function after library-size normalization of each cell using NormalizeData function with default parameters).

We identify features that are variable in the samples with (FindVariableFeatures function) and Principal component analysis (PCA) dimensionality reduction was run using the top 2000 features identified. Number of the included components (PCs) was assessed using the ElbowPlot function and fifteen PCs were conserved. Graph-based clustering approach was used to cluster the cells using FindNeighbours (k = 20) and FindClusters functions (0.25 < res < 2 with 0.25 difference). For visualization of the data, the dimensional reduction technique t-distributed stochastic neighbor embedding (t-SNE) and Uniform Manifold Approximation and Projection (UMAP) were applied using the RunTSNE and RunUMAP functions from Seurat.

In murine single cell sequencing cells positive for CD45 (*Ptprc* gene) and negative for *Epcam* were removed. Inside the *Epcam*+ cells, we defined epithelial subtypes based on canonical markers: *Cd24a*, *Krt8* and *Krt18* for luminal cells; *Trp63*, *Krt5* and *Krt14* for basal cells and *Pax2*, *Pate4* and *Calml3* for semi-vesical cells⁵². For xenograft sc-RNAseq data, all the analysis were performed after regression of *nCount_RNA* and *nFeature_RNA* features, due to differences between the samples.

Single-Cell Sequencing data processing. Differentially expressed genes (DEGs) were identified using FindAllMarkers or FindMarker functions. Genes were identified as differentially expressed in a particular set of cells if FDR < 0.05 and minimum expression in at least 30% of cells.

Pathway analysis were performed using clusterProfiler¹⁷⁵ package: for enrichment analysis we used gseGO (ont = "BP") function while for over-representation analysis we used enrichGO function. The outputs of pathway analysis were simplified using simplify function with cutoff=0.6 and gene set were considering significant with FDR<0.05. In case of multiple comparisons for pathway we used compareClusterResult function. To analyze the activity of 14 relevant signaling pathways, we used Pathway RespOnsive GENes (PROGENy)¹⁷⁶ analysis. To study senescence heterogeneity, all the analysis were performed using exclusively the genes found up-regulated in total senescent cells compared to not senescent cells (3757 genes).

ss-GSVA scores for different published gene sets and cell death pathways were calculated using gsva¹⁷⁷ function (method="ssgsea"). Correlation analysis was performed using cor.test function (alternative = two.sided , method = "pearson", conf.level = 0.95) and adjusted pvalues were calculated with p.adjust function using (method = "BH" . For classification of cells based on Mcl1 expression levels we classified into quartiles: Mcl1+ corresponds to first, second and third quartiles, while for Bcl2 expression we used the median levels to classify in Bcl2+ and Bcl2-. All plots were designed using Seurat package and ggplot2 package.

For out-of-clustering methods, the cells were projected to the combination of twelve senescence-related available datasets using scmap-cluster v1.16.0 (threshold = 0.15) and the function SingleR from SingleR package with default parameters.

Signatures. Apoptotic pathway: Bcl2, Bcl2l1, Mcl1, Bcl2l12, Bcl2a1a, Cflar, Gadd45a, Traf1, Ptpn13, Birc3 (anti- apoptosis), Bbc3, Bcl2l11, Pmaip1, Bak1, Bax, Pidd1, Bid, Apaf1, Fas, Tnfrsf10b (pro-apoptosis); Ferroptosis pathway: Map1lc3a, Atg5, Atg7, Ncoa4, Alox15, Lpcat3, Acsl4, Vdac2, Vdac3, Cybb, Gpx4, Gss, Gclc; Necroptosis pathway: Mlkl, Trpm7, Ripk1, Ripk3; Parthanatos pathway: Aifm1, Rnf146, Parp1, Parg; Pyroptosis pathway: Gsdmd, Nlrp3, Nlr4, Aim2, Casp1, Pycard, Mefv; Sasp signature: Cxcl1, Cxcl2, Hc, Csf3, Csf2, Csf1, Il10, Il13, Il6, Cxcl13, Cxcl10, Icam1, Ccl2, Cxcl15, Ccl20, Vegfc, Vegfa, Inhba, Il1a, Il1b, Bmp2, Gdf15, Tgfb1, Tgfb2, Tgfb3, Bmp6, Ogg1^{125,178}

Quantification and statistical analysis. All experiments were performed on biological replicates as mentioned in the respective figure legends. Sample size for each experimental group/condition is reported in the appropriate figure legends. All data points are presented for quantitative data, with an overlay of the mean with SEM. Statistically significant differences between control and experimental groups were determined using Multiple Student's t-tests (two-tailed, unpaired), one-

way ANOVA with Tukey multiple comparison difference test, Wilcoxon test, and log-rank (Mantel-Cox) test as indicated in the appropriate figure legend and text. All statistical analyses were performed using GraphPad Prism 8, Microsoft Excel 2016 or R-Studio.

4 RESULTS

4.1 Identification of senescent cancer cells in different mouse models of prostate cancer

In order to examine prostate cancer senescent cells at the single-cell level, we collected the epithelial fraction of four prostate tumors from two different mouse models of prostate cancer, the *Pten*-null prostate conditional (*Pten*^{pc-/-}) and the *Pten*^{pc-/-}; *Timp1*^{-/-} mouse models^{28,50}. These mouse models develop prostate tumors that progress into locally invasive and metastatic prostate cancer, respectively. Previous studies have shown that both *Pten*^{pc-/-} and the *Pten*^{pc-/-}; *Timp1*^{-/-} prostate tumors contain either a proliferative or a senescent population, as indicated by positive staining for SA-β-Gal and the expression of various senescence markers at protein levels (Fig. 1a, Supplementary Fig. 1a). The epithelial tumor cells, positive for Epcam marker, were isolated through Fluorescence-activated cell sorting (FACS) and analyzed by 10X Genomics single-cell RNA sequencing. Almost 4000 cells were analyzed for their transcriptomic profiles (Fig. 1b, Supplementary Fig. 1b). By using a graph-based clustering method and Uniform Manifold Approximation and Projection (UMAP), we identified distinct epithelial cell clusters (Supplementary Fig. 2a-d). Since during tumor dissection normal cells may be incorporated in the sample and sequenced, to study exclusively prostate cancer cells, the three main prostate epithelial populations, including luminal, basal and semi-vesical cells were identified using canonical markers. We used Cd24a, Krt18 and Krt8 for luminal cells; Krt14, Trp63 and Krt5 for basal cells; while for semi-vesical cells Calml3, Pate4 and Pax2. Cluster 8 and 9, composed of normal semi-vesical cells, were removed from future analysis (Supplementary Fig. 2a-d).

In order to identify senescent tumor cells at the level of individual cells, we created a senescence signature by combining the expression of *p16*^{INK4a}, *p15*^{INK4b}, *p19*^{Arf}, *p21*^{Waf1/Cip1}, *p27*^{Kip1} and *PAI-1*, which are the most highly upregulated senescence markers detected in whole tumor lysates using western blot analysis^{31,179} (Fig. 1c, Supplementary Fig. 1a).

Among the different clusters identified, clusters 3, 4, 5 and 7 showed higher expression levels of the senescence signature (Supplementary Fig. 2e). Of note, while cluster 3,4,5 defined luminal prostate tumor cells, cluster 7 represented basal prostate tumor cells, thereby demonstrating that senescence can equally occur in cells of both phenotypes (Supplementary Fig. 2b).

As one prominent characteristic of senescent tumor cells is their lack of cellular

proliferation^{31,32,179,180}, we defined three cell cycle arrest signatures to analyze the different clusters identified. Particularly, single-sample Gene Set Enrichment Analysis (ssGSEA) was applied by utilizing three available gene sets of cell proliferation (Kegg, Reactome, WikiPathway) (Fig. 1d and Supplementary Fig. 2f). ssGSEA is a computational method used to assess the enrichment or activity of predefined gene sets in individual samples or cells. Indeed, the enrichment score is obtained by comparing the expression levels of genes within a signature to the expression levels of all genes in a cell, reflecting the up/down-regulation of the signature on the whole transcription profile.

Combining the senescence signature (Fig. 1c) with each of the three cell cycle arrest signatures allowed us to establish three different Senescence Scores (SK, SR, SWP) (Fig. 1e, Supplementary Fig. 2g). Cancer cells were identified as senescent if they had the highest score level of all the senescence scores SK, SR and SWP, simultaneously (Fig. 1f). This newly defined algorithm, based on the abundance of specific genes and cell cycle arrest markers, was named Senescence Index Tool (SIT for short).

Mathematically, The SIT development consisted of four steps:

- Definition of senescence signature, given by the average expression of known senescent markers $p16^{INK4a}$, $p15^{INK4b}$, $p19^{Arf}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $PAI-1$ using AddModuleScore function of Seurat package.
- Definition of cell cycle arrest signatures using ssGSVA and available cell cycle gene set

$$(1) \text{ Cell Cycle arrest signature}_i = -ssGSVA \text{ score}_i \text{ with } (i = K, WP, R)$$
- Verification of the co-occurrence of senescence signature and cell cycle arrest signatures and the definition of senescence scores K, WP and R

$$(2) \text{ Senescence score}_i = \text{Cell cycle arrest signature}_{i \text{ norm}(0-1)} + \text{Senescence signature}_{\text{norm}(0-1)}$$

$$\text{with } (i = K, WP, R)$$
- Identification of senescent cells: cells with senescence scores K, WP and R belonging to the fourth quartile (highest value) are considered senescent cells

To validate the reliability of the SIT, we next investigated the differences between senescent and non-senescent prostate tumor cells at the gene expression level. By performing Gene Set Enrichment Analysis (GSEA) on biological processes from the Gene Ontology collection, we observed selective activation and suppression of different pathways. Among the pathways positively activated in senescent cells, though with higher normalized-enrichment-score (NES), wound healing and migration (*locomotion, cell motility, epithelium migration, tissue migration*), which are features characteristic of senescent cells^{31,103,181}, appear. Conversely, pathways related to oxidative

phosphorylation and mitochondrial respiration were suppressed, in line with previous findings demonstrating that senescent cells have dysfunctional mitochondria and rely for their survival on the tricarboxylic acid (TCA) cycle¹⁸²⁻¹⁸⁴ (Fig. 1g).

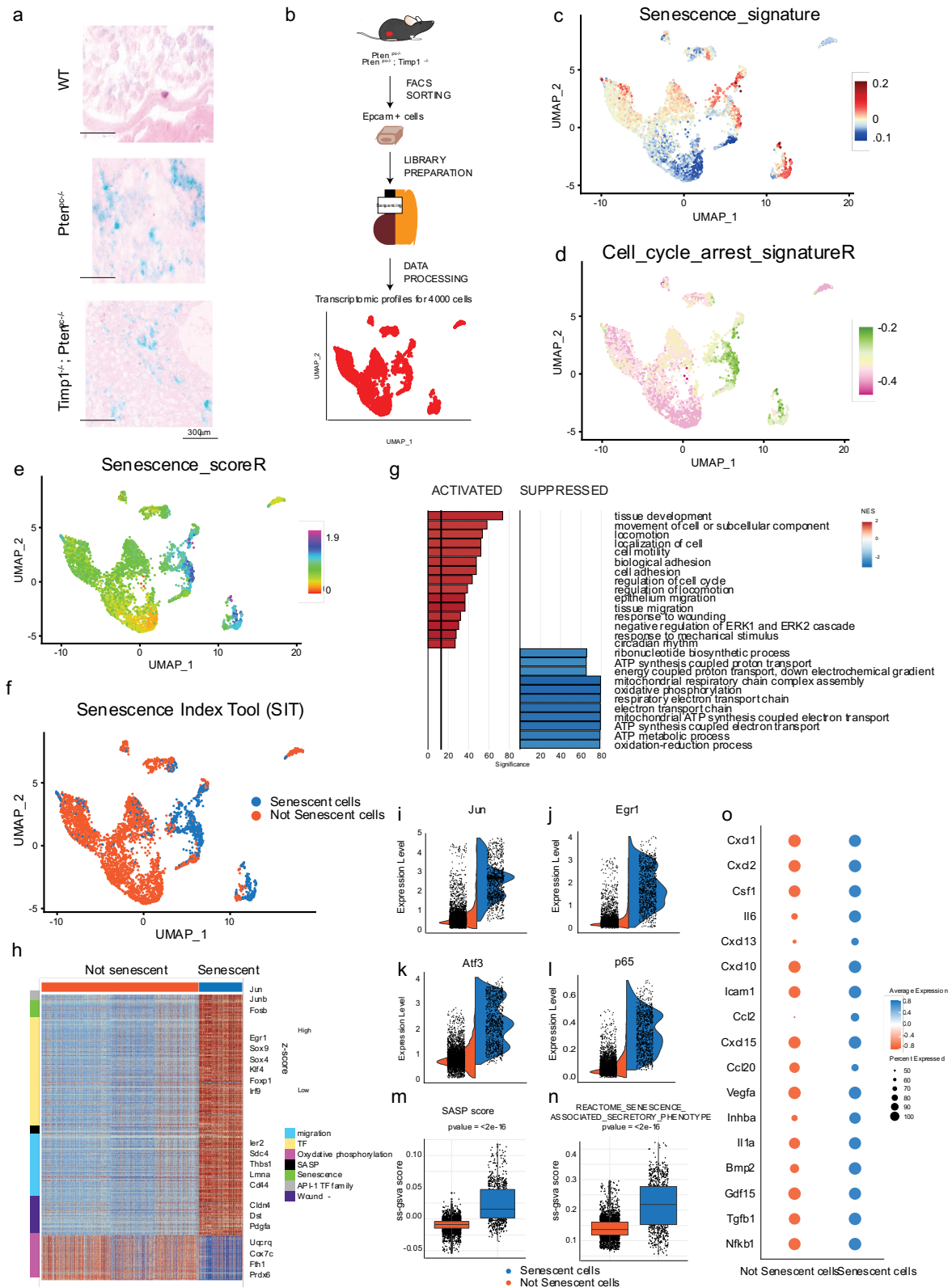


Figure 1 : Identification of senescent cells in prostate cancer with scRNA-seq

a Representative images of SA- β -Gal in WT, Pten^{pc-/-} and Pten^{pc-/-}; Timp1^{-/-} genotypes (Scale bar 300 μ m). Data are representative of two independent experiments. **b** Schematic representation of single cells isolation and sequencing (n = 4 mice). **c** UMAP plot of cancer epithelial cells colored by Senescence_signature. **d** UMAP plot of cancer epithelial cells colored by Cell_cycle_arrest_signature. **e** UMAP plot of cancer epithelial cells colored by Senescence_score. **f** UMAP plot of cancer epithelial cells showing senescent cells (blue) and not senescent cells (orange) found through SIT. **g** Bar plot showing enrichment pathway analysis of senescent cells compared to not senescent cells. gseGO function results: Weighted Kolmogorov Smirnov (WKS) test followed by FDR correction. **h** Heatmap of differentially expressed genes between senescent and not senescent cells. Row annotation showing the functions of different genes (migration, SASP, wounding, TF). Wilcoxon Rank Sum test followed by FDR correction. **i** Violin plot of the most upregulated gene in senescent cells: Jun. **j**, **k** Violin plot of key transcription factors involved in senescence transcriptional program: Egr1, and Atf3. **l** Violin plot showing expression of p65/RelA in senescent cells. **m** Boxplot showing the SASP score of senescent and not senescent cells (two sided Wilcoxon-test: p value < 2.2e-16; N = 3992 cells, Not senescent cells (left) minimum = -0.0537, lower-quartile = -0.0146, median = -0.0088, upper-quartile = -0.0039, maximum = 0.0408, Senescent cells (right) minimum = -0.0353, lower-quartile = 0.0018, median = 0.0154, upper-quartile = 0.0466, maximum = 0.1172). **n** Boxplot showing ss-gsva score of published gene set of Reactome collection77 (senescence_associated_secretory_phenotype) in senescent and not senescent cells (two-sided Wilcoxon-test: pvalue < 2.2e-16; N = 3992 cells, Not senescent cells (left) minimum = 0.0634, lower-quartile = 0.1183, median = 0.1363, upper-quartile = 0.1607, maximum = 0.2952, Senescent cells (right) minimum = 0.0527, lower-quartile = 0.1527, median = 0.2183, upper-quartile = 0.2768, maximum = 0.4215). **o** Expression of fundamental SASP genes where dot size and color represent the percentage of the cell expressing and the averaged scaled expression value, respectively.

Differential expression analysis between senescent and non-senescent prostate tumor cells revealed significant deregulation of genes involved in transcription regulation, wound healing, SASP and oxidative phosphorylation (Fig. 1h). Notably, among transcriptional factors, *c-Jun* was the first up-regulated genes (with fold change higher than 4 times). *c-Jun* has been recently described as a key transcription factor (TF) and regulator of senescence¹⁸⁵, as it critically controls the senescence epigenetic reprogramming. Additional genes encoding proteins that interact with *c-Jun* to form the AP-1 complex, including c-Fos and Atf3, and other members of AP-1 transcription factor family were found upregulated in senescent cells (Fig. 1i-k, Supplementary Fig. 2h). Accordingly, AP-1 downstream targets genes were significantly up-regulated in the senescent cells (Supplementary Fig. 2i), proving the transcriptional reprogramming of these cells by activation of AP-1 TF cascade.

Upon examining the distinctive pathways associated with cellular senescence, we finally observed that senescent tumor cells overexpressed genes signatures linked to autophagy, NF- κ B pathway activation, the SASP and other validated senescence signatures¹⁸⁶⁻¹⁹⁰ (Supplementary Fig. 2j). The up-regulation of the previously validated senescence signatures provides evidence of the reliability of SIT, since they are not prostate cancer related or PICS related.

Of note, the SASP, one of the hallmarks of senescence, is regulated by NF- κ B transcription factor. Consistently we found that *RelA/p65* was the most upregulated gene of the NF- κ B pathway in senescent cells^{191,192}. In addition, SASP factors including *Cxcl1*, *Cxcl15*, *Il6* and *Il1a* exhibited enhanced expression levels as reported by previous data from our and different research teams (Fig.

1l-o).

Furthermore, different out-of-clustering methods were used to validate the consistency of SIT. To this aim, scmap¹⁹³ and SingleR¹⁹⁴ software were applied. Both methods are commonly used in sc-RNA seq data analysis to assist in cell type annotation, cell type prediction, and identification of biological states. As both methods require a query dataset and reference dataset, for this work we used as reference a dataset composed of 12 different datasets from both available single cell and bulk RNA seq (Table 1).

<i>Reference</i>	<i>#Samples</i>	<i>Description</i>	<i>PMID</i>
GSE115301	480	10 sc-RNAseq of ER:RasG12V IMR90 (+4OHT) co-culture with GFP+ cells	31018144
GSE115301	141	smart-Seq2 of 100 growing and 41 senescent IMR90	31018144
GSE61130	6	Non-senescent cells (EV - 4OHT) and Senescent cells (EV + 4OHT) IMR90 ER:RAS cells	26280535
GSE130727	37	WI-38, IMR-90, HUVEC, HAEC cells characterized by replicative exhaustion, ionizing radiation or doxorubicin treatment and expression of HRASG12V.	31251810
GSE102639	10	A549 cells treated with alisertib, barasertib, tozasertib or etoposide and untreated	29045843
GSE132369	6	Proliferating or senescent (IR) profiling of IIMR90 cells	32001510
E-MTAB-9970	60	13 cancer cell lines treated with a lisertib or etoposide or proliferative	34320349
GSE98440	8	proliferative and senescent MSC cell line	29706538
GSE98440	6	proliferative and senescent HUVEC cell line	29706538
GSE98440	8	proliferative and senescent IMR90 cell line	29706538
GSE109270	12	PDX2 KRAS mut NSCLC tumors treated with vehicle or with SHP099	29808006
GSE158743	10	U2OS cells 7 days post IR or untreated	33459422

Table 1: Datasets used for out-of-clustering analysis

The two tools differ in the prediction step. In scmap, the mapping process used a machine learning approach to compare the expression profiles of cells in the query dataset with those in the reference dataset, calculating a similarity score. The latter indicates the most likely cell type for each cell in the query dataset, based on specific expression markers for each cell type. By comparing the similarity score, scmap infers the cell types in the query dataset.

On the other hand, singleR defines a similarity score comparing the expression of genes in the query dataset to those in the reference dataset using the Spearman correlation coefficient. The cells are assigned based on the similarity score, indicating the closest match to the gene expression profile of the query cell to the reference dataset. The analysis reported that the SIT and the scmap overlap. Indeed, 52% of senescent tumor cells recognized by the SIT were also recognized as senescent by the scmap. Note that 26% of cells classified as senescent using the SIT, results unassigned with the scmap (Fig. 2a-b). However, these unassigned cells upregulate canonical senescent markers and the

Fridman_senescence signature (Fig. 2c). Thus, the lack of assignment in the senescent category was probably due to the different experimental models we used for the comparison. Moreover, by comparing the SIT with SingleR the results were even better. Indeed, 88.5% of senescent tumor cells recognized by the SIT were also categorized as senescent by using this method (Fig. 2d-f).

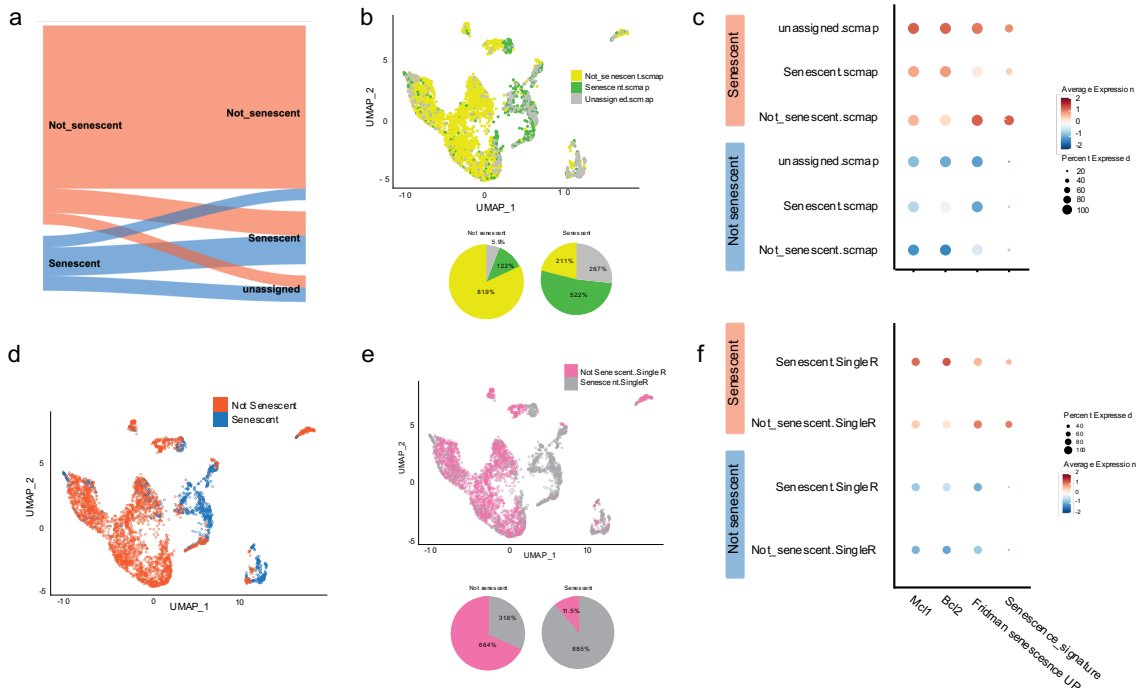


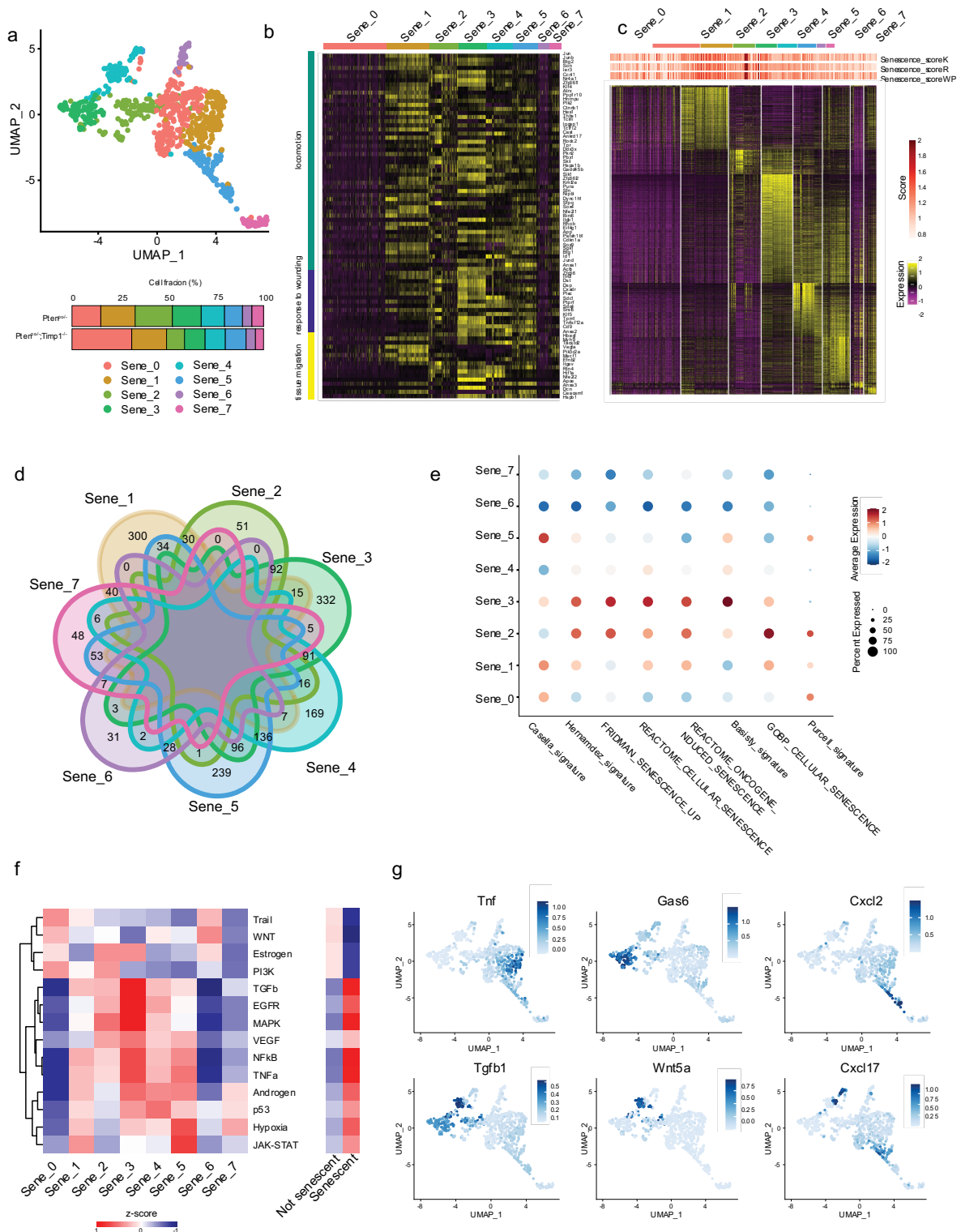
Figure 2: Out-of-clustering analysis for validation of Senescence Index Tool.

a Scmap cluster projection of the SIT classification to the senescence datasets of Table 1. **b** UMAP plot showing the scmap results. Yellow dots = not senescent cell for scmap (N= 2746 cells); green dots = senescent cells for scmap (N= 831 cells); grey dots = unassigned cells for scmap (N=415 cells). (Bottom) Pie-charts of percentage of scmap results in SIT classification. **c** Dotplot showing expression of Mcl1, Bcl2, Fridman_senescence signature and Senescence signature in scmap result. **d** UMAP plot of cancer epithelial cells showing senescent cells (blue) and not senescent cells (orange). **e** UMAP plot showing the SingleR results. Pink dots = Not senescent cells for SingleR (N= 2243 cells); grey dots = Senescent cells for SingleR (N=1749 cells). (Bottom) Pie-charts of percentage of SingleR results in SIT classification. **f** Dotplot showing expression of Mcl1, Bcl2, Fridman_senescence signature and Senescence signature in SingleR results.

4.2 Senescent tumor cells differently segregate at single cell RNA-seq level based on their transcriptional profile

The SIT was employed to investigate the potential heterogeneity among senescent tumor cells. The analysis revealed that these cells formed eight distinct subpopulations, irrespective of the genetic background (Fig. 3a). By performing differential expression analysis, commonalities and significant differences between the eight subpopulations were outlined. Specifically, Sene_1, Sene_3 and Sene_5 exhibited a peculiar gene expression program compared to the other populations (Fig. 3b-

d).

**Figure 3 : Senescent cells are a heterogeneous population**

a UMAP view of only senescent cells, colored by clusters found with FindCluster function (res = 0.5) (top) and percentage of clusters in the two genotypes (bottom). **b** Heatmap showing expression of differentially expressed genes found in total senescent cells related to pathways activated in total senescent cells. **c** Heatmap showing

differentially expressed genes between various senescent clusters (Sene_clusters) using FindAllMarker function. Wilcoxon Rank Sum test followed by FDR correction. **d** Venn diagrams showing the differentially expressed genes in each Sene_cluster. Overlapping areas indicate the number of genes commonly modulated among subpopulations. The numbers report only unique differentially expressed genes or transcripts common between two clusters. **e** Dotplot showing ssGSEA of previously published senescence signature (Casella_signature, Hernandez_signature, Fridman_senescence_up, Basisty_signature, Purcell_signature, Cellular senescence from Gene Ontology collection, Oncogene-induced senescence and Cellular senescence gene sets from Reactome collection) in all the senescent clusters. **f** Heatmap showing pathway activity of 14 relevant signaling pathways calculated using PROGENy between different senescent subpopulations (left) and between senescent and not senescent cells (right). **g** FeaturePlot showing expression levels of different secreted factors upregulated in specific Sene_clusters and not expressed in the other senescent cells.

Notably, the senescent clusters displayed different levels of expression for different senescence signatures¹⁸⁶⁻¹⁹⁰ (Fig. 3e). By investigating the pathway activity inside the different senescent clusters, JAK-STAT, NF- κ B, p53, MAPK and TNF α signaling, known to be upregulated in senescence tumor cells, were mostly activated in the Sene_1-5 clusters.

Instead, Sene_0 and Sene_6 clusters were built on upregulation of WNT and PI3K pathways associated with premature senescence¹⁹⁵⁻²⁰¹ (Fig. 3f). Intrigued by these data, we exploited the difference among the SASP factors. Interestingly certain SASP genes such as *Tnf*, *Cxcl2*, *Cxcl17*, *Gas6*, *Wnt5a* and *Tgfb1* were specifically expressed by some of the Sene_clusters being undetectable in others (Fig. 3g). Previous data have highlighted the importance of Cxcl2 and Cxcl17 as crucial chemokines involved in myeloid cells' recruitment, which represents the most abundant immune population in CRPC^{125,202,203}.

In summary, these findings revealed the transcriptional heterogeneity of senescent tumor cells and, for the first time, the SASP factors, typically upregulated during senescence, have been identified within specific sub-clusters rather than being present in all senescent cells.

4.3 Mcl-1 is the key factor that antagonizes cell death in senescent prostate tumor cells

The SIT allowed the detection of senescent tumor cells and the investigation of senescent heterogeneity. However, in order to identify new therapeutic vulnerabilities, we then moved to investigate the common genetic pathways expressed by these kinds of cells. Particularly, senescent cells are known to be resistant to programmed cell death, attributed to the increased expression of BCL2 and BCL-XL^{31,32}. However, a comprehensive analysis of the cell death modalities in senescent prostate tumor cells was missing. Therefore, aiming to identify a shared vulnerability, we utilized the SIT to annotate the deregulated pro-survival pathways in senescent prostate tumor cells (Fig. 4a). We decided to investigate the state of various molecular mechanisms of cell death to dissect

modalities of survival of senescent cells. We studied the major cell death pathways, including apoptosis, ferroptosis, necroptosis, parthanatos and pyroptosis (Fig. 4b-c). Apoptosis and necroptosis were identified as the most enhanced cell death pathways. Moreover, by separating the genes involved in pro-apoptotic and anti-apoptotic pathways, we observed significant upregulation of the latter in senescent tumor cells. This finding was in line with the capability of senescent cells to bypass cell death.

Therefore, we conducted a more in-depth analysis at the gene level, investigating many of the genes involved in the pro-survival pathway, with the aim of identifying the genes with the greatest significance and impact. We correlated the pro-survival genes (n=47) (Supplementary Fig. 3a) to the senescence scores (SK, SR, SWP) identified with SIT, and only 12 genes positively correlated with all senescence scores with Pearson's coefficient R higher than 0.4 and FDR <0.05 (Fig. 4d, Supplementary Fig 3b). As a positive control of the goodness of the analysis, Bcl2, the main target for the elimination of senescent cells, was included in the shorted list of impacting genes^{108,204}.

Then, the senescent tumor cells were classified based on Bcl2 expression (Bcl2⁺ and Bcl2⁻) (Fig. 4e). Surprisingly, we found that roughly 50% of senescent tumor cells were not expressing Bcl2 at high levels (Fig. 4e). On the contrary, Mcl-1, a member of the BCL2 gene family, was the most correlated gene and was more upregulated than Bcl2²⁰⁵ (Supplementary Fig.3b-c). Moreover, Mcl-1 was expressed both in the Bcl2⁺ and Bcl2⁻ clusters and, when studying the co-expression of Bcl2 and the other pro-survival genes, Mcl-1 emerged as the gene mostly expressed by all the senescent cells (Fig. 4f-h, Supplementary Fig. 3d).

We subclustered the senescent cells based on Bcl2 and Mcl1 expression in Bcl2⁺Mcl1⁺, Bcl2⁻Mcl1⁺, Bcl2⁺Mcl1⁻ and Bcl2⁻Mcl1⁻, and we interrogated the difference in term of gene expression, with particular focus on SASP factors and the pathways. Both Bcl2⁺Mcl1⁺ and Bcl2⁻Mcl1⁺ senescent cells overexpressed genes involved in the regulation of angiogenesis and migration, and were enriched in SASP genes (Fig. 4i-j and Supplementary Fig. 3e-g).

Altogether, these data suggest that the majority of senescent tumor cells rely on elevated levels of Mcl-1 and that these cells activate gene pathways that may contribute to tumor progression through diverse mechanisms.

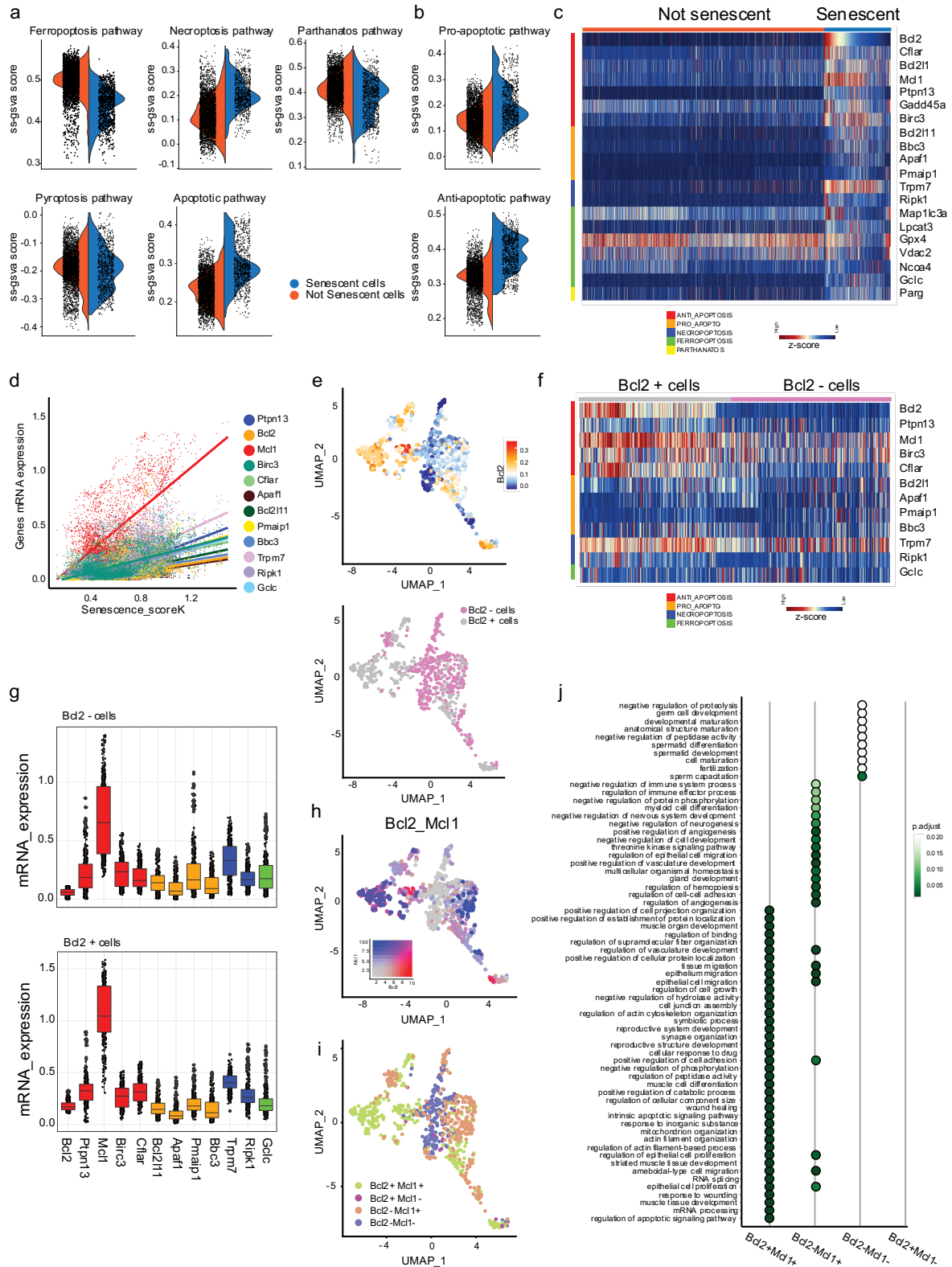


Figure 4 : Mechanisms of survival in senescent cells

a Violin plot showing ss-GSVA score activation of different cell death modalities between senescent and not senescent cells. **b** Violin plot showing ss-GSVA score activation of apoptosis between senescent and not senescent cells, separating in pro and anti-apoptotic genes. **c** Heatmap of 20 differentially expressed genes of different cell death modalities. FDR < 0.05. **d** Correlation plot between Senescence_scoreK and survival related-genes with Pearson

correlation coefficient higher than 0.4 and FDR < 0.05. **e** UMAP plot showing Bcl2 expression levels in senescent cells (top) and classification of them in Bcl2+ or Bcl2- (bottom). **f** Heatmap showing z-score expression levels of 12 survival related-genes, positively correlated with Senescence_scoreK,WP,R, in Bcl2+ and Bcl2- senescent cells. **g** mRNA expression levels of 12 survival related-genes in Bcl2+ and Bcl2- senescent cells (N = 862 cells, Bcl2 - cells: Bcl2 median = 0.0596; Ptpn13 median = 0.1857; Mcl1 median = 0.654; Birc3 median = 0.234; Cflar median = 0.160; Bcl2l11 median = 0.140; Apaf1 median = 0.068; Pmaip1 median = 0.165; Bbc3 median = 0.091; Trpm7 median = 0.331; Ripk1 median = 0.169; Gclc median = 0.175. Bcl2 + cells: Bcl2 median = 0.169; Ptpn13 median = 0.324; Mcl1 median = 1.045; Birc3 median = 0.274; median = 0.314; Bcl2l11 median = 0.145; Apaf1 median = 0.083; median = 0.178; Bbc3 median = 0.113; Trpm7 median = 0.402; Ripk1 median = 0.261; Gclc median = 0.179). **h** Co-expression of Bcl2 and Mcl1 in senescent cells. **i** UMAP plot showing the classification of senescent cells based on Bcl2 and Mcl1 expression levels. **j** Dotplot showing over-representation results of biological process (GO) pathway analysis by considering senescent cell subclassification. Hypergeometric test followed by FDR correction.

4.4 Inhibition of Mcl-1 works as potent senolytic therapy

Intrigued by these findings, we next checked whether cells treated with drugs that cause therapy-induced senescence (TIS) had high expression levels of Mcl-1. We took advantage of various human (PC3, PC3 shTIMP1 and LNCaP) and mouse (TrampC1, TC1, TrampC1Pten^{-/-}, TC1Pten^{-/-}, and RapidCap) prostate tumor cells. To assess senescence, the different cell lines were treated with two different pro-senescence drugs, Docetaxel and Palbociclib (Fig. 5a-d, Supplementary Fig. 4a,b). The induction of senescence was established by checking the upregulation of senescence markers. After TIS, Mcl-1 and Bcl2 upregulation occur both at protein and mRNA levels (Supplementary Fig. 4a,b). To evaluate the role of Mcl-1 in the survival of senescent tumor cells, we treated them with either S63845, a potent Mcl-1 inhibitor^{206,207} or the Bcl2 inhibitor ABT263^{108,204}. Of note, S63845 proved to be more efficient than ABT263 in driving senolysis, both in terms of selectivity and potency. These data were collected through different assays such as SA- β -Gal staining, quantification of proliferation by crystal violet and Incucyte imaging analysis (Fig. 5a-d, Supplementary Fig. 4c). The removal of senescent cells by S63845 led to apoptosis as shown by upregulation of Cleaved Caspase 3 at protein level (Supplementary Fig. 4d). Further validation was performed using two additional Mcl-1 inhibitors, UMI77 and AZD5991^{208,209} (Supplementary Fig. 4e-h).

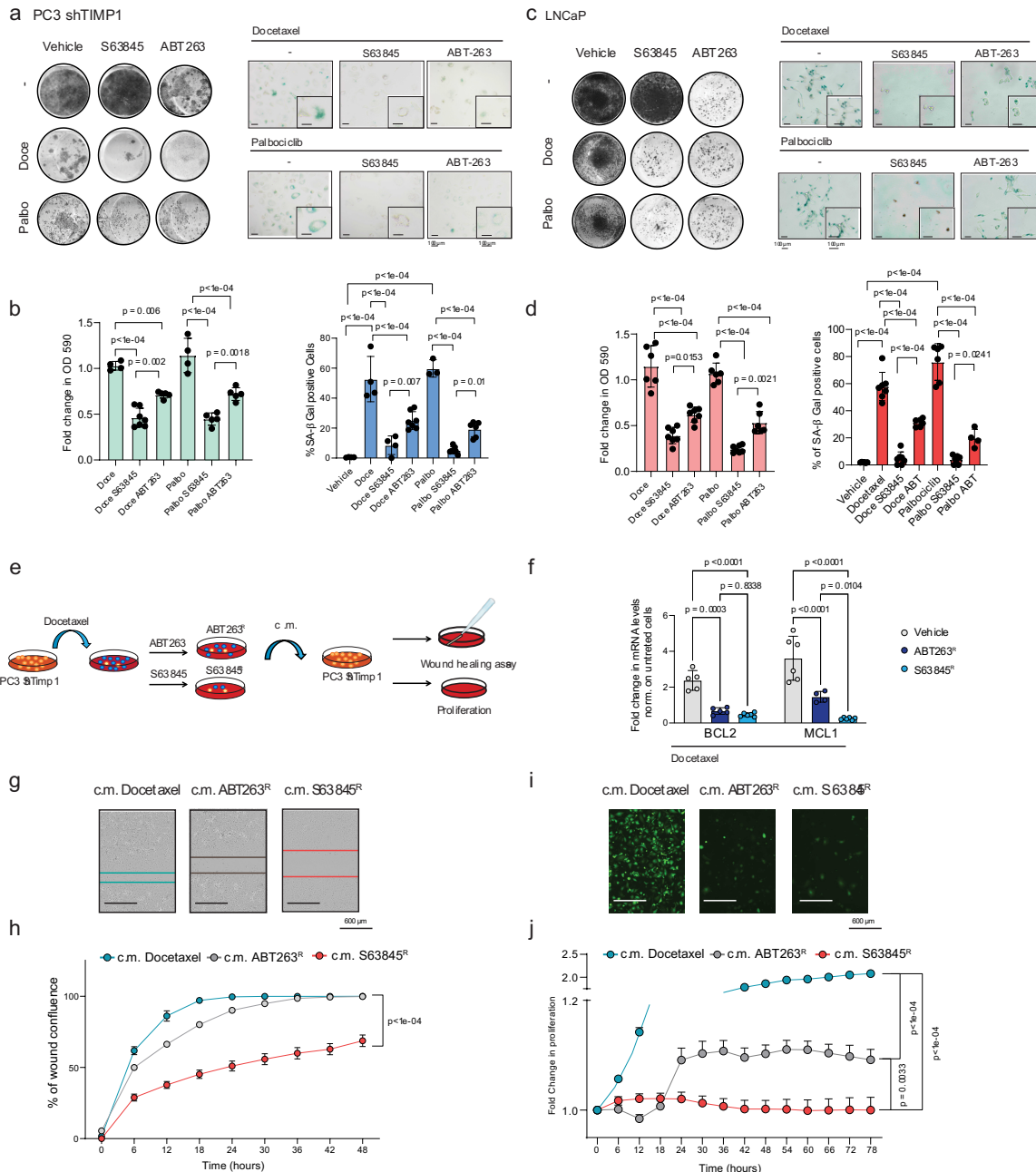


Figure 5 Mcl-1 targeting as senolytic anti-migratory therapy

a Representative pictures of crystal violet and SA-β Gal of PC3 shTIMP1 treated cells (Scale bar 100 μm). Data are representative of three independent experiments. **b** Cristal violet and SA-β Gal quantification (Cristal violet n = 4 for Docetaxel and Palbociclib, n = 5 for Docetaxel ABT263, Palbociclib S63845 and Palbociclib ABT263, n = 7 for Docetaxel S63845; SA-β Gal n = 4 for Vehicle, Docetaxel, Docetaxel S63845, n = 3 for Palbociclib, n = 6 for Palbociclib ABT263 and n = 7 for Docetaxel ABT263 and Palbociclib S63845, n = biological independent samples from three independent experiments). **c** Representative pictures of crystal violet and SA-β Gal of LNCaP treated cells (Scale bar 100 μm). Data are representative of three independent experiments. **d** Quantification of crystal violet and SA-β Gal staining (Cristal violet n = 6 for Docetaxel, Palbociclib and Palbociclib ABT263, n = 7 for Docetaxel S63845, Docetaxel ABT263 and Palbociclib S63845; n = biological independent samples, SA-β-Gal n = 6 for Vehicle, Docetaxel ABT263 and Palbociclib, n = 7 for Docetaxel, Docetaxel ABT263 and Palbociclib S63845, n = 4 for Palbociclib ABT263, n = biological independent samples from three independent experiments). **e** Schematic representation of the experimental design. **f** RT-qPCR analysis of Docetaxel treated cells and the remaining clones upon ABT263 (ABT263^R) and S63845 (S63845^R) treatment (for Bcl2 n = 5 for Vehicle and ABT263R, n = 6 for S63845R; for Mcl1 n = 6 for Vehicle and S63845^R and n = 4 for ABT263^R, n = biological independent samples from three experiments). **g** Representative pictures of wound healing assay (Scale bar 600 μm). Data are representative of two independent

experiments. **h** Percentage of wound confluence over time normalized to time 0 (for c.m. Docetaxel and c.m. ABT263^R n = 8, for c.m. S63845^R n = 6, n = biological independent samples from two independent experiments). **i** Representative pictures of proliferation assay (Scale bar 600 μ m). Data are representative of two independent experiments. **j** Fold change in proliferation normalized to time 0 (for c.m. Docetaxel, c.m. ABT263^R and c.m. S63845^R n = 6, n = biological independent samples from two independent experiments). **b, d, f, h, j** The p values were determined by one way ANOVA followed by Tukey's multiple comparison test. **b, d, f** Data are represented as mean $\pm$ SD. **h, j** Data are represented as mean $\pm$ SEM.

Interestingly, after ABT263 administration, a population of ABT263 resistant (ABT263^R) cells, still SA- β -Gal positive, persisted, whereas the treatment with S63845 reduced the percentage of surviving senescent tumor cells (S63845^R) (Fig. 5a,c). ABT263^R cells exhibited higher mRNA levels of Mcl-1, whereas S63845^R cells did not (Fig. 5e, f), suggesting that Mcl-1 can be a new target for the elimination of ABT263^R senescent cells.

Using an inducible shMCL1 in LNCaP and RapidCap cells, we validated Mcl-1 genetically as a target for senolysis (Supplementary Fig. 4i). The levels of Mcl-1 in both the cell lines were efficiently reduced after Doxycycline administration (Supplementary Fig. 4j,m). Of note, the silencing of Mcl-1 in cells treated with TIS properly eliminated senescent tumor cells (Supplementary Fig. 4k-l, n-o). In cells treated with Doxycycline and S63845 alone, the killing of senescent cells was comparable (Supplementary Fig. 4l,o). However, in cells treated with the combination of Doxycycline and S63845, the percentage of death cells was not increased (Supplementary Fig. 4l,o). These data indicate that Mcl-1 is essential for the survival of senescent cells and that S63845 is a specific Mcl-1 inhibitor.

Additionally, we explored the potential effect of senescent tumor cells resistant to senolytic therapy on the proliferation and migration of neighboring tumor cells. In line with our previous findings²⁸, Conditioned media (c.m.) from senescent PC3 shTIMP1 cells treated with Docetaxel enhanced the migration and proliferation of parental PC3 cells, while c.m. from S63845 treated cells completely blocked the proliferation and migration of PC3 parental cells. This resulted in an improved outcome than that seen in cells treated with ABT263 (Fig. 5g-j).

Intrigued by the fact that senescent tumor cells can migrate by themselves, we performed co-culture experiments using Docetaxel-treated GFP⁺ PC3 ShTIMP1 cells and untreated parental PC3. We observed that a small percentage (15%) of senescent cells could migrate, as shown by the overlap of SA- β -Gal⁺ and GFP⁺ cells. This phenomenon was abolished in S63845 treated cells (Supplementary Fig. 5a-e). Similar pro-tumorigenic effects were observed in murine Tomato⁺RapidCap cells. These cells were treated either with c.m. from Docetaxel, ABT263, or S63845 treated cells or were co-cultured with parental untreated cells. Also in murine cell lines, S63845 treatment demonstrated superior results compared to ABT263 administration (Supplementary Fig. 5f-n).

In summary, these results indicate that senescent tumor cells treated with TIS can migrate and promote the proliferation and migration of neighboring prostate tumor cells. In addition, senolytic therapy with S63845 can significantly attenuate these pro-tumorigenic effects *in vitro*.

4.5 Mcl-1 inhibition increases the efficacy of conventional treatment in prostate cancer

To further corroborate our data, we subcutaneously injected PC3 Luc-shTIMP1 in NRG mice. As previously demonstrated by our group, this tumor cell type upon TIS exhibits migratory and invasive behavior, leading to metastases due to the elevated activity of MMPs²⁸.

Once the tumors reached the volume of 100 mm³, mice were treated with Docetaxel followed by either S63845 or ABT263 treatments (Fig. 6a,b). As expected, Docetaxel induced senescence in primary prostate tumors, whereas S63845 treatment properly eliminated senescent prostate tumor cells, as confirmed by a reduction in SA- β -Gal and MCL-1 positive cells and a increase in Cleaved Caspase 3 positive tumor cells (Fig. 6c,d). It should be noted that S63845 therapy killed senescent tumor cells more efficiently than ABT263. These findings were in line with the bioinformatic and *in vitro* analysis showing that senescent cells rely on Mcl1 more than Bcl2. However, the two therapies were comparable in tumor growth progression.

Since the interconnection between senescence and metastases has recently emerged¹³³, we took advantage of the xenograft model and of the luciferase expression in PC3 Luc-shTIMP1 cells to perform immunohistochemistry (IHC) analysis in different metastatic organs.

Importantly, while mice treated with Docetaxel developed metastases to the lungs and the liver, mice treated with the combination of Docetaxel and S63845 showed a stronger reduction in metastases formation. This effect was superior in mice treated with Docetaxel in combination with ABT263 in terms of the number of metastases (Fig. 6e, f).

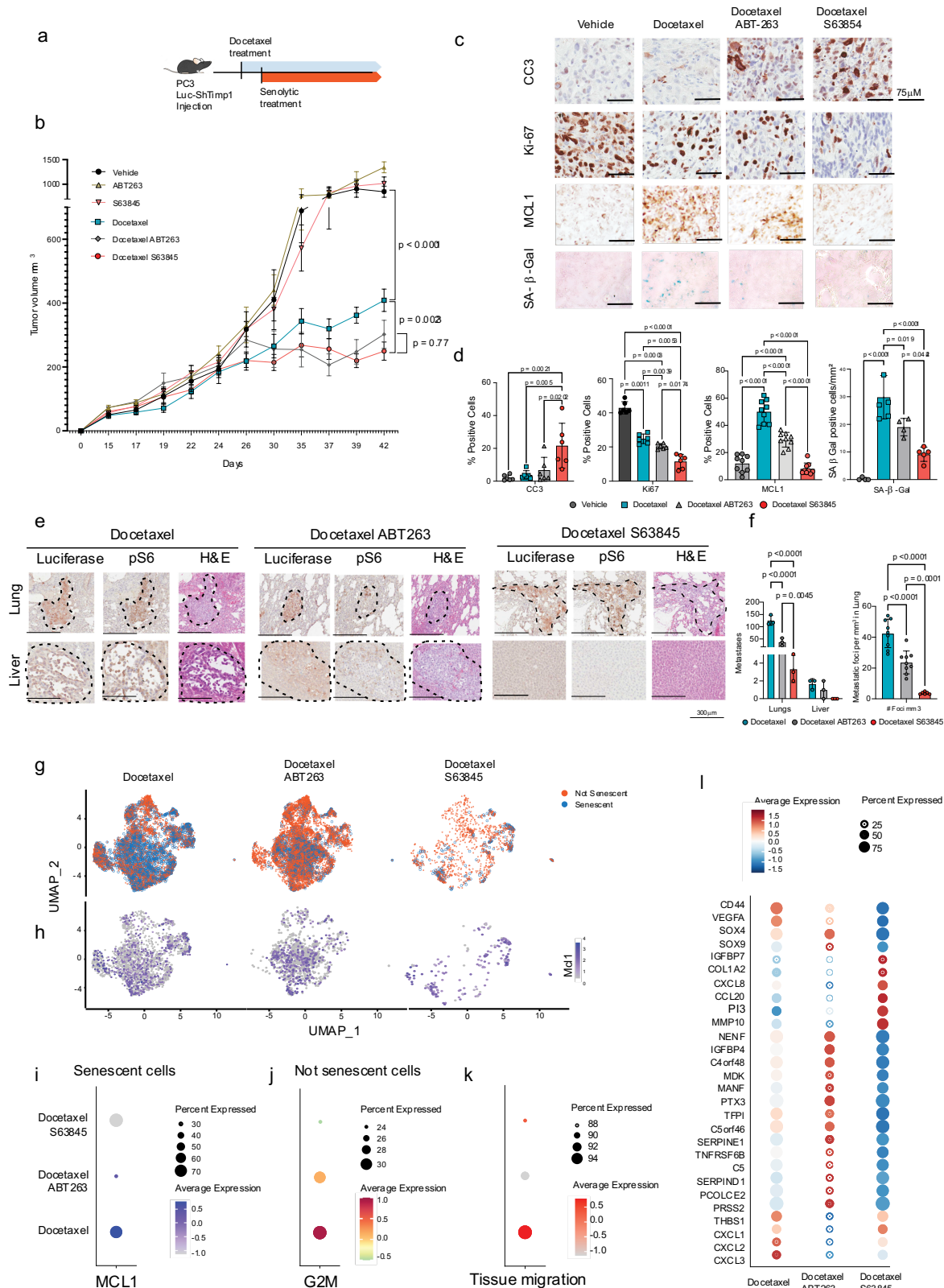


Figure 6 : S63845 is a potent senolysis in vivo in PCa.

a Schematic representation of the experimental design. **b** Growth curve of tumors in vivo in mm³ (n = 5 for Untreated, ABT263, S63845, Docetaxel, Docetaxel + ABT263, Docetaxel + S63845, n = independent animals from one experiment). The p values were determined by two-way ANOVA followed by Tukey's multiple comparison test at time point 39 days post injection, separately for single treatment (Untreated, Docetaxel, ABT263, S63845) and Docetaxel

treated groups (Docetaxel, Docetaxel + ABT263, Docetaxel + S63845). Data are represented as mean $\pm$ SEM. **c** Representative pictures of immunohistochemistry for Cleaved Caspase 3 (CC3), Ki67, MCL-1 and SA- β Gal. Data are representative of one experiment. **d** From left, quantification in percentage (CC3 $n = 6$, Ki67 $n = 6$, MCL-1 $n = 9$ and SA- β Gal $n = 6$; $n =$ multiple areas of four animals from one experiment. The p values were determined by One-way ANOVA test followed by Tukey's multiple comparisons test). Data are represented as mean $\pm$ SD. **e** H&E, Luciferase and phospho-S6 immunohistochemical staining in lung and liver metastases in Docetaxel, Docetaxel + ABT263 and Docetaxel + S63845 treated NRG mice. Data are representative of one experiment. **f** Bar graph representing metastases quantification ($n = 9$ for Docetaxel and Docetaxel + ABT263, $n = 6$ for Docetaxel + S63845; = multiple areas of 4 animals from one experiment). For metastases count the p values were determined by Two-way ANOVA followed by Šídák's multiple comparisons, while for the count of metastases foci per mm³, the p values were determined by one Way ANOVA followed by Tukey's multiple comparisons test. Data are represented as mean $\pm$ SD. **g** UMAP plot of senescent and not senescent cells, defining using SIT, in xenograft PC3 shTIMP1 cells treated with Docetaxel alone or in combination with ABT263 or S63845 (Docetaxel % senescent cells = 35.5%, Docetaxel + ABT263 % senescent cells = 15.6%, Docetaxel + S63845 % senescent cells = 14%). **h** UMAP plot of senescent cells showing MCL-1 expression in xenograft PC3 shTIMP1 cells treated with Docetaxel alone or in combination with ABT263 or S63845. **i** MCL-1 expression for each treatment, where dot size and color represent the percentage of cells expressing the indicated gene and the average scaled expression value, respectively. **j** G2M signature for each treatment, where dot size and color represent percentage of cells expressing and the averaged scaled expression value, respectively. **k** ss-GSVA of tissue migration signature for each treatment, where dot size and color represent percentage of cell expressing and the averaged scaled expression value, respectively. **l** Marker genes expressions for each treatment, where dot size and color represent percentage of cell expressing and the average scaled expression value, respectively.

Intrigued by the effect of these treatments on tumor cells at single-cell resolution, we performed 10X Genomics single-cell RNA sequencing. We sorted prostate tumor cells by taking advantage of the GFP signal since the ShTIMP1 plasmid was carrying this fluorescent reporter, and we applied the SIT to these data.

The use of the SIT in 10X Genomics data of primary senescent prostate tumors cells indicates that S63845 treatment led to a stronger elimination of senescent tumor cells when compared to ABT263 treatment (Fig. 6g, Supplementary Fig. 6a). Of note, sc-RNA seq data showed that in prostate tumor treated with Docetaxel and ABT263, the remaining senescent tumor cells increased MCL-1 levels and upregulated genes linked to angiogenesis, cell migration and wound healing (Fig. 6h,i, Supplementary Fig. 6b), mirroring the more invasive capability of these cells.

Since senescent cells coexisted with other tumor cells, we investigated the proliferative and pro-migratory capability at single-cell levels. Indeed, the non-senescent cells resistant to Docetaxel and ABT263 had an increased proliferation and pro-migratory capability compared to the non-senescent cells remaining after the combination of Docetaxel and S63845 (Fig. 6j-l).

These results were in line with the reduction in Ki67 staining and the decreased number of metastases found in mice treated with S63845 (Fig. 6c-f). Validation of the elimination of the senescent tumor cells and the down-regulation of metastasis-related genes in mice treated with Docetaxel and S63845 was obtained through RT-qPCR of whole tumor lysates (Supplementary Fig. 6c,d).

To corroborate these findings, the same experiment was performed in *Pten^{pc/-}* mice (Supplementary Fig. 7a). Gland scoring analysis with IHC showed normalization of prostate glands in mice treated with Docetaxel and S63845 compared to the other mice. (Supplementary Fig. 7b and Supplementary Fig. 8a).

To assess the effectiveness of S63845 and ABT263 in eliminating senescent cells in the tumors, p16^{INK4a} and SA-β-Gal immunohistochemistry analysis, along with RT-qPCR for p21^{Waf1/Cip1} and PAI-1, were conducted. Mice treated with both S63845 and ABT263 showed a decrease in senescent cells (Supplementary Fig. 7c-e). However, S63845 demonstrated a stronger senolytic effect and a robust apoptotic response compared to ABT263. This reduction in senescence markers was accompanied by a decrease in MCL-1 and BCL2 levels (Supplementary Fig. 7e).

Furthermore, treatment with S63845 and ABT263 led to a decrease in tumor-infiltrated MDSCs and tumor-associated macrophages (TAMs), which are the two most prominent immune populations in prostate tumors characterized by PTEN loss^{99,210} (Supplementary Fig. 7f-g and Supplementary Fig. 8b). Interestingly, a notable upregulation of Perforin, a marker of T and NK cell activation, was also observed (Supplementary Fig. 7h).

4.6 SIT application on human scRNA-seq data reveals up-regulation of MCL-1 and pro-tumorigenic SASP factors

The study reported above aimed to develop a bioinformatic tool for identifying senescent tumor cells inside prostate cancer samples and discovering new targets for senolytic therapy. The majority of the study was based on human and murine cell lines or murine GEMMs. The SIT, the algorithm specifically developed to spot senescent cells, was developed starting from epithelial tumor cells of murine GEMMs. However, we wanted to expand the applicability of the SIT in human prostate cancer biopsies, investigating published scRNA-seq data collected from CRPC patients²¹¹.

We conducted an analysis on 6 biopsies from CRPC patients sequenced with 10x Genomics scRNA-seq. Using canonical markers, we classified the different cell types (epithelial, immune and stromal cells) in the samples, and then we focused the attention on epithelial cells (Fig. 7a and Supplementary Fig. 9a).

Among these patients, three had received the first-line therapy of the LHRH analog goserelin combined with the AR inhibitor bicalutamide, two had undergone surgical castration along with bicalutamide, and the remaining patient was treated with the chemotherapeutic drug docetaxel (Table 2).

Patient ID	Age	PSA level at diagnosis (ng/ml)	The Gleason score at diagnosis	First-line therapy	Second-line therapy	Time from treatment start to CRPC (mo)	Time from CRPC to now (mo)
Patient #1	82	8.88	4 + 5 = 9	Goserelin, bicalutamide	None	9	0.5
Patient #2	82	56.53	4 + 3 = 7	Goserelin, bicalutamide	None	15.9	3.2
Patient #3	86	55.16	4 + 4 = 8	Goserelin, bicalutamide	None	15.9	3.7
Patient #4	78	>149	4 + 3 = 7	Bilateral orchidectomy, bicalutamide	Docetaxel, Abiraterone	12.5	18.6
Patient #5	65	15.6	4 + 4 = 8	Bilateral orchidectomy, bicalutamide	None	27.8	2.6
Patient #6	70	72.72	NEPC	Docetaxel	None	14.8	3.7

Table 2: Clinical data of CRPC patients

Various studies suggested that both ADT and chemotherapies can induce senescence²¹²⁻²¹⁴. Therefore, the application of SIT had a comprehensive relevance in this dataset.

The clinical story, the line of treatment, the genetic background, and also the quality of the sample after the biopsy could influence the classification of SIT. However, due to the higher heterogeneity of human samples, we applied the SIT for each patient separately. We identified a range of senescent cells, varying between 3% and 19% (Fig. 7b-c and Supplementary Fig. 9b).

We next investigated the differences between senescent and non-senescent prostate tumor cells in each patient. Interestingly, MCL1 was predominantly over-expressed in the senescent cells of Patient1, Patient5 and Patient6, with elevated average expression in the senescent compartment overall (Fig. 7d). On the other hand, BCL2 was expressed in less than 25% of cells and in lower levels (Fig. 7d).

Since the SASP reprogramming impacts the tumor's aggressiveness, the common SASP factors up-regulated in at least two patients were studied. Among them, we spot the overexpression of GDF15, CCL2, CXCL1, CXCL8, CXCL2 and others in the senescent population (Fig. 7e). Our team demonstrated that the GDF15 gene is part of the pro-tumorigenic SASP in the metastatic model of *Pten^{pc/-}; Timp1^{-/-}*²⁸. Moreover, it is known that CXCL1 is a SASP factor involved in the formation of the premetastatic niche¹⁴⁰ and CXCL8, named also IL-8, is a major secreted factor involved in EMT transition and angiogenesis in several cancer¹³⁸.

In sum, these data suggested that human senescent cells identified through SIT increased the expression of secreted factors that promote invasive and metastatic cancer.

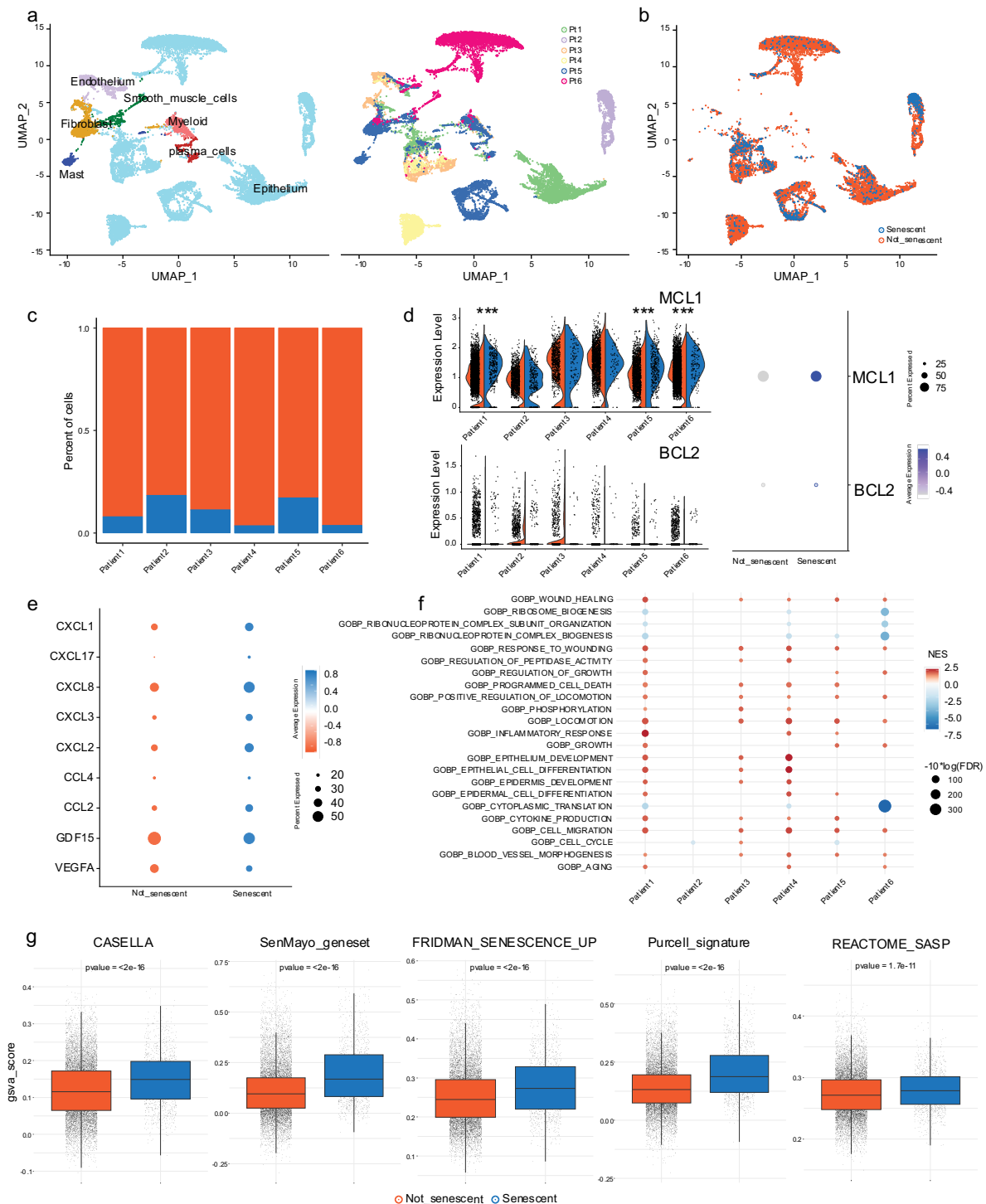


Figure 7 Application of SIT on human CRPC samples

a (Left) UMAP plot showing the different cell type of 6 CRPC human samples (Right) UMAP plot colored by patient IDs. **b** UMAP plot indicating the cell classified as senescent (blue) or not senescent (orange) with SIT. **c** Barplot of percentage of senescent and not senescent cells in each patient. **d** (Left) Violin Plot with expression of MCL1 and BCL2 in each patient between the two populations (Right) Dotplot of MCL1 and BCL2 expressions in senescent and not

senescent cells of all patients. **e** Dotplot of SASP factors. **f** Dotplot representing the results of pathway analysis between senescent and not senescent cells in each patient. Dimension of dot is $-10 \times \log FDR$. Color is representative of the NES. Only pathway with $FDR < 0.05$ are reported. **g** Boxplot of ss-GSVA scores for gene sets related to senescence.

Additionally, pathway analysis of senescent cells of each patient revealed activation of pathways associated with cell aging and cell growth, together with signaling related to migration and wound healing (Fig. 7f). The transcriptomic profile of senescent cells indicated a pro-migratory phenotype, consistent with the findings of SIT on murine samples and with the in vitro and in vivo experiments. Ribosome-related pathways were suppressed during senescence (Fig. 7f).

Moreover, upon examining the distinctive features of cellular senescence, we focused on different senescence-related signatures. Casella signature, Fridman_senescence_up, Purcell_signature and Reactome_SASP gene list were over-expressed in all senescent cells compared to other tumor cells (Fig. 7g and Supplementary Fig. 9c). Notably, a recently developed gene signature called SenMayo²¹⁵, designed explicitly within the context of aging, demonstrated increased activity in senescent cells, both when considering them collectively or when evaluating each patient separately.

In conclusion, the application of SIT in human CRPC patients under different treatments confirmed the potential role of MCL1 in senescence and highlighted the possible migratory phenotype of senescent human cells. These findings advance the understanding of senescence mechanisms and provide valuable insights into potential therapeutic targets for senolytic therapy in human prostate cancer patients.

5. DISCUSSION

Prostate cancer is a widely prevalent disease affecting men worldwide. Most of the PCa diagnosed cases exhibit a non-aggressive nature and they are treated with therapeutic strategies, such as prostatectomy and ADT treatment, which can be sufficient to abolish the advancement of the tumor^{3,216-218}. However, a significant percentage of PCa patients demonstrate an aggressive phenotype that advances into metastatic prostate cancer as tumor cells disseminate to distant organs^{3,219}. To treat these tumors, a range of different therapeutic approaches has been identified to enhance patient outcomes and overall survival rates²¹⁶⁻²¹⁸. Nevertheless, these treatments include chemotherapies that often present substantial side effects and may give the possibility of PCa developing resistance to treatment²²⁰. The chemotherapies Docetaxel and Cabazitaxel, used for the treatment of mCRPC cases, together with ADT treatments, are also classified as "pro-senescence" therapies²²¹. Thereby cellular senescence is induced^{84,104}.

However, the mechanism behind cellular senescence establishment, maintenance and survival remains an object of intense investigation. In contrast to the well-understood cell death induction, our understanding of how tumor cells may undergo senescence and persist within the tumor microenvironment remains limited. Recent findings reveal that pro-senescence approaches initially reduce the tumors' growth and proliferation through senescence¹⁸¹; however, if senescent cells are not promptly removed by the immune system, their prolonged persistence can be adverse in promoting more aggressive tumor and even metastases formation^{28,31,103,133}. Our team, in one publication where I have contributed, demonstrated that in prostate cancer, the dual deletion of PTEN and TIMP1, a metalloproteinases inhibitor, promotes a senescent phenotype and initiates metastases formation²⁸. Indeed, the lack of TIMP1 promotes the release of pro-tumorigenic SASP, which favor the EMT phenotype and, as a consequence, the initiation of metastatic spread²⁸.

Thus, the genetic background of PCa may contribute to the establishment, reprogramming and functional impact of senescence, which can increase tumor aggressiveness. For these and other reasons, the development of senolysis to specifically eliminate senescent cells is needed.

Most of the currently available senotherapies for cancers are restricted to BCL-2 targeting^{84,104,108,204}. In TIMP1 work, we have demonstrated that the administration of senolytic therapy ABT-263, BCL-2 and BCL-XL inhibitor, eliminates senescent cells and reduces the number of metastases²⁸. However, the administration of BCL-2 inhibitors shows many side effects in clinics, opening a window for the discovery of new therapeutic targets and agents for elimination of senescent cells^{113,114}.

In my Ph.D. project, I took advantage of single-cell technologies and I developed a bioinformatic tool,

Senescence Index Tool, able to spot senescent cells in two different mouse models, $Pten^{pc-/-}$ and $Pten^{pc-/-}; Timp1^{-/-}$. The SIT classifies cells as senescent based on two of the main features of senescence: the overexpression of senescence markers and the positivity to different signatures of cell cycle arrest. The high expression levels of AP1 TF family members, the upregulation of SASP factors and of the main pathways related to senescence, such as NF- κ B, wound-healing and different senescence-related signatures, confirmed the reliability of SIT.

In addition, using the SIT, we have demonstrated that within tumors of identical genetic backgrounds, senescent tumor cells can exhibit heterogeneity in terms of expression profile, pathway activation and, last but not least, SASP factors release. This complexity may present challenges in the design of therapies aimed at removing senescent tumor cells and could impact the future development of senolytic treatments in clinical trials.

In this study, we discovered a subset of senescent tumor cells that survive independently from Bcl-2, the major target for senolysis. In addition, even after exposure to the Bcl-2 inhibitor Navitoclax, the remaining cells maintain their ability to drive tumorigenesis through the SASP mechanism.

Irrespective of the heterogeneity observed in senescence, this study identifies Mcl-1 as a novel target for effectively eliminating senescent tumor cells. Mcl-1, belonging to the BCL2 family of BH3 proteins that collectively regulate apoptosis together with Bcl-2, Bcl2-L10, Bcl-W, and Bcl-xL, possesses a distinct structural composition²²². While ABT263 efficiently inhibits Bcl-2 and Bcl-xL, it lacks the capacity to target Mcl-1²²². On the contrary, the second and third generations of Mcl-1 inhibitors exhibit high selectivity and are currently tested in certain clinical trials²²³.

Our findings reveal that the effectiveness of Docetaxel treatment, a standard therapy for metastatic castration-resistant prostate cancer (mCRPC) patients, can be enhanced when administered together with Mcl-1 inhibitors, both in in vitro and in vivo settings. Furthermore, treatment involving various Mcl-1 inhibitors successfully eliminates senescent tumor cells and completely suppresses the migratory phenotype observed in bystander non-senescent tumor cells, which is orchestrated by the SASP in both transgenic and xenograft models.

Notably, this combination of pro-senescence therapy and S63845 demonstrates superior efficacy compared to the combination of Docetaxel with ABT263. Significantly, in the transgenic mouse model, the elimination of senescent tumor cells promotes the reactivation of the tumor immune response, as indicated by reduced MDSC infiltration and heightened perforin levels, a marker of T and NK cell activation.

In addition, we expanded the application of SIT on human scRNA-seq data of prostate cancer

patients. Examining six patient biopsies²¹¹, SIT identified senescent cells (varying from 3-19% per patient) and found MCL1 to be upregulated in multiple patients' senescent cells, contrary to BCL2. Moreover, the high expression of factors linked to invasive and metastatic cancer and the activation of pro-migratory pathways could explain the progression and advancement of CRPC patients into metastatic tumors.

In summary, while senescent cells exhibit significant heterogeneity, their survival ultimately depends on Mcl-1, a pro-survival factor. Notably, this study strongly supports the efficacy of Mcl-1 inhibitors as potent senolytic agents.

Intriguingly, different groups had identified an interconnection between MCL1 and senescence in various contexts. A prior breast cancer study unveiled that the sensitivity of senescent cells to ABT263 is influenced by NOXA, an Mcl-1 inhibitor²²⁴. Cells with high NOXA levels respond to ABT263, whereas those with lower levels are resistant and react to Mcl-1 inhibition, thereby corroborating our findings across different contexts. In addition, Kohli et al.²²⁵ showed that senescent melanocytes, which can progress to melanoma, exhibit prolonged survival due to high expression of anti-apoptotic protein BCL-W but they are resistant to ABT-263 treatment, targeting BCL-W. However, they rely on MCL-1 and the combination of ABT-263 and MCL-1 inhibitors showed potential as a non-invasive therapy to eliminate senescent melanocytes and nevi.

Furthermore, senescent tumor cells demonstrate common attributes with aged cells, as demonstrated by the SenMayo signature²¹⁵ in human CRPC scRNA-seq data. Thus, if validated in additional models, these results could potentially have relevance in ameliorating aging and age-related pathologies.

In conclusion, this work expands senescence understanding and identifies potential senolytic therapy target for prostate cancer patients. Our findings provide a starting point towards more effective, targeted, and personalized approaches for treating prostate cancer and related conditions. In addition, these therapies could also be expanded to other therapeutic areas in which senescence plays a crucial role.

6. EVALUATING THE LIMITATIONS OF THE SIT FOR ENHANCING THE APPLICABILITY

The SIT is developed to identify senescent cells in prostate cancer models. The tool allows us to spot the senescent tumor cells, to study their mechanisms of survival and to identify a new target for the selective elimination of this kind of cells. However, there are also some points that could be improved in the tool. The SIT is based on three major steps: 1) the calculation of senescence signature and the cell cycle arrest signatures, 2) the definition of the three senescence scores and 3) the identification of the senescent tumor cells based on quartile values.

Regarding step 1, for the establishment of the cell cycle arrest status at single cell resolution we have used a tool called Gene Set Variation Analysis (GSVA) with the method “ssGSEA” (single sample Gene Set Enrichment Analysis)²²⁶. To compute the ssGSEA, an enrichment score is calculated using the Empirical Cumulative Distribution Functions (ECDF) of the genes within the gene set and those outside it and the enrichment score is determined by integrating the difference between these ECDFs. In particular, for a given gene set G of size N and a single sample S from a dataset comprising N genes, the genes are reassigned according to their rank based on expression, resulting in a list $L=(r_1, r_2, \dots, r_N)$. The enrichment score $ES(G, S)$ is computed by summing (integrating) the discrepancy between a weighted ECDF of the genes within the gene set and the ECDF of the remaining genes. This calculation is repeated for each signature and each sample in the dataset²²⁶. However, the algorithm was developed for bulk RNA data analysis and some works have shown that bulk-sample-based techniques for signature scoring are more vulnerable to dropout-related issues²²⁷. The dropout is a phenomenon occurring in single cell RNA sequencing where the expression of certain genes is not detected due to the physiological cyclic production of mRNA in cells. In the past year, many signature scoring methods were developed for single cell RNA-seq data. Between them, there are AUCell²²⁸, UCell²²⁹, that effectively address dropout effects by focusing on expressed genes. We will try to use these methods for the definition of the cell cycle arrest signatures and to compare the results, improving the SIT.

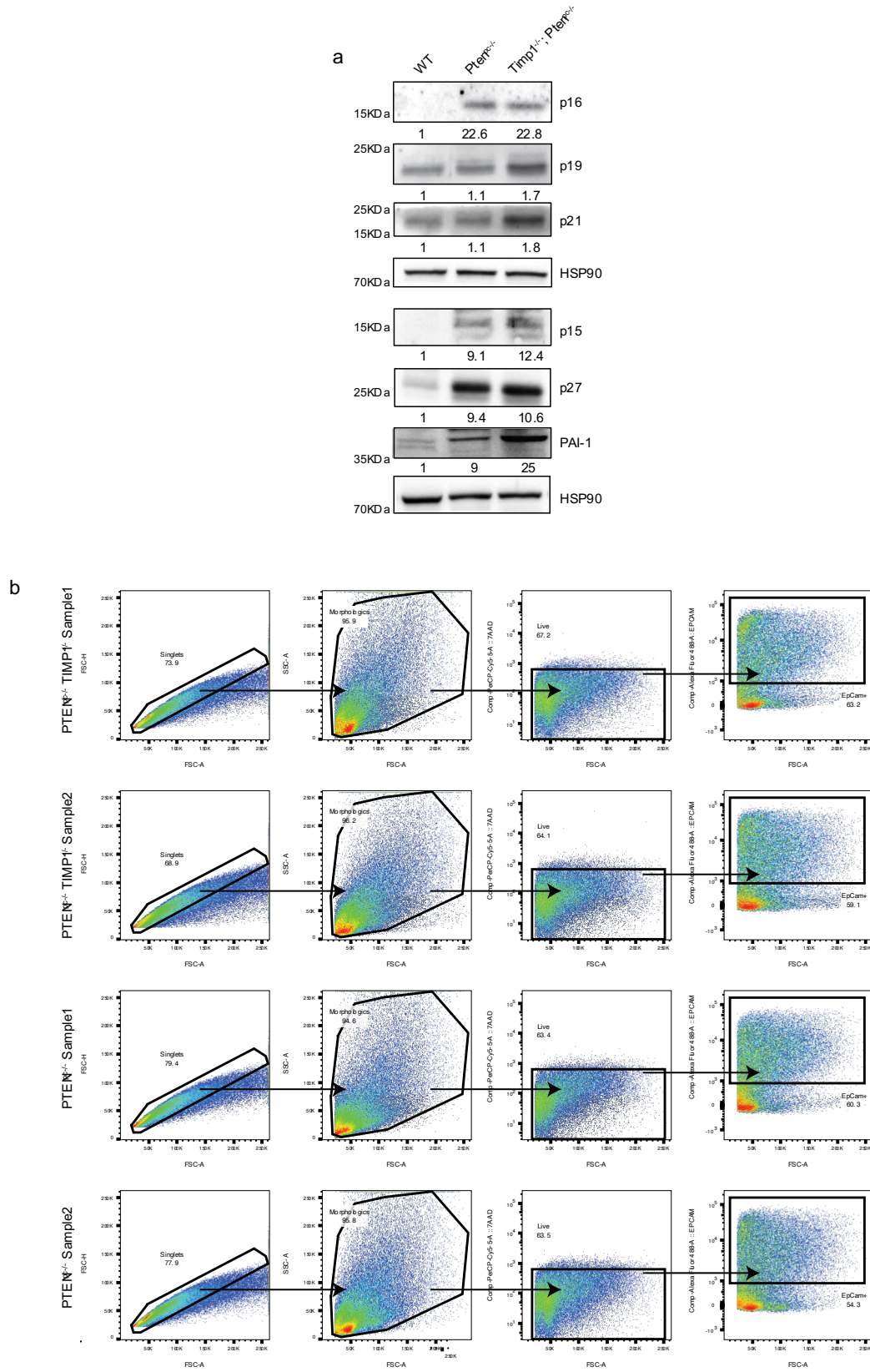
Moreover, in the step 2) the senescence scores are defined with the equation

$$(2) \text{ Senescence score}_i = \text{Cell cycle arrest signature}_{i \text{ norm}(0-1)} + \text{Senescence signature}_{\text{norm}(0-1)} \\ \text{with } (i = K, WP, R)$$

However, in the normalization of the senescence signature between 0 and 1 some negative values, corresponding to a lack of expression of senescent biomarkers, could give a positive contribution in the senescence scores. For this reason, in the step 3) where there is the definition of senescent cells

we will introduce more criteria for the selection, as the exclusion of cells identified as senescent if the senescence signature, defined as average expression of the senescent markers, is not positive. In addition, the identification of a cell as senescent is based on the quartile division of the senescence scores. The type of division can be adjusted on the basis of the biological knowledge. To conclude, we are exploring the possibility to apply the SIT in scenarios other than prostate cancer. In this view we should acknowledge that in the step 1) we have performed an average expression of six senescent markers, which could be not expressed in the various types of senescence in all the different organs. We propose to improve the definition of the senescent markers by considering genes overexpressed in multiple senescent cell types, normal and cancer, in order to expand the applicability of the SIT to other organs and other senescence inducers. In addition, in collaboration with another group studying the senescence in kidney acute injury, we have noticed that the exclusively definition of the senescence signature is sufficient to detect senescent cells in organs that are not proliferating. To conclude, we will improve the algorithm of SIT in order to expand the applicability of the tools.

Supplementary Figures

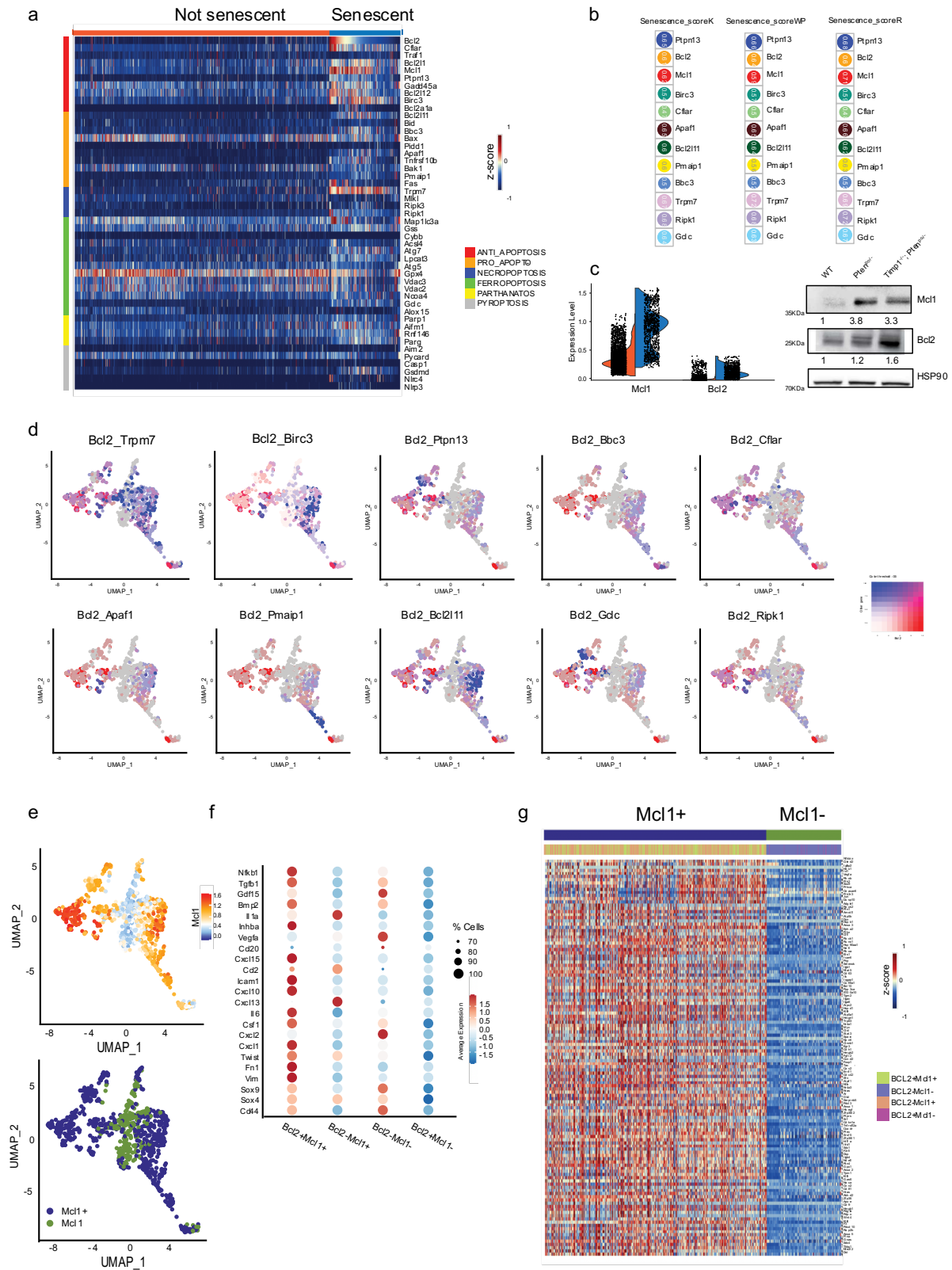


Supplementary Figure 1 : Markers of senescence in prostate cancer and sorting strategy for 10X Genomics analysis.

a, Western blots with quantification for the senescence markers (*p16^{INK4a}*, *p15^{INK4b}*, *p19^{Arf}*, *p21^{Waf1/Cip1}*, *p27^{Kip1}* and *PAI-1*) in the indicated genotypes. Data are representative of three independent experiments. **b**, Gating strategy of the epithelial tumor cells of the different genotypes identified by the EPCaM antibody. Data are representative of 4 animals from one experiment.

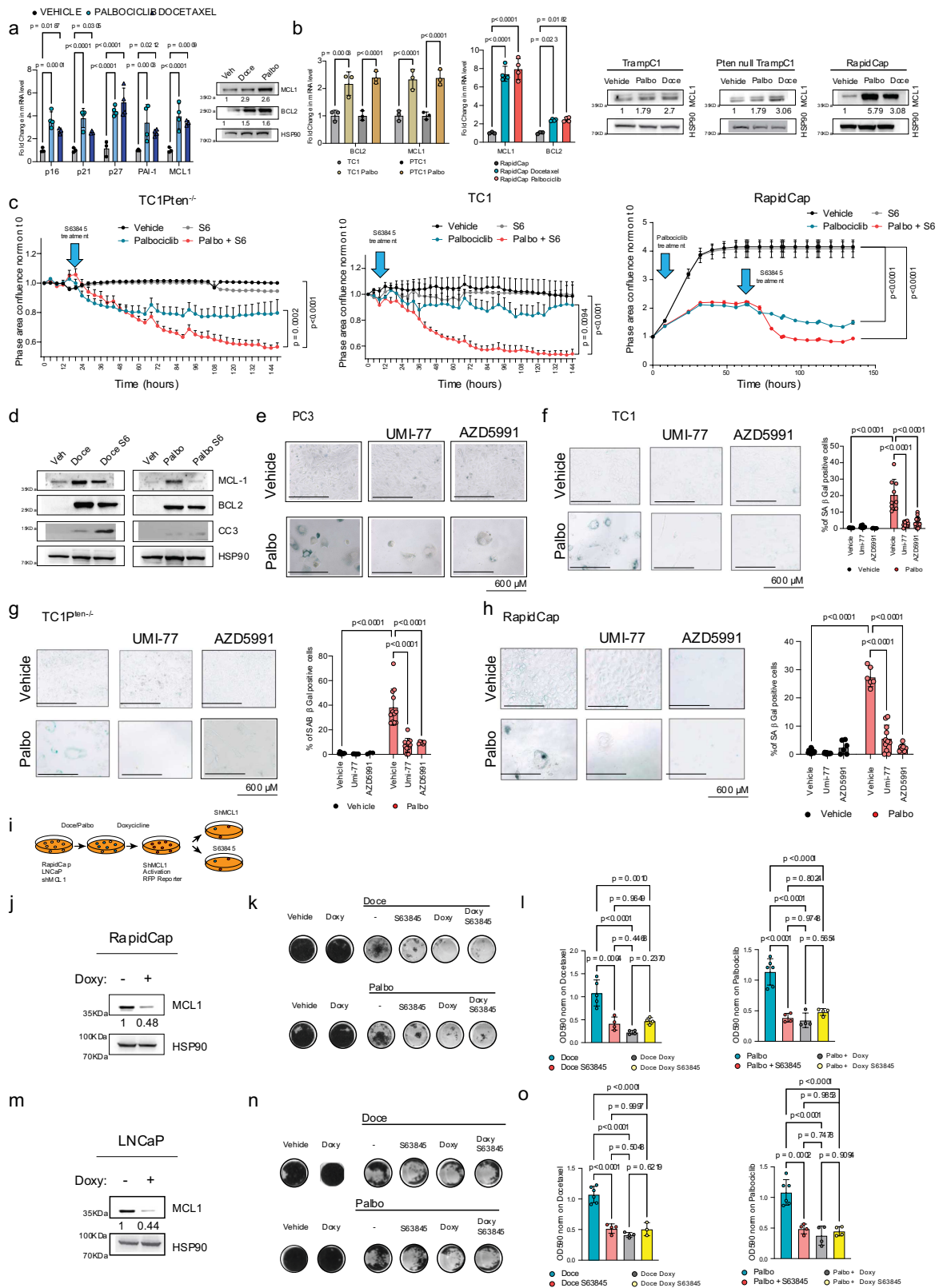
75

a, UMAP plots of 10X Genomics integrated data of *Pten*^{PC-/-} and *Pten*^{PC-/-}; *Timp1*^{-/-} mouse models. **b**, Epithelial marker genes expressions for each cluster of the 12 ones found with *FindClusters* function. Semi-vescical markers: Calml3, Pate4, Pax2. Luminal markers: Krt18, Krt8, Cd24a. Basal markers: Krt14, Trp63, Krt5. **c**, UMAP plot showing 12 clusters found with *FindClusters* function. **d**, UMAP plot of classification of cells as cancer cells and semi-vescical cells. **e**, Violin plot of Senescent signature between the 10 cancer cell clusters. **f**, UMAP plot of cancer epithelial cells colored by Cell_cycle_arrest_signatureWP and signatureK. **g**, UMAP plot of cancer epithelial cells colored by Senescence_scoreWP (SWP) and Senescence_scoreK (SK). **h**, Violin plot of the up-regulated genes in senescent cells belonging to the AP-1 TF family. **i**, Heatmap of target genes of TF upregulated in senescent cells. **j**, Boxplot of ss-GSVA scores for gene sets related to senescence: Senescence and autophagy in cancer (Wikipathway), NFKB pathway (Biocarta), Casella_signature, Hernandez_signature, Fridman_senescence_up, Basisty_signature, Purcell_signature, Cellular senescence from Gene Ontology collection, Oncogene-induced senescence and Cellular senescence genesets from Reactome collection. (N= 3992 cells, two-sided Wilcox-test: Senescence and autophagy in cancer p-value < 2.2e-16; Not senescent cells (left) median = 0.617 Senescent cells (right) median = 0.806; NFKB pathway p-value < 2.2e-16; Not senescent cells (left) median = 0.472, Senescent cells (right) median = 0.757; Casella_signature p-value = 4.6e-181; Not senescent cells (left) median = 0.226, Senescent cells (right) median = 0.287; Hernandez_signature p-value < 2.2e-16; Not senescent cells (left) median = 0.286, Senescent cells (right) median = 0.335; GO_cellular_senescence p-value < 2.2e-16; Not senescent cells (left) median = 0.242, Senescent cells (right) median = 0.302; Fridman_senescence_up p-value = 2.13e-95; Not senescent cells (left) median = 0.28, Senescent cells (right) median = 0.318; Reactome_cellular_senescence p-value = 3e-285; Not senescent cells (left) median = 0.353, Senescent cells (right) median = 0.396; Reactome oncogene_induced_senescence p-value = 1.5e-291; Not senescent cells (left) median = 0.396, Senescent cells (right) median = 0.462; Basisty_signature p-value = 1.9e-93; Not senescent cells (left) median = 0.493, Senescent cells (right) median = 0.52; Purcell_signature p-value = 3.6e-35; Not senescent cells (left) median = -0.053, Senescent cells (right) median = -0.013.



Supplementary Figure 3 : Cell-death related genes and senescence.

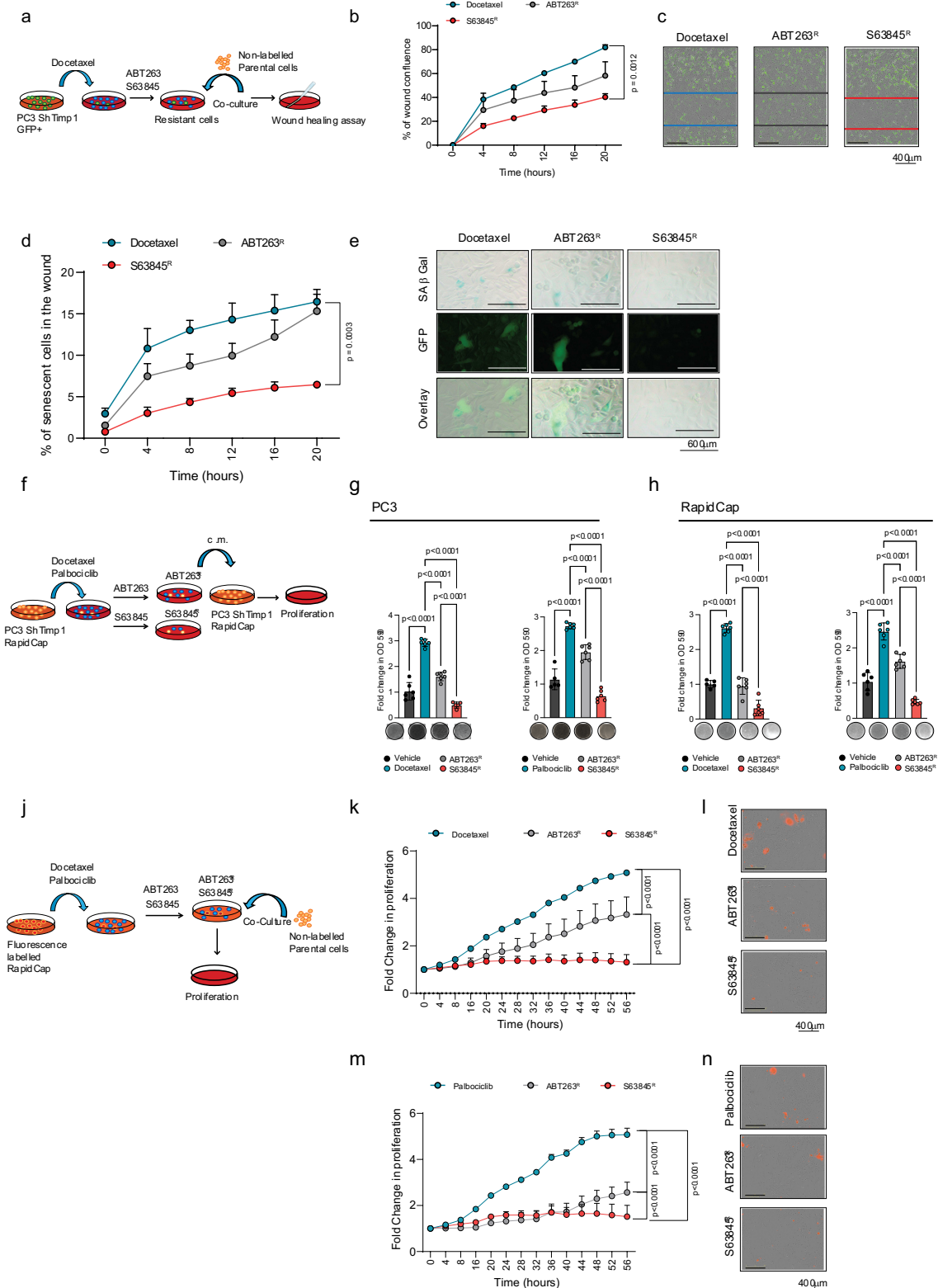
a, Heatmap of 47 cell death related-genes between senescent and not senescent cells. **b**, Correlation matrix showing Pearson correlation coefficients (R higher than 0.4) between cell death-related genes and Senescence_scoreK, Senescence_scoreWP and Senescence_scoreR. Only genes with FDR<0.05 are reported. **c**, Left panel, violin plot of Mcl-1 and Bcl2 in senescent cells and not senescent cells. Right panel, Western blots for Mcl-1 and Bcl2 in the three different genotypes. Western blot data are representative of three experiments. **d**, Co-expression of Bcl2 and selected cell death-related genes in senescent cells. Red cells mostly express Bcl2, blue cells mostly express the gene of interest while purple cells co-express both targets. **e**, UMAP plot showing Mcl1 expression levels in senescent cells (top) and classification of them in Mcl-1⁺ or Mcl-1⁻ (bottom). **f**, Dotplot showing SASP genes and metastatic related genes between the different senescent subpopulations. **g**, Heatmap of differentially expressed genes related to migration, mobility and locomotion in senescent cells Mcl-1⁺ and Mcl-1⁻.



Supplementary Figure 4 : Validation of senolytic activity of MCL-1 inhibition with pharmacological and genetic approaches.

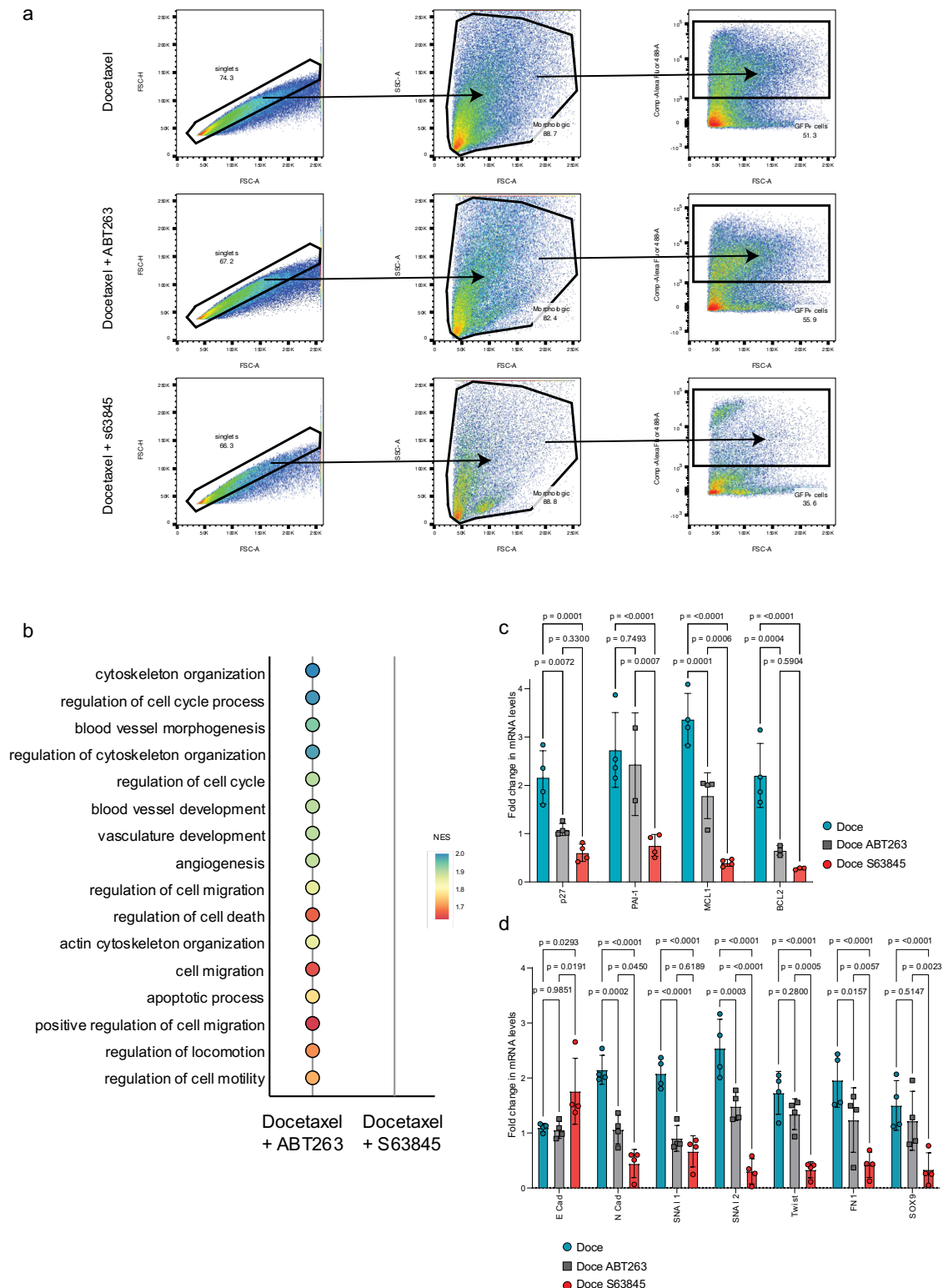
a, RT-qPCR for senescence markers and western blot of pro-survival proteins of PC3 shTIMP1 upon TIS (for Vehicle sample n=3 for each gene, for Palbociclib n = 4 for each gene, for Docetaxel n = 3 for p16, p21 and MCL1 while n = 4 for p27 and PAI-1, n= biological independent replicates from two experiments). **b**, RT-qPCR and western blots of senescent murine cell lines (TC1, TC1^{Pten}^{-/-} and RapidCap) for Mcl-1 (n=3 for Vehicle, Docetaxel and Palbociclib in TC1, TC1^{Pten}^{-/-} and n=3 for Vehicle and n =4 for Docetaxel and Palbociclib in RapidCap, n= biological independent replicates from two experiments) and for Bcl2 (n=3 for Vehicle, Docetaxel and Palbociclib in TC1, TC1^{Pten}^{-/-} and n=3 for Vehicle and n =4 for Docetaxel and Palbociclib in RapidCap, n= biological independent replicates from two experiments). **c**, Incucyte Growth curve of TC1, TC1^{Pten}^{-/-} and RapidCap subjected to Palbociclib first and S63845 after (in TC1 n=7 for Vehicle, n= 3 for Palbociclib and S63845 and n = 5 for Palbociclib + S63845; in TC1^{Pten}^{-/-} n=6 for Vehicle and Palbociclib and n = 3 for Palbociclib + S63845 and S63845). **d**, Western blots of PC3 shTIMP1 treated with TIS and S63845. Data are representative of one experiment. **e**, Representative images of SA-b-Gal of PC3. Data are representative of two experiments. **f**, Representative images of SA-b-Gal of TC1 treated with TIS and two different MCL1 inhibitors and its quantification (in Vehicle category (black dots) n=12 for Vehicle and UMI-77 , n = 8 for AZD5991; in Palbociclib treatment (pink dots) n=9 for Palbociclib, n= 11 for UMI-77 and n=12 for AZD5991 ; n=biological independent replicates from three experiments); **g**, Representative images of SA-b-Gal of TC1^{Pten}^{-/-} treated with TIS and two different MCL1 inhibitors and its quantification (in Vehicle category (black dots) n=11 for Vehicle, n = 12 for UMI-77, n = 3 for AZD5991; in Palbociclib treatment (pink dots) n=12 for Palbociclib, n= 11 for UMI-77 and n=6 for AZD5991) ; n=biological independent replicates from three experiments). **h**, Representative images of SA-b-Gal of RapidCap treated with TIS and two different MCL1 inhibitors and its quantification (in Vehicle category (black dots) n=12 for Vehicle and UMI-77, n = 6 for AZD5991; in Palbociclib treatment (pink dots) n=6 for Palbociclib, n= 11 for UMI-77 and n=7 for AZD5991) ; n=biological independent replicates from three experiments); **i**, Experimental design. **j**, Western blot analysis of shMCL1 in the murine RapidCap cells, and its quantification. Data are representative of two experiments. **k**, Representative pictures of crystal violet images of the treated RapidCap cells. Data are representative of two experiments. **l**, Fold change of crystal violet quantification in RapidCap cells subjected to Docetaxel treatment (left; n=5 for Docetaxel and n=4 for Docetaxel S63845, Docetaxel Doxy and Docetaxel Doxy S63845) and to Palbociclib (right; n= 6

for Palbociclib and n=4 for Palbociclib S63845, Palbociclib Doxy and Palbociclib Doxy S63845; n= biological independent replicates from two experiments). **m**, Western blot of shMCL1 in the human LNCaP cells, and in its quantification. Data are representative of two experiments. **n**, Representative pictures of crystal violet images of the treated LNCaP cells. Data are representative of two experiments. **o**, Fold change of crystal violet quantification in LNCaP cells to Docetaxel treatment (left; n=6 for Docetaxel, n=4 for Docetaxel S63845 and Docetaxel Doxy and n= 3 for Docetaxel Doxy S63845) and to Palbociclib (right; n= 6 for Palbociclib and n=4 for Palbociclib S63845, Palbociclib Doxy and Palbociclib Doxy S63845; n= biological independent replicates from two experiments). **a,b,f,g,h,i,o**, Data are represented as mean $\pm$ SD. **c**, Data are represented as mean $\pm$ SEM. **a,b,c,f,g,h**, The p-values were determined by two Way Anova followed by Tukey's multiple comparisons test. **i,o**, The p-values were determined by one Way Anova followed by Tukey's multiple comparisons test.



Supplementary Figure 5 : Mcl-1 inhibition revert proliferation and migration driven by the SASP.

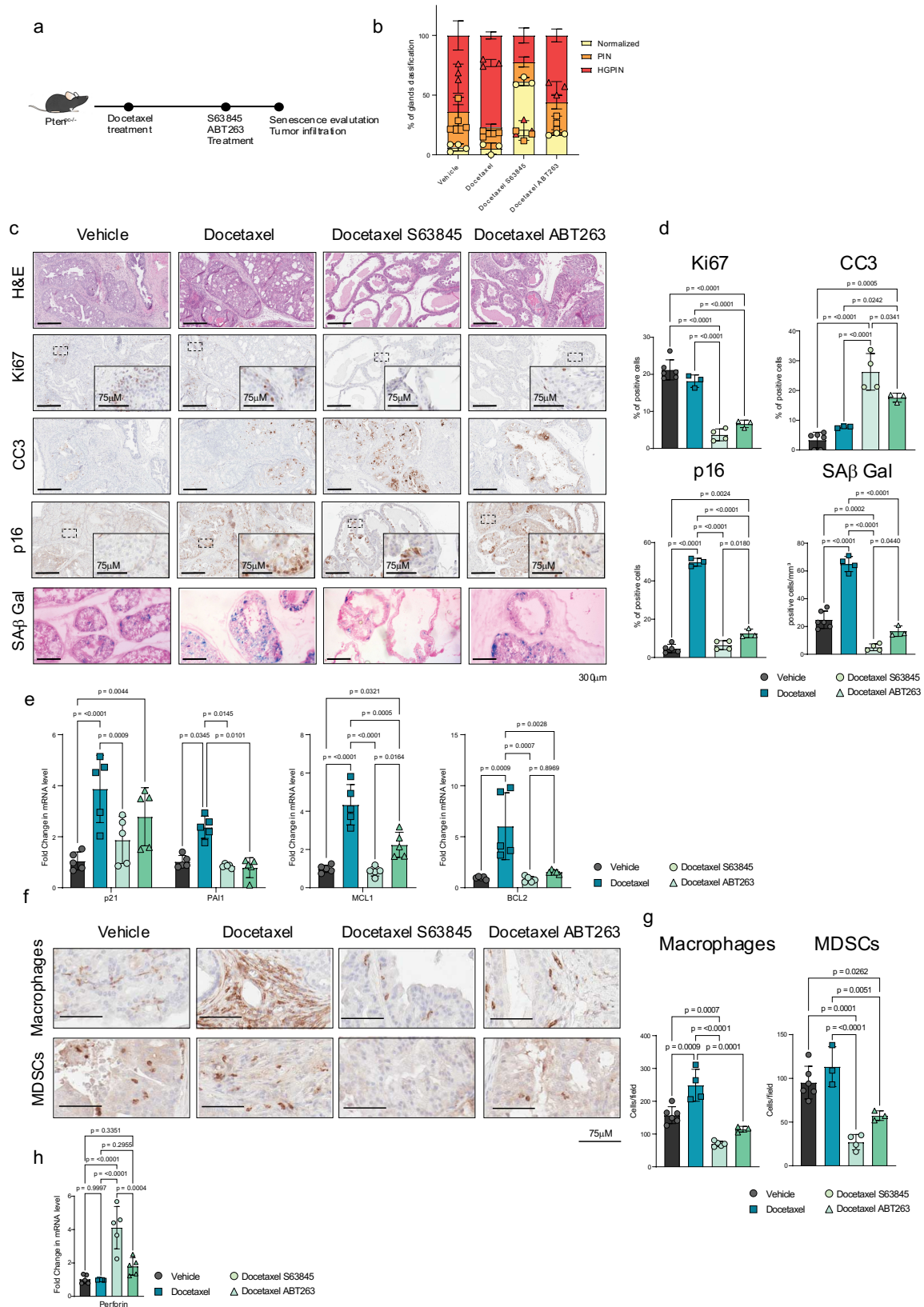
a Schematic representation of experimental design. **b**, Percentage of wound closure measured by Incucyte (n=2 for Docetaxel, n=4 for S63845^R and ABT263^R; n=biological independent replicates from two experiments). **c**, Representative pictures of wound closure upon 20 hours. Scale bar 400 μ m. Data are representative of two experiments. **d**, Graph showing the percentage of senescent GFP⁺ cells into the wound (n=2 for Docetaxel, n=3 for S63845^R and n=4 for ABT263^R; n=biological independent replicates from one experiment). **e**, Representative images showing in bright field the SA-b Gal positive cells, in fluorescence channel the positivity to GFP and the merge of the two channels. Scale bar 600 μ m. Data are representative of one experiment. **f**, Schematic representation of experimental design. **g**, Bar graph representing the fold change of crystal violet absorbance, and representative images below in PC3 shTIMP1 after TIS with Docetaxel (left panel n=6 for Vehicle, Docetaxel, and ABT263^R, n=5 for S63845^R; n=biological independent replicates from two experiment) and Palbociclib (right panel n=6 for Vehicle, Palbociclib, ABT263^R and S63845^R; n=biological independent replicates from two experiments) and **h**, RapidCap after TIS with Docetaxel (left panel n=5 for Vehicle, n=6 for Docetaxel, ABT263^R, and S63845^R; n=biological independent replicates from two experiments) and Palbociclib (right panel n=6 for Vehicle, Palbociclib, ABT263^R and S63845^R; n=biological independent replicates from two experiments). **j**, Iconographic representation of experiment layout. **k**, Fold change in proliferation of parental RapidCap upon co- culture of Docetaxel treated cells (n=6 for Docetaxel and S63845^R, n=5 for ABT263^R; n=biological independent replicates from two experiments). **l**, Representative images of co-culture of Docetaxel treated Tomato⁺-RapidCap and parental untreated and un-labelled cells. Scale bar 400 μ m. Data are representative of two experiments. **m**, Fold change in proliferation of parental RapidCap after co- culture with Palbociclib treated cells (n=9 for Palbociclib, n=5 for S63845^R and n=3 for ABT263^R, n=biological independent replicates from two experiments). **n**, Representative images of co-culture with Palbociclib treated Tomato⁺-RapidCap and parental un-labelled cells. Scale bar 400 μ m. Data are representative of two experiments. **b,d,k,m**, Data are represented as mean $\pm$ SEM. **b,d,k,m**, The p-values were determined by two Way Anova followed by Tukey's multiple comparisons test; **g,h**, The p-values were determined by one Way Anova followed by Tukey's multiple comparisons test



Supplementary Figure 6 : Removal of pro-metastatic features upon S63845 treatment.

a, Gating strategy for the sorting of tumor cells isolated by using the GFP constitutively expressed by PC3shTIMP1 cell line. Data are representative of one experiment. **b**, Dotplot showing enrichment results of biological process (GO) pathway analysis by considering senescent cell

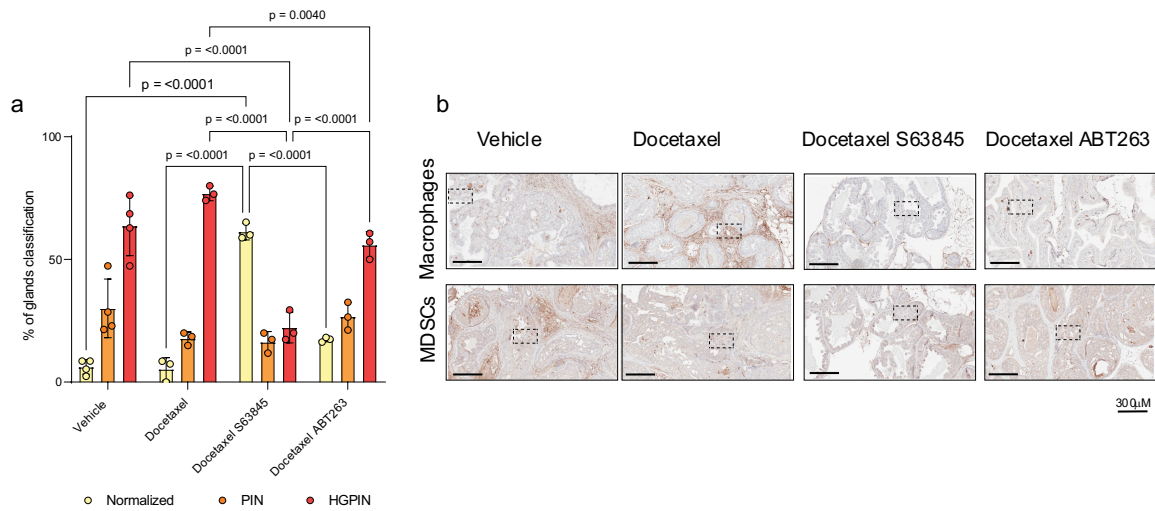
treated with S63845 or ABT263. Color scale reports normalized enrichment score (NES). **c**, RT-qPCR for transcriptional markers of senescence (for p27 and MCL-1 n=4 for Docetaxel, Docetaxel + ABT263 and Docetaxel + S63845; for PAI-1 n=4 for Docetaxel and Docetaxel + S63845 and n=2 Docetaxel + ABT263; for BCL2 n=4 for Docetaxel and n =3 for Docetaxel + S63845 and Docetaxel + ABT263; n=biological independent replicates from one experiment). **d**, RT-qPCR for pro-migratory features (n=4 for Docetaxel, Docetaxel + ABT263 and Docetaxel + S63845; n=biological independent replicates from one experiment). **c,d**, The p-values were determined by Two Way Anova followed by Tukey's multiple comparisons test **c,d**, Data are represented as mean $\pm$ SD.



Supplementary Figure 7 : Senolytic treatment in transgenic *Ptenpc*^{-/-} prostate cancer model.

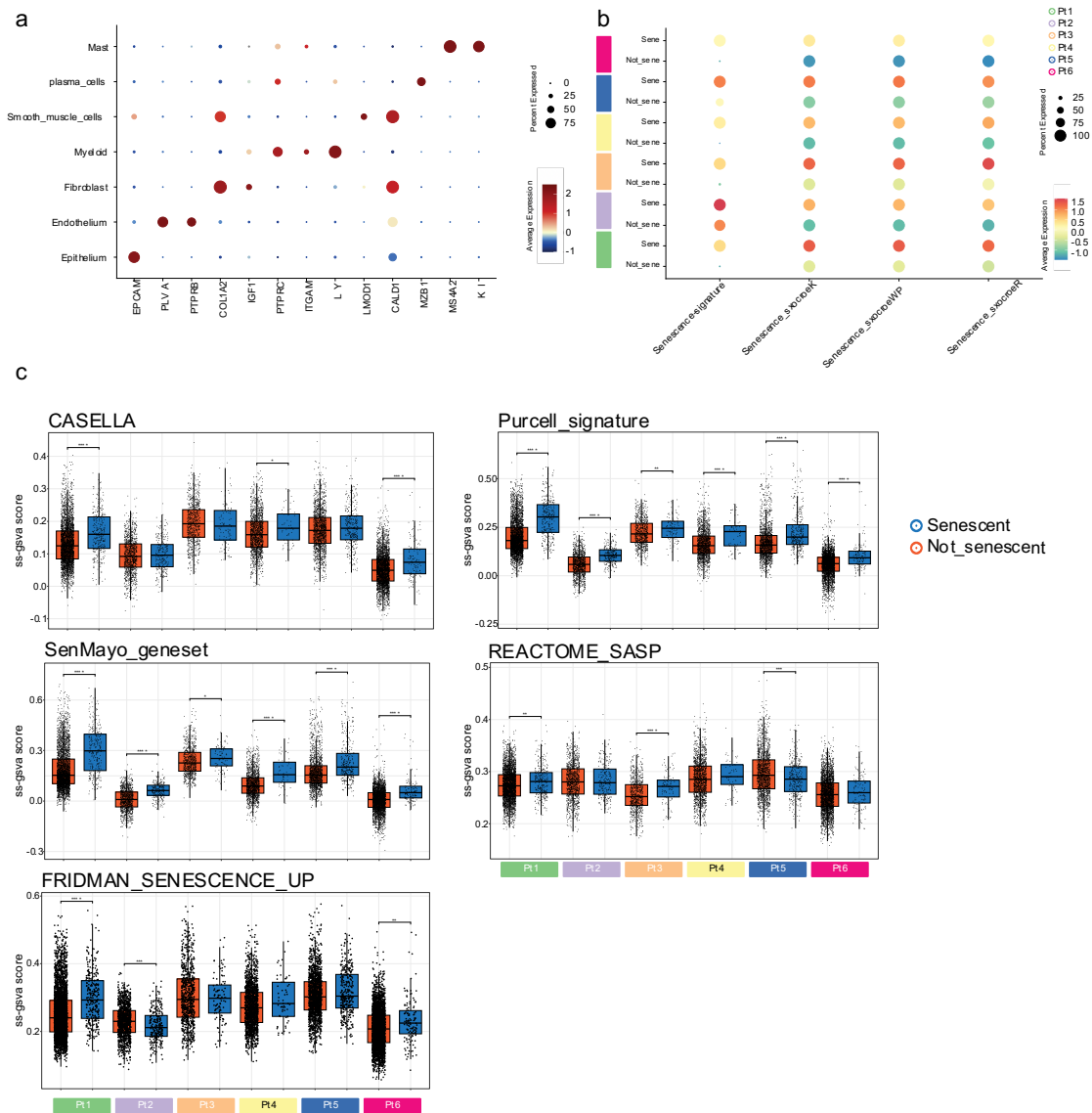
a, Schematic representation of the experimental design. **b**, Gland scoring expressed in percentage in the different treatment (n= 4 for Vehicle, n = 3 for Docetaxel, Docetaxel + ABT263 and Docetaxel

+ S63845, n= independent animals from one experiment). **c**, Representative pictures of the immunohistochemical analysis of H&E, proliferation marker Ki-67, apoptotic marker Cleaved Caspase 3 (CC3), and senescence markers p16 and SA-b Gal (Scale bar 300µm; insert 75µm). Data are representative of one experiment. **d**, Immunohistochemistry and SA-b Gal quantification (for Ki67 n= 6 for Vehicle, n=3 for Docetaxel and Docetaxel ABT263, n=4 for Docetaxel S63845, for CC3 n= 6 for Vehicle, n=3 for Docetaxel and Docetaxel ABT263, n=4 for Docetaxel S63845, for p16 n= 5 for Vehicle, n=3 for Docetaxel and Docetaxel ABT263, n=4 for Docetaxel S63845, for SA-b- Gal n= 6 for Vehicle, n=3 for Docetaxel ABT263, n=4 for Docetaxel and Docetaxel S63845, n= areas from 5 animals from one experiment). **e**, mRNA levels of marker of senescence (p21 and PAI-1) and the pro-survival factors (Mcl-1 and Bcl2) (for all genes and all conditions n=5 biological samples from 5 independent animals from one experiment). **f**, Representative pictures of immunohistochemical analysis of the immune subpopulations: Macrophages (F4/80) and Myeloid- derived suppressor cells (Gr1). Data are representative of one experiment. **g**, Quantification of immunohistochemistry of panel f (for macrophages n= 6 for Vehicle, n=3 for Docetaxel, n= 3 for Docetaxel ABT263, n= 5 for Docetaxel S63845, for Myeloid-derived suppressor cells n= 6 for Vehicle, n=3 for Docetaxel and Docetaxel ABT263, n=4 for Docetaxel S63845, n= areas from 5 animals from one experiment). **h**, mRNA levels of Perforin expression (n=5 biological replicates from 5 independent animals from one experiment). **d,e,g,h**, The p-values were determined by One Way Anova followed by Tukey's multiple comparisons test. **b,d,e,g,h**, Data are represented as mean $\pm$ SD.



Supplementary Figure 8 : S63845 treatment has an effect on prostate gland and the immune infiltrate.

a, % of glands scoring in the different treatments (n= 4 for Vehicle, n = 3 for Docetaxel, Docetaxel + ABT263 and Docetaxel + S63845 from one experiment). The p-values were determined by two Way Anova followed by Tukey's multiple comparison test. Data are represented as mean $\pm$ SD. **b**, Zoom out of pictures related to immune subsets infiltrating the prostate tumors in Supplementary Figure7f (Scale bar 300µm). Dashed squares represent areas visualized in Supplementary Figure7f. Data are representative of one experiment.



Supplementary Figure 9 : Exploiting the senescence in human CRPC samples

a, Dotplot showing the expression of canonical marker for the different cell type in human scRNA-seq samples. **b**, Dotplot showing the average expression of the different signatures used

during SIT application in each patient, comparing Sene and Not sene cells. **c**, Boxplot of the five senescence-related signatures investigated divided by senescent and not senescent cells in each patient. The p-values were determined by multiple Wilcox tests followed by Bonferroni multiple comparison correction.

Supplementary Table

Primers	Sequence
H_MCL1 Fw	CCA AGA AAG CTG CAT CGA ACC AT
H_MCL1 Rev	CAG CAC ATT CCT GAT GCC ACC T
H_BCL2 Fw	GATGTGATGCCTCTGCGAAG
H_BCL2 Rev	CATGCTGATGTCTCTGGAATCT
H_E Cadherin Fw	CGAGAGCTACACGTTACGG
H_E Cadherin Rev	GGGTGTCGAGGGAAAAATAGG
H_N Cadherin Fw	TCAGGCGTCTGTAGAGGCTT
H_N Cadherin Rev	ATGCACATCCTTCGATAAGACTG
H_FN1 Fw	CGGTGGCTGTCAGTCAAAG
H_FN1 Rev	AAACCTCGGCTTCCTCCATAA

H_Snai1 Fw	TCGGAAGCCTAACTACAGCGA
H_Snai1 Rev	AGATGAGCATTGGCAGCGAG
H_Snai2 Fw	CGAACTGGACACACATACAGTG
H_Snai2 Rev	CTGAGGATCTCTGGTTGTGGT
H_Twist Fw	GCAAGAAGTCGAGCGAAGAT
H_Twist Rev	GCTCTGCAGCTCCTCGAA
H_p16 Fw	ACCAGAGGCAGTAACCATGC
H_p16 Rev	TGTCGTTGCGGGGCGCAACTG
H_p21 Fw	TGTCCGTCAGAACCCATGC
H_p21 Rev	AAAGTCGAAGTTCCATCGCTC
H_p27 Fw	TAATTGGGGCTCCGGCTAACT
H_p27 Rev	TGCAGGTCGCTTCCTTATTCC
H_PA1-1 Fw	TGCTGGTGAATGCCCTCTACT

H_PA1-1 Rev	CGGTCATTCCCAGGTTCTCTA
H-b Actin Fw	GAGAAAATCTGGCACCACACC
H-b Actin Rev	ATACCCCTCGTAGATGGGCAC
m_MCL1 Fw	AAGAGGCTGGGATGGGTTTGT
m_MCL1 Rev	AGTCCCCTATTGCACTCACAAG
m_BCL2 Fw	TACCGTCGTGACTTCGCAGAG
m_BCL2 Rev	GGCAGGCTGAGCAGGGTCTT
R18s Fw	ACCGCAGCTAGGAATAATG
R18s Rev	GCCTCAGTTCCGAAAACCA
GADPH Fw	AGGTCGGTGTGAACGGATTG
GADPH Rev	TGTAGACCATGTAGTTGAGGT
m_PA1-1 Fw	GCCAGATTTATCATCAATGA
m_PA1-1 Rev	GGAGAGGTGCACATCTTTCTCAAAG

m_p27-1 Fw	AACGTGCCGAGTGTGTCTAACGG
m_p27-1 Rev	CCTCTAGGGGTTTGTGATTCT
m_Perforin-1 Fw	AGCACAAGTTCGTGCCAGG
m_Perforin-1 Rev	GCGTCTCTCATTAGGGAGTTTTT

Supplementary Table 1 : list of primers

Acknowledgements

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Annex I



ARTICLE


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OPEN

Single-cell transcriptomics identifies Mcl-1 as a target for senolytic therapy in cancer

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Cells subjected to treatment with anti-cancer therapies can evade apoptosis through cellular senescence. Persistent senescent tumor cells remain metabolically active, possess a secretory phenotype, and can promote tumor proliferation and metastatic dissemination. Removal of senescent tumor cells (senolytic therapy) has therefore emerged as a promising therapeutic strategy. Here, using single-cell RNA-sequencing, we find that senescent tumor cells rely on the anti-apoptotic gene Mcl-1 for their survival. Mcl-1 is upregulated in senescent tumor cells, including cells expressing low levels of Bcl-2, an established target for senolytic therapy. While treatment with the Bcl-2 inhibitor Navitoclax results in the reduction of metastases in tumor bearing mice, treatment with the Mcl-1 inhibitor S63845 leads to complete elimination of senescent tumor cells and metastases. These findings provide insights on the mechanism by which senescent tumor cells survive and reveal a vulnerability that can be exploited for cancer therapy.

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Annex II

List of publications

CDCP1 overexpression drives prostate cancer progression and can be targeted in vivo.

Alajati A, D'Ambrosio M, **Troiani M**, Mosole S, Pellegrini L, Chen J, Revandkar A, Bolis M, Theurillat JP, Guccini I, Losa M, Calcinotto A, De Bernardis G, Pasquini E, D'Antuono R, Sharp A, Figueiredo I, Nava Rodrigues D, Welte J, Gil V, Yuan W, Vlainic T, Bubendorf L, Chiorino G, Gnetti L, Torrano V, Carracedo A, Campese L, Hirabayashi S, Canato E, Pasut G, Montopoli M, Rüschoff JH, Wild P, Moch H, De Bono J, Alimonti A.

J Clin Invest. 2020 May 1;130(5):2435-2450. doi: 10.1172/JCI131133. PMID: 32250342

Retinoic Acid Sensitivity of Triple-Negative Breast Cancer Cells Characterized by Constitutive Activation of the notch1 Pathway: The Role of Rarβ.

Paroni G, Zanetti A, Barzago MM, Kurosaki M, Guarrera L, Fratelli M, **Troiani M**, Ubezio P, Bolis M, Vallerga A, Biancardi F, Terao M, Garattini E.

Cancers (Basel). 2020 Oct 18;12(10):3027. doi: 10.3390/cancers12103027. PMID: 33081033

Senescence Reprogramming by TIMP1 Deficiency Promotes Prostate Cancer Metastasis.

Guccini I, Revandkar A, D'Ambrosio M, Colucci M, Pasquini E, Mosole S, **Troiani M**, Brina D, Sheibani-Tezerji R, Elia AR, Rinaldi A, Pernigoni N, Rüschoff JH, Dettwiler S, De Marzo AM, Antonarakis ES, Borrelli C, Moor AE, Garcia-Escudero R, Alajati A, Attanasio G, Losa M, Moch H, Wild P, Egger G, Alimonti A.

Cancer Cell. 2021 Jan 11;39(1):68-82.e9. doi: 10.1016/j.ccell.2020.10.012. Epub 2020 Nov 12. PMID: 33186519

Commensal bacteria promote endocrine resistance in prostate cancer through androgen biosynthesis.

Pernigoni N, Zagato E, Calcinotto A, **Troiani M**, Mestre RP, Calì B, Attanasio G, Troisi J, Minini M, Mosole S, Revandkar A, Pasquini E, Elia AR, Bossi D, Rinaldi A, Rescigno P, Flohr P, Hunt J, Neeb A, Buroni L, Guo C, Welte J, Ferrari M, Grioni M, Gauthier J, Gharaibeh RZ, Palmisano A, Lucchini GM, D'Antonio E, Merler S, Bolis M, Grassi F, Esposito A, Bellone M, Briganti A, Rescigno M, Theurillat JP,

Jobin C, Gillessen S, de Bono J, Alimonti A.

Science. 2021 Oct 8;374(6564):216-224. doi: 10.1126/science.abf8403. Epub 2021 Oct 7. PMID: 34618582

HER3 Is an Actionable Target in Advanced Prostate Cancer.

Gil V, Miranda S, Riisnaes R, Gurel B, D'Ambrosio M, Vasciaveo A, Crespo M, Ferreira A, Brina D, **Troiani M**, Sharp A, Sheehan B, Christova R, Seed G, Figueiredo I, Lambros M, Dolling D, Rekowski J, Alajati A, Clarke M, Pereira R, Flohr P, Fowler G, Boysen G, Sumanasuriya S, Bianchini D, Rescigno P, Aversa C, Tunariu N, Guo C, Paschalis A, Bertan C, Buroni L, Ning J, Carreira S, Workman P, Swain A, Califano A, Shen MM, Alimonti A, Neeb A, Welte J, Yuan W, de Bono J; PCF/SU2C International Prostate Cancer Dream Team.

Cancer Res. 2021 Dec 15;81(24):6207-6218. doi: 10.1158/0008-5472.CAN-21-3360. Epub 2021 Nov 9. PMID: 34753775

Single-cell transcriptomics identifies Mcl-1 as a target for senolytic therapy in cancer.

Troiani M, Colucci M, D'Ambrosio M, Guccini I, Pasquini E, Varesi A, Valdata A, Mosole S, Revandkar A, Attanasio G, Rinaldi A, Rinaldi A, Bolis M, Cippà P, Alimonti A.

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Author Correction: Single-cell transcriptomics identifies Mcl-1 as a target for senolytic therapy in cancer.

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Mitochondrial DNA released by senescent cells triggers immunosuppression in cancer

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