Universidade de Aveiro Departamento de Química

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MÁRCIO FILIPE MAGALHÃES DE SOUSA

TERAPIAS DIRIGIDAS PARA O CANCRO DA MAMA TRIPLO-NEGATIVO

TARGETED THERAPIES FOR TRIPLE-NEGATIVE BREAST CANCER

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Márcio Filipe Magalhães de Sousa

Terapias dirigidas para o cancro da mama triplo-negativo

Targeted therapies for triple-negative breast cancer

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica – ramo Métodos Biomoleculares, realizada sob a orientação científica do Doutor Rodrigo Leite de Oliveira, Investigador do Netherlands Cancer Institute

“The object of life is not to be on the side of the majority, but to escape finding oneself in the ranks of the insane” – Marcus Aurelius, Meditations

o jurí

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palavras chave cancro da mama triplo-negativo, “synthetic lethality”, hipóxia, angiogénese, terapias anti-angiogénicas, resistência e senescência

resumo

O cancro da mama triplo-negativo (TNBC) é uma heterogénea e complexa doença que actualmente ainda não dispõem de terapias direcionadas devido à falta de alvos terapêuticos presentes em outros tipos de cancro da mama, tais como a presença de receptores hormonais (receptores de progesterona (PR) e receptores de estrogénio (ER)) assim como de receptores de factores de crescimento humano 2 (HER2). Muitos dos pacientes de TNBC respondem muito bem aos tratamentos comuns dados aos pacientes que sofrem com cancros, mas infelizmente quando esses tratamento não são eficazes e TNBC volta, já se torna um tipo de cancro muito difícil de tratar. Neste projecto de investigação temos o objectivo de melhorar as terapias direcionadas para TNBC via duas abordagens conceptualmente diferentes. A primeira consiste na identificação de genes essenciais à sobrevivência de TNBC sob condições de hipóxia, onde hipotetizamos que a inactivação de tais genes seja “synthetic lethal” com terapias antiangiogénicas (AA).

Na segunda parte, olhamos para a senescência das células cancerosas como uma vulnerabilidade que pode ser explorada usando um “one-two punch model” como abordagem terapêutica. Em primeiro lugar induzindo senescência via uma combinação de tratamento com Palbociclib com uma diminuição de cinase-dependente de ciclina 2 (CDK2), seguido de um tratamento com ABT-263, um agente senolítico que promove apoptose prefencialmente em células senescentes sobre células não-senescenctes.

Com este projecto pretendemos contribuir, não apenas para o melhoramento de terapias direcionadas para pacientes com TNBC, mas também para outros tipos de cancros, uma vez que estes conceitos podem ser extendidos a diferentes tipos de cancro.

keywords triple negative breast cancer, synthetic lethality, hypoxia, angiogenesis, antiangiogenic therapies, resistance and senescence

abstract

Triple-negative breast cancer (TNBC), a heterogeneous and complex disease entity, lacks targeted therapies due to the absence of common therapeutic targetable features that exist in other types of breast cancer (BC). TNBC patients generally respond very well to standard chemotherapy, but when TNBC recurs is often very hard to treat. In this project, we aimed at improving TNBC targeted therapy via two conceptually different approaches. The first one consisted of the identification of essential genes for the survival of TNBC cancer cells under hypoxia, where we hypothesized that inactivation of such genes would be then synthetic lethal with antiangiogenic therapies. Thus contributing to the improvement of this class of drugs to which patients often acquire resistance.

In the second part of this project, we aimed at exploring senescence as a vulnerability in cancer cells that can be therapeutically targeted using a one-two punch model. In a first step senescence is induced via a combination of Palbociclib (CDK4/6 inhibitor) treatment with Cyclin-dependent kinase 2 (CDK2) impairment; and in a second step, TNBC induced senescence cells were treated with ABT-263, a senolytic agent that promotes apoptosis preferentially on senescence cells over non-senescence cells.

With this project, we contributed not only to the so needed improvement of target therapies for TNBC patients but also to other tumors, once these different approaches can be extended to other cancer types.

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List of abbreviations

ASXL2	putative Polycomb group protein ASXL2
BC	breast cancer
CBFA2	core-binding factor subunit alpha-2
CBF	core-binding factor
CDK2/4/6/7	cyclin-dependent kinase 2/4/6/7
CBF	core binding factor
CF	colony formation
CRISPR	clustered regularly interspaced short palindromic repeats
DNA	deoxyribonucleic acid
DSB	DNA double-strand break
DCIS	ductal carcinoma in situ
EC	endothelial cells
ER	estrogen receptor
FACS	fluorescent activated cell sorting
HIF1AN	hypoxia-inducible protein 1-alpha inhibitor
HER2	human epidermal growth factor receptor 2
HIF	hypoxia-inducible factor
IDC	invasive ductal carcinoma
IHC	immunohistochemistry
ILC	invasive lobular carcinoma
MAPK	mitogen-activated protein kinase
NTC	non-targeting control
O_2	oxygen
$p16^{INK4a}$	cyclin-dependent kinase inhibitor 2A
p21	cyclin-dependent kinase inhibitor 1A
p53	tumor suppressor protein
PCR	polymerase chain reaction
PD	palbociclib
PIGF	placental growth factor
pO_2	partial pressure of oxygen

PR	progesterone receptor
p-RB	retinoblastoma-associated protein
RBBP7	histone-binding protein RBBP7
RFP	red fluorescent protein
RNA	ribonucleic acid
RUNX1	runt-related transcription factor 1
SAβ-gal	senescence-associated β-galactosidase
SAHF	senescence-associated secretory phenotype
SASP	senescence-associated heterochromatin foci
sgRNA	single guide RNA
shRNA	short hairpin RNA
TNBC	triple-negative breast cancer
TRC	The RNAi Consortium
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WT	wild-type

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1. Introduction

1.1. Cancer

1.1.1. Cancer overview

Cancer is a collection of related diseases caused when cells in the body grow in an uncontrolled and abnormal fashion compromising normal cells' function in the process.[1–3] Cancer cells, in contrast to normal cells, undergo a process of loss of specialization in which gain the ability of continuous proliferation. In a healthy organism, cells' self- sacrifice — apoptosis— is the rule that enables survival. This suicide mechanism allows metazoans to eliminate cells that put at risk whole body's survival.[4,5] From this perspective, cancer cells possess the capability to disrupt the most fundamental rules of cell behavior that underlie the basis for the building and maintaining the homeostasis of multicellular organisms.[6]

Our understanding of the carcinogenesis process has increased significantly in the last two decades. It is now clear that cancer is the result of a multistep mutagenic process.[7] Such process often leads to mutations in genes that control important pathways of the cell cycle, cell proliferation, and survival, thus creating a deregulation on these pathways that are essential for tumor establishment.[5,8] There are many types of genetic alterations accountable for the genetic basis of cancer that result in widespread deregulation of gene expression profiles, such as, subtle changes like deletions or insertions, as well as alterations in chromosome number, chromosome translocations and gene amplifications.[6–10] Genetic instability has proved to be, in many cases, the motor of tumor progression and heterogeneity. Some mutations in certain genes are more prone to lead to cancer than others. Usually, they are grouped in two classes, the proto-oncogenes, and the tumor suppressor genes. While in the first cancer is driven by the gain of a function mutation, in the second the opposite happens, therefore there is a loss of function mutation.[6,11]

Cancer represents the leading cause of deaths in developed countries and the second in developing countries. The forecasts that in the next years' cancer diagnose will increase have to do mostly with population growth and aging.[12,13]

There are many factors which can promote the necessary mutations to tumor emergence. For instance, adoption of cancer-causing behaviors, such as smoking and sun exposure (X-rays and ultraviolet radiation), unhealthy diets, obesity and physical inactivity

are significant risk factors.[14–16] Clearly, age plays a decisive role here, among the elderly population cancer incidence is increased. Several factors contribute to this observation, such as the decreased immune surveillance, accumulation of genetic mutations, a longer lifetime of exposure to carcinogens and age-related hormonal alterations.

Epigenetics, along with genetic abnormalities also represents a very important process in the earliest stages of neoplasia. There are many data showing that almost in every type of human cancer there is a hypermethylation of the promoter regions of key regulatory genes, which is linked with inappropriate transcription leading to gene silencing.[17,18]

Modern cancer biology proposes that tumor progression is the result of a multi-gene, multi-step clonal selection within the original clone, following a Darwinian adaptive system. This selection can occur from a single cell of origin[19] and leads to a 'refinement' of cancer cells with more and more proliferation and survival advantage, therefore leading to more aggressive subpopulations.[20–22] This is easily understandable against current knowledge that populations of tumor cell are more genetically unstable than of normal cells, so in the primary tumor there is a mix of subclonal populations constantly and sequentially being selected to an increasing genetically and biological abnormal state (Figure 1).[19]

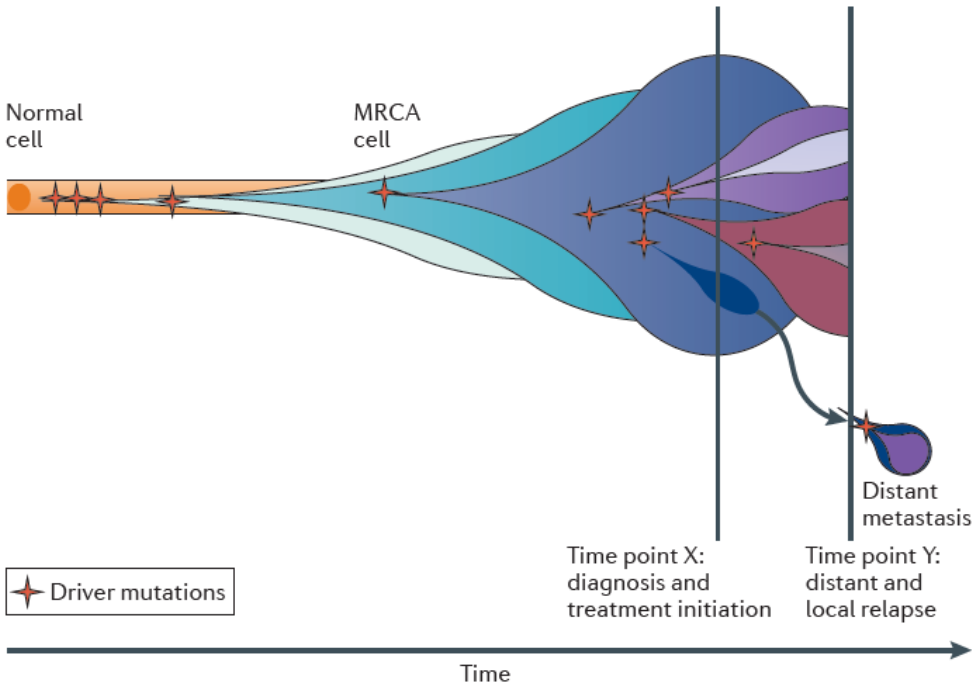

Figure 1: Clonal evolution from normal cells to primary tumors and metastases.[23]
Each colored area represents a cell colony with a specific set of mutations accumulated in the progeny of a single most recent common ancestor (MRCA) cell. Time progresses to the right. The height of each colored

area represents the quantity of cells in the colony. New mutations are represented by stars and may originate from an established colony. The figure shows that these new mutations can originate in any part of the tumor and then competes for space and resources with the others.

Evidence shows that metastatic dissemination is usually the last step in the primary tumor progression.[24] It can take years until the genetic and epigenetic changes in the primary tumor to occur before it becomes mature enough to spread.[25] Metastasis only occurs when the tumor has grown to a size that has likely allowed a clonal selection of cell mutations that give cancer cells that ability,[25] and it is the cause of the majority of cancer deaths.[26]

1.1.2. Hallmarks of cancer

Weinberg et al[24], believe that the complexity of cancer can be reduced to a small number of underlying principles. These principles are what we call the hallmarks of cancer. Currently, they consist of eight common traits to all cancers, complementary to each other, that together govern the transformation of normal to cancer cells. Namely, they are self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, reprogramming of energy metabolism and evading immune destruction. Alongside with these hallmarks, genome instability and tumor-promoting inflammation are described as two enabling characteristics for the acquirement of these hallmarks.[27,28]. In Figure 2 is depicted an overview of the hallmarks of cancer.

A progressive and multistep acquisition of activating mutations in oncogenes or inactivating mutations in tumor suppressor genes are largely the drivers of tumor progression despite the variability of the genetic and epigenetic landscapes across different cancers.[29] In fact, studies[30,31] have shown that it is often the case that cancer diseases are “addicted” to the activation and maintenance of activity of oncogenes in order to keep the malignant phenotype.

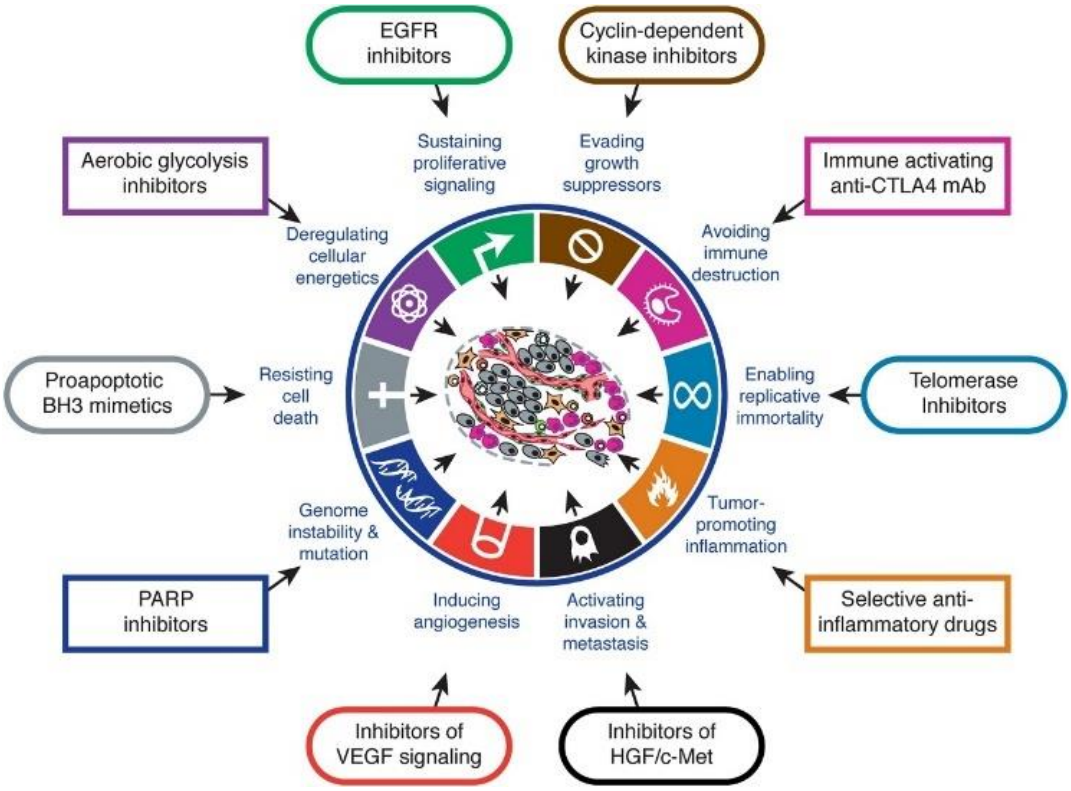

Figure 2: The hallmarks of cancer and its therapeutic targets.[28]
Drugs that interfere with each of the acquired capabilities necessary for tumor growth and progression have been developed and are in clinical trials or in some cases approved for clinical use in treating certain forms of human cancer. Additionally, drugs are being developed to target each of the enabling characteristics and emerging hallmarks, which also hold promise as cancer therapeutics. The drugs listed are but illustrative examples; there is a deep pipeline of candidate drugs with different molecular targets and modes of action in development for most of these hallmarks.

1.1.3. Cancer treatment

The most common cancer therapies can be divided into two categories, one being (1) treatments targeting general cancer mechanisms, which are not tumor/tissue-specific; and (2) treatments targeting tumor/tissue-specific mechanisms/phenotypes, which are referred as targeted therapies.[32]

Currently, the majority of the treatments fall into the first category, which is comprised by surgery, chemotherapy, and radiotherapy, these are the oldest treatments and over the decades they have been highly effective in extending the survival of cancer patients and even eradicating some sorts of tumors.[33] The second category includes targeted therapy and hormone therapy which have been greatly developed in recent years. Usually, most of

the oncology patients receive a combination of these treatments, and the type of treatment given depends on the type and stage of the disease.[34]

When possible, surgery is performed with the intent to remove the entire tumor, but sometimes that is not feasible, due to the damage that the complete removal would make in an organ, and only a part of the tumor is removed. When performed in early stages surgery remains to be the most effective treatment of primary tumors.[35]

Radiotherapy was first used in the 20's and since then it was improved significantly.[36] Not much later, in the 40's, the principle that the systematical administration of drugs could induce tumor regression was established in when Goodman and Gilman tested the first chemotherapy treatment by injecting a 'nitrogen mustard' compound in patients with advanced non-Hodgkin's lymphoma and observed that the mediastinal and lymphatic masses regressed.[37] Oftentimes, these two treatments are used together as they complement each other. Despite the great advances made in these treatment techniques efficiency, there is still a major drawback that cannot be ignored, which is the fact that they are not tumor specific, implying that normal cells are also targeted and thus leading to undesirable side effects more or less severe.[35] Both treatments consist of inducing DNA damage that in principle impairs cell division and triggers apoptosis. Furthermore, the treatment for a first cancer is associated with an increased risk of developing a second primary cancer when compared with the general population.[38]

Targeted therapy refers to the more recent anti-cancer drugs (set apart of the common chemotherapeutical drugs) that can be separated into two types: small-molecule inhibitors and humanized monoclonal antibodies.

With regard to the small-molecule inhibitors, these drugs are designed to interfere with specific molecular targets that play key roles in cancer progression (particular features common to cancer cells).[39] They do so by taking advantage of the metabolic differences between normal and cancer cells, therefore such drugs can interfere with altered cell programs, mainly active in cancer cells, limiting the damage to the normal cells.[40]

These therapies work in one of four ways: (1), blocking signals/pathways that cancer cells use to make new cancer cells, (2) delivering toxic substances that kill or damage cancer cells, (3) stimulating immune cells to destroy cancer cell and (4) blocking the growth of new

blood vessels around cancer cells, starving the cancer cells from nutrients essential for their growth. In sum, we can say that they are developed towards specific molecular targets directly involved in the development of the hallmarks of cancer.[28] In Figure 2 is possible to see some examples of correlations between the drugs and the effects on the hallmarks of cancer. Thus, the challenges regarding targeted therapy consist in identifying valid targets as well as the subsequent drug development in such a way that the toxicity and off-target effects are very limited. Unfortunately, tumors that were initially sensitive to targeted therapies often end up developing drug resistance.[41] Mutations during treatment and other adaptive responses, such as an increase in the expression of the therapeutic target or concomitant activation of alternative signaling pathways are one of the reasons for drug resistance.[42,43] These phenomena have to do with the heterogeneity of the tumor, what happens is that upon targeted treatment the tumor is put under a pressure that leads to the selection, and later proliferation, of a minor drug-resistance cell subpopulation among the numerous tumor cells present in the original tumor.[28,43]

With the increasing of genetic tools researchers start to use the concept of synthetic lethality in order to tackle this gain of drug resistance to small-molecule inhibitors targeted therapy. Two genes are said to be synthetic lethal if a mutation of either alone is compatible with viability but mutation of both leads to death.[44] From this concept, one can foresee that targeting a gene that is synthetic lethal to a cancer-relevant mutation should lead to a more efficient elimination of cancer cells while sparing normal ones.[9,45] A great example proving this concept was discovered in breast cancer where researchers found toxicity of Poly (ADP-ribose) polymerase (PARP) 1 and 2 inhibitors specifically in BRCA1 and BRCA2 mutant cells. This observation has already been clinically validated with success.[46,47]

Immunotherapy is another promising, exciting and ground-breaking therapy field, for both advanced and metastatic cancers. For many decades' researchers had hoped to modulate the body natural immune system to efficiently identify and attack cancer cells.[48] Establishing this type of anti-cancer defense is hard because the immune system by itself has evolved to recognize and eliminate foreign agents while leaving body's own tissue untouched. The problem is evident, cancer cells are also native to the body, therefore, in many ways, indistinguishable from normal cells[3] and often coexist in an equilibrium with the immune system.[49] Furthermore, there are cases during the progression of the disease, in which cancer cells are capable of high jacking the immune system converting it into a

support structure for cancer development.[50] Howbeit, regardless of the lack of understanding on the precise mechanism through which the immune system interacts with cancer, the immunotherapy objective is both to boost and to restore the immune system with the ability to detect and attack cancer cells.[51,52] Recent studies have already allowed a shift on the standard of care for patients with advanced melanoma disease (metastatic), considered incurable until the date, that now can be treated with potentially curative instead of palliative intent.[53] With this promising results, it is expected that immunotherapy will still gain more and more relevance in the next years as new results from clinical trials are known, as well as the possibility of combining immunotherapy with other types of therapies.[48,54]

Despite all the promising results, in general, target therapies suffer from one same important aspect that contributes to the lack of durable success of these types of therapy. That aspect is the lack of biomarkers that allow a proper stratification of patients to receive the most appropriated targeted therapy.[55] Hopefully, the foreseen era of stratified and personalized therapy will enable tailored therapies to improve the outcomes and to reduce toxicity, but that future is still dependent on numerous challenges to be overcome.

1.2. Breast Cancer

1.2.1. Breast cancer overview

Breast cancer (BC) is the type of cancer more commonly diagnosed (30% of the new cases) and the second leading cause of cancer death in women in the United States.[56] Breast cancer is a collection of diseases with diverse origins, clinical characteristics, responses to therapy and outcomes. Over the years BC has been subdivided into different types and sub-types in an attempt to better classify different patient's prognosis and consequently to give them the better treatment option available that best suits the different BC types. BC can arise from different parts of the breast. The majority of them originates in the breast lobules, which are composed of glands that produce the milk, and in the milk ducts, the "pipes" that connect lobules to the nipple (Figure 3). These distinct origins cause that there is a multitude of different histological types of BC.

Ductal carcinoma is the most common malignancy of BC, as well as the primary cause of death among women worldwide[57] with an incidence of 50-80%.[58] Ductal carcinomas can be divided into two types: the Ductal carcinoma in situ (DCIS) and the invasive ductal carcinoma (IDC). DCISs are considered to be a noninvasive form of BC. In this type of BC, cancer cells replace normal epithelial cells of the ducts, making them and the lobules expand, but without ever grow beyond the layer where they originated. IDCs, in its turn, are invasive forms of BC, which means that these BC cells manage to expand beyond the walls of the glands or ducts in which they originated.

The second most prevalent subtype of BC is the invasive lobular carcinoma (ILC) representing between 5-15 %.[58] In the others BC subtypes are included categories such as medullary, neuroendocrine, tubular, apocrine, metaplastic, mucinous, inflammatory, adenoid cystic and micropapillary types.[59]

Breast cancer can be staged using the TNM (tumor node metastasis) classification system accordingly to the stage of disease they are in; the T refers to the primary tumor size, the N to the near lymph nodes that are involved and M to the metastasis stage (presence or absence).[60] With the TNM method patients receive a classification from 0 (*in situ* tumor, cancer is still at a very early stage) to IV (invasive cancer already spread to distant parts of the body).

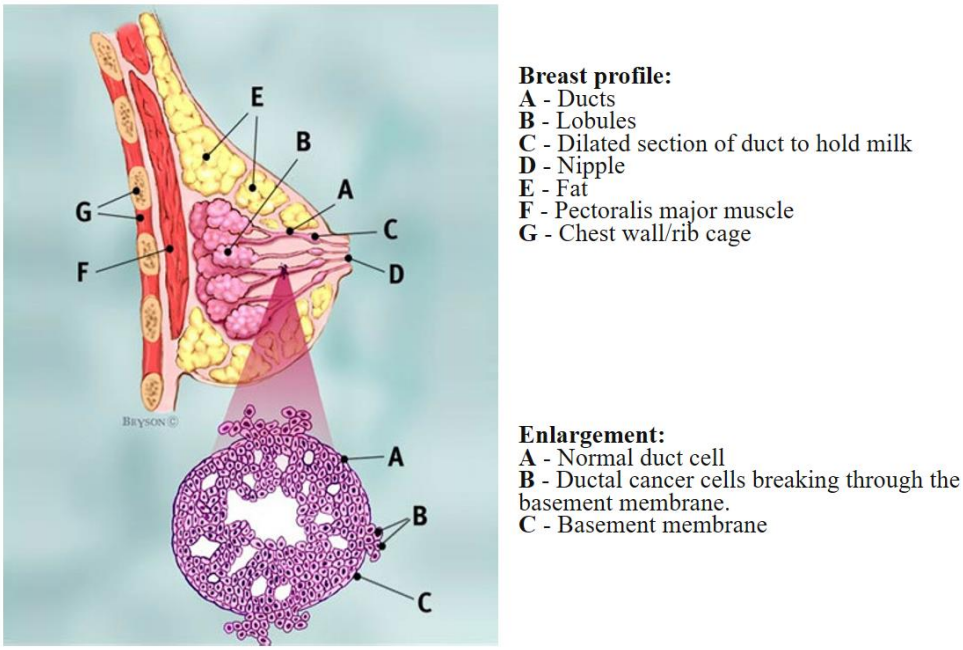

Figure 3: Normal breast with invasive ductal carcinoma (IDC) in an enlarged cross-section of the duct.[57] Scheme of the women's breast anatomy and the location of the IDC.

High-throughput technologies, such as gene expression microarray studies, have led to a new taxonomy of breast cancer based on their molecular characteristics. The more traditional molecular distinctions take into consideration the status (presence or absence) of hormone receptors —estrogen receptor (ER) and progesterone receptor (PR)— and, the amplification of the growth-promoting protein, human epidermal growth factor receptor 2 (HER2).[61–63] Studies based on the gene expression revealed five subtypes of breast cancer: luminal A (HR+ and/or PR+/HER2-) and B (HR+ and/or PR+/HER2+/-), basal-like/ triple-negative (HR- and PR-/HER2-), HER2-enriched (HR- and PR-/HER2+) and normal-like (similar to luminal A).[64] Basal-like BC definition was not yet internationally accepted, whereas some groups base their definition recurring to immunohistochemistry (IHC) studies, some others opt by microarray profiling.[64]

In the last two decades' mortality rates had suffered reductions, due mainly to the development of early detection, prevention and novel treatments. Nevertheless, the statistics show that incident rates have been increasing, which is believed to be closely related to the increasing and improvement of diagnostic techniques.[65]

1.2.2. Triple-negative breast cancer (TNBC)

TNBC owns its name to the fact that this subtype of BC is negative to all the three common markers in breast cancer already described (ER, PR and HER2).[64,66,67] Meaning that the proliferation and survival of this type of BC are neither dependent on hormones (estrogen and progesterone) neither on the amplification of HER2 receptors. A direct implication of this phenotype is its resistance to conventional hormonal therapy, such as tamoxifen or aromatase inhibitors, as well as to therapies targeting HER2 receptors, the case of trastuzumab.[68–70] Due to the lack of recognizable molecular targets, chemotherapy, radiotherapy and surgery are still the best treatment, both in early or advanced stages of TNBC.[66,71,72] In the cases which TNBC becomes resistant to these treatments the tumors often become more aggressive and unresponsive making of TNBC a type of cancer associated with a poor outcome,[66] only 30% of metastatic TNBC cancer patients survive 5 years.[72] TNBC tumors account for 10-17% of all BC carcinomas[64,67] and represent about 15% of all invasive breast cancers.[67] Unlike others low-grade BC carcinomas, risks of TNBC are higher with increasing parity and waist-to-hip circumference ratio, suggesting that genetic and social factors come together in an intimate way, observed by the fact that TNBC has a higher prevalence among young black and Hispanic women.[67]

An early and correct diagnosis of TNBC is of paramount importance, once it has implications in the choice of systemic therapy to give to the patients, so for instance, by avoiding false diagnosis, patients with positive expression of one of those receptors could have benefits from endocrine therapies or HER2 targeted drugs. The diagnose is done by IHC, where the levels of protein expression of PR, ER and HER2 are assessed. Thus, now the challenge is to be able to subtype TNBC to better identify molecular-based therapies. Due to the great heterogeneous disease that characterizes TNBC this type of BC displays a complex genomic landscape that results in a wide spectrum of transcriptional, histological and clinical differences.[72] From such differences, inevitably, there is the emergence of tumors with different grades of aggressiveness; and although TNBCs are, in its majority, considered as high-grade tumors, there is also a subset of well-characterized low-grade TNBCs that displays a favorable outcome.[73] So it is of pivotal importance to improve the subtyping of TNBCs.[74–76]

1.3. Hypoxia and angiogenesis

Capillary blood vessels are composed of endothelial cells and pericytes.[77,78] In embryogenesis blood vessels arise via two different processes, namely, vasculogenesis, in which endothelial cells arise from progenitor cell types, and angiogenesis, the process by which new capillaries sprout from existing vessels.[78,79] In physiological conditions, the vasculature is at a quiescent state in adult mammals with the exception of female reproductive cycles processes, such as ovulation, menstruation, implantation and pregnancy, in which new blood vessels form through angiogenesis.[78] It is a complex and highly regulated process, but when unregulated it is the cause of diseases such as arthritis and diabetes.[79]

Like normal cells, in order to survive, tumor cells also need to have access to oxygen and nutrients as well as an ability to evacuate metabolic wastes and carbon dioxide.[28] Today there is no doubt about the importance of angiogenesis in the role of solid tumor development. Evidence shows that a tumor that grows to a small size of few millimeters in diameter requires the induction of new capillary blood vessels[80] and, on the other hand, in the absence of an adequate vasculature tumor cells undergo necrosis and/or apoptosis resulting in tumor impairment.[78] Thus, in order to overcome this growth inhibition, tumor cells induce the formation of new blood vessels from pre-existing ones.[81] This process is known as tumor angiogenesis and is not only essential for primary tumor growth but also for the metastatic spread, since the presence of a new vasculature in the tumors fosters the entrance of tumor cells into circulation, increasing metastasis occurrence chances and thus is associated with poor prognostic outcomes.[80–82] During this process, there is an 'angiogenic switch'. This switch consists in a transition from a small-sized avascular tumor mass to a state where blood vessels develop promoted by the release of diffusible activators of angiogenesis by the tumor.[78,83] This process is initiated when cancer cells experience hypoxia conditions, which is characterized by a cellular or tissue level of oxygenation lower than normal that triggers a physiological stimulus to which the organism tries to respond accordingly to the demands of the respiring tissues.[82,84,85] Hypoxic regions are present in many tumors types as a result of rapid proliferation and deviating angiogenesis. The O_2 levels in tumors are very heterogeneous with some regions having a partial pressure of oxygen (pO_2) of less than 5 mmHg, compared to a pO_2 of 30-50 mmHg in the same, non-

cancerous, organs.[86] In the case of BC, reported to be one most hypoxic types of cancer, nearly 60% of the tumors have a pO_2 lower than 2.5 mmHg, whereas in healthy tissue values lower than 12.5 mmHg were not found.[87]

The response to hypoxia conditions is mainly mediated by the hypoxia-inducible factors (HIFs).[82,88] HIFs are a family of transcription factors that control important pathophysiological pathways and, in particular, emerged as the master regulators of oxygen tension homeostasis.[89] There are around 1000 genes that have their regulation dependent on HIF, most of them involved in the adaptations to hypoxia. These include genes regulating metabolism, blood-vessel growth, cell division and inflammation. HIF-1 is a heterodimeric protein comprising a constitutively expressed β subunit and a α subunit tightly regulated by oxygen availability.[84,90]

Under hypoxic condition, HIF is activated and upregulates the expression of a set of genes with pro-angiogenic functions. One of those genes is the vascular endothelial growth factor (VEGF) family (VEGF-A, VEGF-B, VEGF-C, VEGF-D and PIGF (placental growth factor)) which are signal proteins secreted by both tumor and endothelial cells (ECs) in response to hypoxia.[91] VEGFs bind to the vascular endothelial growth factor receptor (VEGFR) family, signal mainly through VEGF receptor 2 (VEGFR-2)[92] but also through VEGFR-1 and VEGFR-3, three transmembrane cell surface tyrosine kinases, which activate the intracellular pathway leading to permeability, proliferation, migration and survival of ECs (Figure 4).

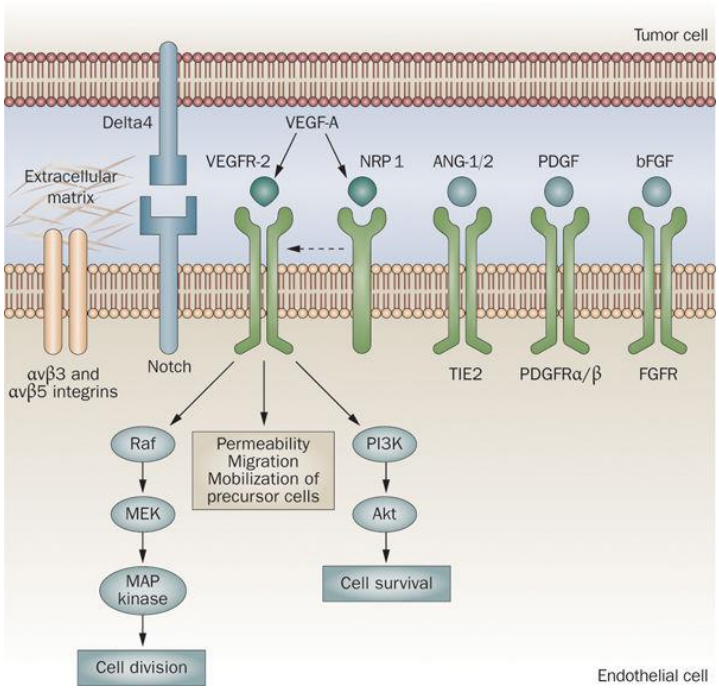

Figure 4: An overview of the activation of the angiogenic pathway.[93]
VEGFR is activated upon binding of VEGF, which results in activation of several downstream pathways, leading to angiogenesis. Neuropilin 1 (NRP1) can, when activated by VEGF-A, potentiate VEGFR-2's function. TIE2 activation through binding of Angiopoietin-1/2 (Ang-1/2) promotes angiogenesis. PDGFR activation upon PDGF binding has a vessel stabilizing effect by increasing pericyte coverage. Activation of the Fibroblast Growth Factor Receptor (FGFR) by the Fibroblast Growth Factor (FGF) promotes angiogenesis and EC proliferation. Furthermore, the pro-angiogenic signaling molecules αvβ3 and αvβ5 integrins are activated through several components of the extracellular matrix. Notch activation by the Delta4 ligand on the tumor cell membrane has a pro-angiogenic effect as well.

1.4. Senescence

The word senescence derives from the Latin *senex*, which means old man or old age. At a cellular level senescence refers to a phenomenon that involves deteriorative processes culminating in the irreversible cell proliferation arrest, to what we call cellular senescence.[94] It is a cellular response, to endogenous or exogenous stresses, that allows cells to maintain alive under the condition of cellular cycle arrest characterized by changes on a morphological and physiological level.

Hayflick and colleagues were the first to describe the cellular senescence phenomenon by showing that primary fibroblasts (normal human cells) had a limited ability to replicate when kept in culture for long periods of time.[95] This work revealed that after many cell doublings, the replicative potential of those cells was exhausted resulting in the presence of viable cells with no capability to proliferate even though they had an abundance of growth factors, nutrients and space.[95] Today it is known that this particular type of cellular senescence is triggered by the telomere shortening and it is called replicative senescence.[96] The dysfunctional telomeres trigger senescence via the p53 tumor suppressor protein (p53) pathway (Figure 5).[94]

One other strong, and telomere independent, senescence inducer mechanism is the DNA double strands breaks (DSBs) lesions.[97] Oxidative stress and other DNA damaging agents can cause DNA base damage and single-strand breaks which, on its turn, can be converted to DBSs during the replication and repair processes. In the context of cancer treatment, the formation of DNA DSBs can arise from ionization radiation, topoisomerases inhibitors and other cytotoxic chemotherapeutic drugs,[98,99] which are notably effective senescence inducers, capable of both inducing it in tumor cells or in the surrounding normal cells.[100] The precise mechanism behind this type of induction is unknown, but such lesions provoke a constant DNA damage response signaling, which is characterized by the long-term presence of nuclear DNA damage foci that contains activated DNA damage repair proteins including p53.

Mitogenic signals can also be strong inducers of senescence.[101] The best examples of this type of induction are the senescence responses that are provoked by certain oncogenes. This phenomenon has come to be known as oncogene-induced senescence. In some cases,

the overexpression of oncogenes induces molecular and morphological changes that cause the cells to stop dividing, instead of promoting an increase in proliferation. Earlier studies on this type of senescence response were done in mutant HRAS ($HRAS^{V12}$) cells, which chronically stimulates the mitogen-activated protein kinase (MAPK) signaling pathway, shown that it promotes senescence in normal cells.[102] Since then other components of the MAPK pathway have been linked to the induction of senescence when overexpressed or present in oncogenic forms.[103,104] Furthermore, cells can also undergo senescence in response to many other mitogenic stimuli such as the overexpression of growth factor receptors such as the HER2,[105] chronic stimulation by cytokines[106] and other forms of intense mitogenic stimulation.[107] Independently of the initiating event, mitogenic signals end up engaging either or both of the two major tumor suppressor pathways: p53/p21 and the cyclin-dependent kinase inhibitor 2A/Retinoblastoma-associated protein ($p16^{INK4a}$/p-RB) pathways (Figure 5), known to establish and maintain cellular senescence.[108] In fact, the chronic activation or overexpression of p53, p-RB, p21 or $p16^{INK4a}$ is commonly sufficient to induce senescence.[102,109] These are two complex pathways with numerous regulators and effectors that, in addition, can cross-regulate each other.[110,111] Both p53 and p-RB are master transcriptional regulators, thus these pathways control the senescence response mainly through widespread changes in gene expression. p21 is a downstream effector and $p16^{INK4a}$ is a positive upstream regulator of p-RB, and both are cyclin-dependent kinase inhibitors that work as strong negative regulators of the cell cycle. p53, in particular, has a major role as a tumor suppressor[112] and it is regarded as one of the most important regulators of cell fate.[113] It acts by restricting proliferation through numerous cell cycle checkpoints leading the cell to apoptosis or cellular senescence.

As we still struggle to fully understand how senescence works, there is evidence demonstrating that senescence has both beneficial and detrimental roles. On one hand, transient cellular senescence induction followed by tissue remodeling favors the removal of damaged cells and acts as a tumor-suppressor mechanism against oncogenic development. On the other hand, if this process is persistent and it is not followed by the ability to eliminate such cells it will directly influence the renewable of the tissues and becomes a detrimental process.[114] Broadly, it is possible to say that the role of senescence is to allow the organism to remove unwanted cells.

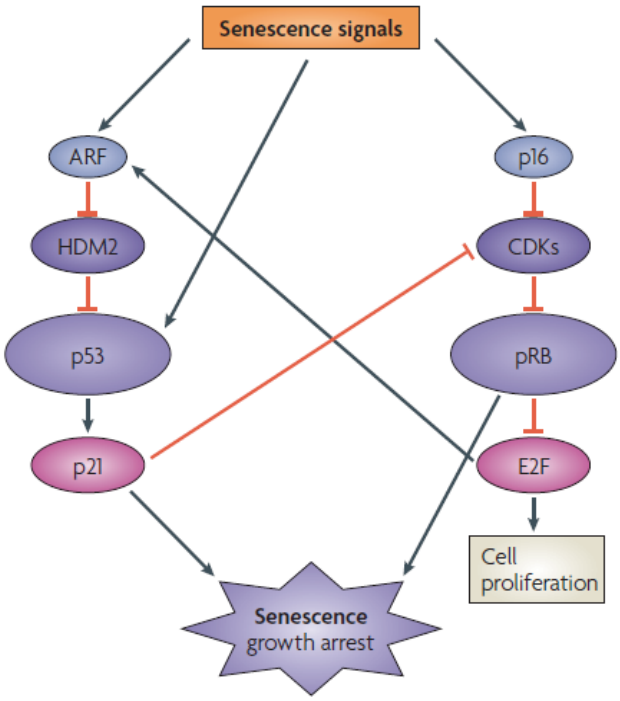

Figure 5: Senescence controlled by the p53 and p16–pRB pathways.[94]
Senescence-inducing signals usually engage either the p53 or the p16/p-RB tumor suppressor pathways. Some signals, such as oncogenic RAS, engage both pathways. p53 is negatively regulated by the E3 ubiquitin-protein ligase HDM2 (MDM2 in mice), which facilitates its degradation, and HDM2 is negatively regulated by the alternate-reading-frame protein (ARF). Active p53 establishes the senescence growth arrest in part by inducing the expression of p21, a cyclin-dependent kinase (CDK) inhibitor that, among other activities, suppresses the phosphorylation and, hence, the inactivation of pRB. Senescence signals that engage the p16–pRB pathway generally do so by inducing the expression of p16, another CDK inhibitor that prevents pRB phosphorylation and inactivation. pRB halts cell proliferation by suppressing the activity of E2F, a transcription factor that stimulates the expression of genes that are required for cell-cycle progression. E2F can also curtail proliferation by inducing ARF expression, which engages the p53 pathway. So, there is reciprocal regulation between the p53 and p16–pRB pathways.

Together with apoptosis —also a cellular response to endogenous or exogenous stimuli but which results in a programmed cell death— these two mechanisms are the most efficient to remove those damaged cells.[114] Both are conceptually identical to each other, it is possible to say that senescence is an apoptosis resistance mechanism, and p53 protein is the major regulator of both processes. Although it is not still very clear what makes a cell to whether undergo a senescence or apoptotic pathway, the cell type seems to be a relevant factor, for instance, damaged epithelial cells and fibroblasts are more prone to senescence than damaged lymphocytes, which tend to undergo apoptosis.[94]

Over the years the characterization of the cellular senescence state has been improving. As is expected there is not a single characteristic to be exclusive of the

senescence state. Instead to distinguish senescent cells from other non-dividing cell processes, such as quiescent or other types of terminally differentiated cells, a set of several features comprising both morphological changes and molecular markers are used.

The absence of proliferation markers is obviously one of those markers. For instance, the absence of the proliferative marker 5-bromodeoxyuridine is an essential condition to certificate the state of senescence.[94] This synthetic nucleoside, analogue to thymidine, is incorporated by the cell during the DNA synthesis in the S phase of the cell cycle, thus by detecting the incorporated chemical it is possible to measure the rate of proliferation.[94]

At a morphological level cellular senescence is clearly visible by the changes in the phenotype, cells become larger, often with doubling in volume, flat and in some cases with a multinucleated appearance.[115] These basic characteristics on their own are a good indication for a potentially cellular senescence state, however, they are still insufficient.

Together with these microscope visible features, the histochemical staining for senescence-associated β-galactosidase (SAβ-gal) activity was one of the first and most consensual biomarkers to be used to identify cellular senescence.[101,116] This activity is based on the overexpressed lysosomal content of senescent cells. It reflects the enlargement of the lysosomal compartment as a consequence of the increased lysosomal biogenesis in senescent cells.[117] This activity is linked with an increase in autophagy, which is an effector mechanism of senescence.[116] For the most of the mammalian cells SA-β-gal activity detection is made at pH 4.0 but for senescence, it is only sensed around pH 6.0.[117] The simplicity of this assay and its apparent specificity for senescent cells have made of this biomarker one of the most extensively used.

Another marker that can be used, is the senescence-associated heterochromatin foci (SAHF) which is a characteristic present in some, but not all, senescent cells.[118] SAHF represents cytologically detectable heterochromatin domains that endorse altered expression of proteins that affect the chromatin structure. This heterochromatin main objective is to repress and contribute to the silencing of proliferation-promoting genes in the senescent cell.[119] SAHF appears to be dependent on $p16^{INK4a}$/p-RB pathway activation and have been strongly correlated with the irreversibility of senescence arrest.[119] Although SAHFs are not present in all senescent cells, it has been proposed that, in cells that do not develop these structures, the $p16^{INK4a}$/p-RB pathway might establish chromatin states that are functionally

equivalent to SAHFs due to the ability of pRB to complex in histone modifying enzymes that form repressive chromatin.[94] Senescent cells can be identified by the cytological markers of SAHFs that are detected through favorable binding of DNA dyes, such as 4′,6-diamidino-2-phenylindole.

Alongside with these changes in chromatin organization and gene expression alterations, it is still necessary to mention yet another important biomarker to the identification of senescence, that is, the senescence-associated secretory phenotype (SASP). Many senescent cells overexpress genes that encode secreted proteins that can alter the tissue microenvironment. These include numerous proinflammatory cytokines, chemokines, growth factors and proteases.[120,121] The complexity of the SASP proteins reflects the myriad of biological activities that it provokes. Some components of SASP can promote cell proliferation[120,122] as well as stimulate new blood vessel formation.[123] SASP secretions of the pro-inflammatory cytokines (interleukin-6 and 8), chemokines and macrophage inflammatory proteins directly or indirectly promote inflammation.[120,124] Some other components, particularly transforming growth factor-beta, are known to trigger senescence in neighboring cells in a paracrine manner.[114] One interesting feature of SASP is that it is primarily a property of cells that senesce owing to genomic damage or epigenomic perturbation, thus a cell that enters senescence via ectopic overexpression of p21 or $p16^{INK4a}$ do not express SASP as senescence feature.[125]

Currently, it is hypothesized that SASP might have evolved together with the senescence response as a mechanism to both suppress the development of cancer and promote tissue repair in young organism[126] through the promotion of an inflammatory microenvironment that stimulates the clearance of damaged and senescence cells by the immune system.[114] However, the “dark side” (already mentioned above dual role of the senescence response) of the SASP secretome is only revealed later in life; when the age-dependent accumulation of senescent cell creates sites of chronic inflammation and remodeling/healing features, which are permissive for the development, or at least the progression, of cancer through SASP factors known to fuel the deleterious effects of senescent cells.[126] Also with age comes the decline of the adaptive immune system making it less likely that senescent cells will be cleared efficiently.[127] So, senescent cells increase with age and can promote cancer initiation through their prominent feature of SASP secretions that, on their turn, can stimulate the infiltration of leukocytes which produce

reactive toxic moieties that can cause DNA damage.[128,129] It seems that at some point, senescent growth arrest starts to lose its evolutive function of preventing cancer development and tissue damage, and instead it contributes to the exact opposite.

An important question then is if the senescent state can be used as a therapeutic target. Recent studies have been emerging with promising results in this field.[130] Nevertheless, cellular senescence remains very ambiguous and still raises a battery of questions remaining to be answered.

1.5. Objectives

TNBC is a type of BC that is a remarkably heterogeneous and complex disease entity that lacks targeted therapies. And though a lot of women respond very well to the surgery/chemotherapy/radiotherapy treatment, when TNBC recurs or spreads it is, unfortunately, more difficult to treat than some other forms of BC. We very much need to find new treatments, both for patients with metastatic or advance TNBC, and for patients with an early stage to do a better job in preventing recurrences.

The objective of this work is the improvement of breast cancer therapy by using two conceptually different approaches: (1) investigating the synthetic lethality with hypoxia; (2) exploiting senescence as a vulnerability in cancer cells that can be therapeutically targeted.

(1) Tumor hypoxia is a common feature in cancer diseases and it can have dual roles. On one hand it can function as a drug resistance and as a driving oncogenic force, and on the other hand, it can prevent the growth of tumor cells. The development of antiangiogenic (AA) treatments, at first very promising, revealed to be a clinical failure conferring no enduring benefits to oncology patients. Thus, there is the need to improve the efficacy of AA therapies. This part of the project aims at doing so and consisted on the validation of a functional genetic CRISPR screen aiming to find synthetic lethal interactions with hypoxia.

(2) A genetic screen aiming to find synthetic lethal partners with Palbociclib treatment in TNBC cell lines revealed not a synthetic lethality interaction but instead the induction of senescence with the cyclin-dependent kinase 2 protein (CDK2). This second part of the project aimed to further expand the validation of this finding to a larger TNBC cell line panel using different approaches (genetic and pharmacological) and subsequently test the efficiency of a senolytic drug (ABT-263) in specifically killing senescent cancer cells.

2. Results and discussion

2.1. Finding synthetic lethal interactions with hypoxia in TNBC

Most cancers grow in hypoxic conditions due to the lack of a proper functional vasculature able to support the physiological needs of the fast-growing malignant cells. It is in these hypoxic tumor areas that we find some of the most malignant cells.[131] There are currently several targeted therapies to target and inhibit angiogenesis. Yet these anti-angiogenic therapies have failed to show enduring anti-cancer responses and thus contributed marginally to improve overall survival of breast cancer patients in clinical trials. Here we aim at contributing to the improvement of anti-angiogenic treatments. To this purpose, we use hypoxia culturing conditions (1% O_2) to mimic the effect of reduced tumor vasculature induced by this treatment in vitro. We choose a TNBC cell model because this type of BC lacks targeted therapies, has a bad prognosis and is, usually, more hypoxic than the other BC subtypes.[132] In this study, we used a CRISPR/Cas9 technology approach to perform a genetic screen aiming at finding synthetic lethal interactions with hypoxia/anti-angiogenic agents. The screen was performed with an epigenetic library since there is little knowledge about the regulation of the epigenome in hypoxia.

The human TNBC line SUM159PT was infected with that library which contained 5230 single guide RNA (sgRNA) sequences targeting chromatin modifiers and epigenetic regulators. The screen was maintained at a complexity of 1000-fold coverage (each sgRNA was present in approximately 1000 cells), and with a multiplicity of infection (MOI) of approximately 0.3 to avoid multiple integrations and thus confounding results due to passenger effect. Cells were seeded in three technical replicates for each arm, normoxia (21% O_2), and hypoxia (1% O_2), and collected also in triplicates in the time-point zero as a reference sample (Figure 6A).

After culturing for 20 days, genomic DNA was isolated and the viral DNA was recovered by polymerase chain reaction (PCR), and analyzed by deep sequencing to identify the number of sgRNA inserts in the cell population. The results were analyzed using the MAGeCK software.[133] Overall sgRNAs targeting essential genes were depleted in comparison to those targeting nonessential genes in both normoxia and hypoxia arms (Figure 6B), indicating that technically the screen performed as intended. The M/A plot in Figure

6C represents the depletion of sgRNA at 1% O_2 versus the mean reads per sgRNA at 21% O_2. The ten candidate genes identified as top hits are indicated in Table 1.

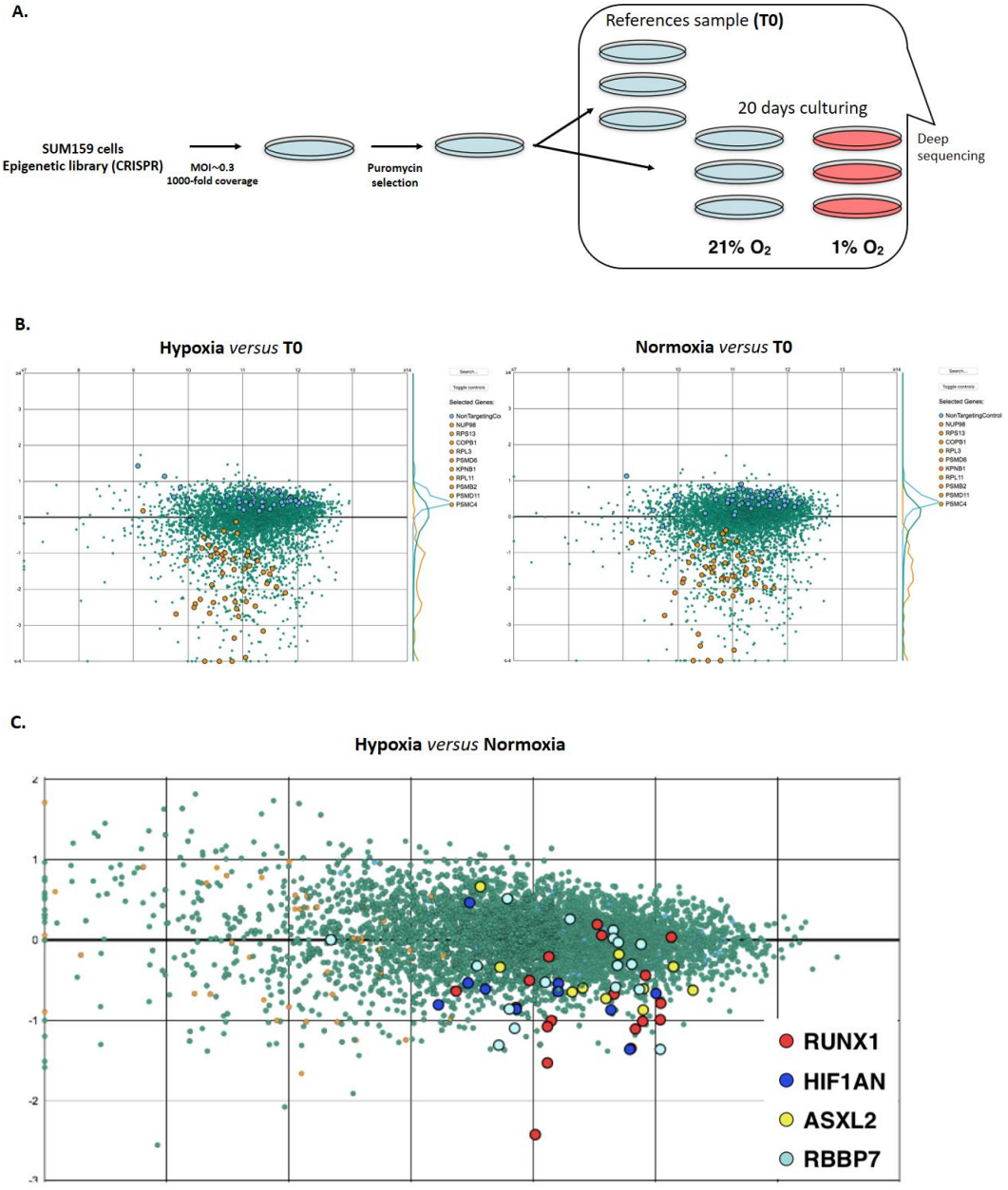

Figure 6: Synthetic lethality dropout screen layout and its hits.
(A.) An epigenomic CRISPR library was used to infect SUM159PT TNBC cell line at a complexity of 1000 and MOI of 0.3. Cells were collected at time-point zero (control) and cultured in 1% and 21% O_2 for 20 days. The screen was designed with three biological replicates per arm and collected in three technical replicates. Lentiviral DNA was captured, PCR-barcoded for each technical replicate and sequenced using Illumina Hi-Seq. **(B.)** *In vitro* screen controls, M/A plots of hypoxia vs. T0 and normoxia vs T0 in which the depletion (relative abundance 1% O_2 / T0 and 21% O_2 T / T0) of the control genes is plotted versus mean read per sgRNA (log 10 scale), non-targeting controls (NTC) are shown in blue and essential genes in orange. The plots show the depletion of essential genes while the non-targeting controls remain in the cloud in both oxygen tensions. **(C.)** M/A plot in which the depletion (relative abundance 1% / 21% O_2) is plotted versus mean read per sgRNA (log 10 scale). The four top hits are shown in different colors (each one with at least 3 different sgRNAs to the same gene), the cut-off criteria for a gene to be considered a hit was a false discovery rate of < 0.003.

Table 1: Top 10 candidate genes hit list after screen analysis.

neg | rank	Gene ID	Nr. of sgRNAs	Neg | fdr
1	RUNX1	18	0.00165
2	HIF1AN	10	0.00165
3	ASXL2	10	0.00165
4	RBBP7	17	0.003713
5	KAT2A	10	0.008911
6	EP300	10	0.009076
7	KDM3B	10	0.170438
8	ZMYND11	15	0.316213
9	TDRD7	10	0.833333
10	SP140	18	0.857921

When I arrived in the Bernards lab, the screen had been already performed and analyzed. I started my internship by validating the top four candidate genes: RUNX1, HIF1AN, ASXL2 and RBPP7.

The first step was to assess the effect of hypoxia on the proliferation of the SUM159PT cell line on a long-term clonogenic assay. In Figure 7A is not possible to appreciate a strong difference in the colony formation assay, but quantification with cell titer blue (CBT) showed a statistically significant difference (Figure 7B) with a p-value of 0,02707 at end stage. This indicated a slight sensitivity of this cell line to hypoxia that we aimed to increase further by interfering with the genes identified in the screen.

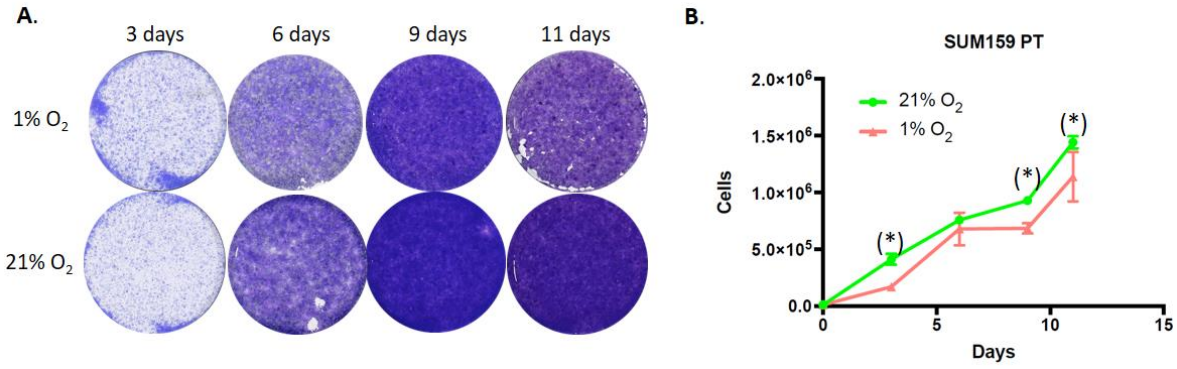

Figure 7: Hypoxia condition (1% O_2) does not significantly impair SUM159PT cell proliferation. SUM159PT cells were seeded at low density in a 6-well plate setting and cultured either at 21% or 1% O_2 conditions. **(A.)** Colony formation assays where cells were fixed and stained in each of the indicated time points. **(B.)** Growth curves from cell counts of the indicated time points (results from two biological replicates counted in duplicate). (Time points where p-values show statistical significance are indicated with (*); T=3, (p = 2,352E-06), T=9 (p =0,00239) and at T= 11 (p = 0,02707)).

2.1.1. RUNX1 validation

Runt-related transcription factor 1 (RUNX1) also known as acute myeloid leukemia 1 protein or Core Binding Factor subunit alpha-2 (CFBA2) is a protein that is encoded by the RUNX1 gene, which showed up as the strongest hit in the screen. CFBA2 can bind to the DNA as a monomer, but its affinity is enhanced 10-fold if it heterodimerizes with the Core binding factor subunit beta (CBFB), forming the Core binding factor (CFB) which is a transcription factor and it can bind to the core element of several different enhancers and promoters. RUNX1 binds DNA and is thought to be involved in the development of normal hematopoiesis.[134]

For the validation of RUNX1, we individually cloned the six sgRNAs targeting RUNX1 that best scored on the screen into the pLentiCRISPR2.1 vector. These were used to produce lentivirus particles that transduced SUM159PT cells. In Figure 8A it is shown RUNX1 protein levels from SUM159PT cells infected with the six different sgRNAs.

From all the six sgRNAs, only the cells infected with the sgRNA-RUNX1-11 showed a significant reduction in the levels of RUNX1 protein at the time-point zero (lysates harvested after puromycin selection). This result seemed to be correlated with the fact that this sgRNA was the one that had the strongest score in the screen (Table 6 in materials and methods section). However, over a period of 15 days, sgRNA-RUNX1-11 levels did not remain diminished and were in fact enriched over time (red boxes in Figure 8A).

This transient loss of RUNX1 protein knockdown levels over time can be explained by the polyclonal population setting in which the experiment was performed. It is known that over time, certain knockouts confer cells with a proliferative disadvantage. What might be happening is that cells that have less RUNX1 knockdown levels take over the population by displaying proliferation advantage over the most efficiently RUNX1 knockdown cells. Furthermore, there is also the possibility that there are cells edited with a silent mutation that does not have impact on the protein synthesis or functions.[135]

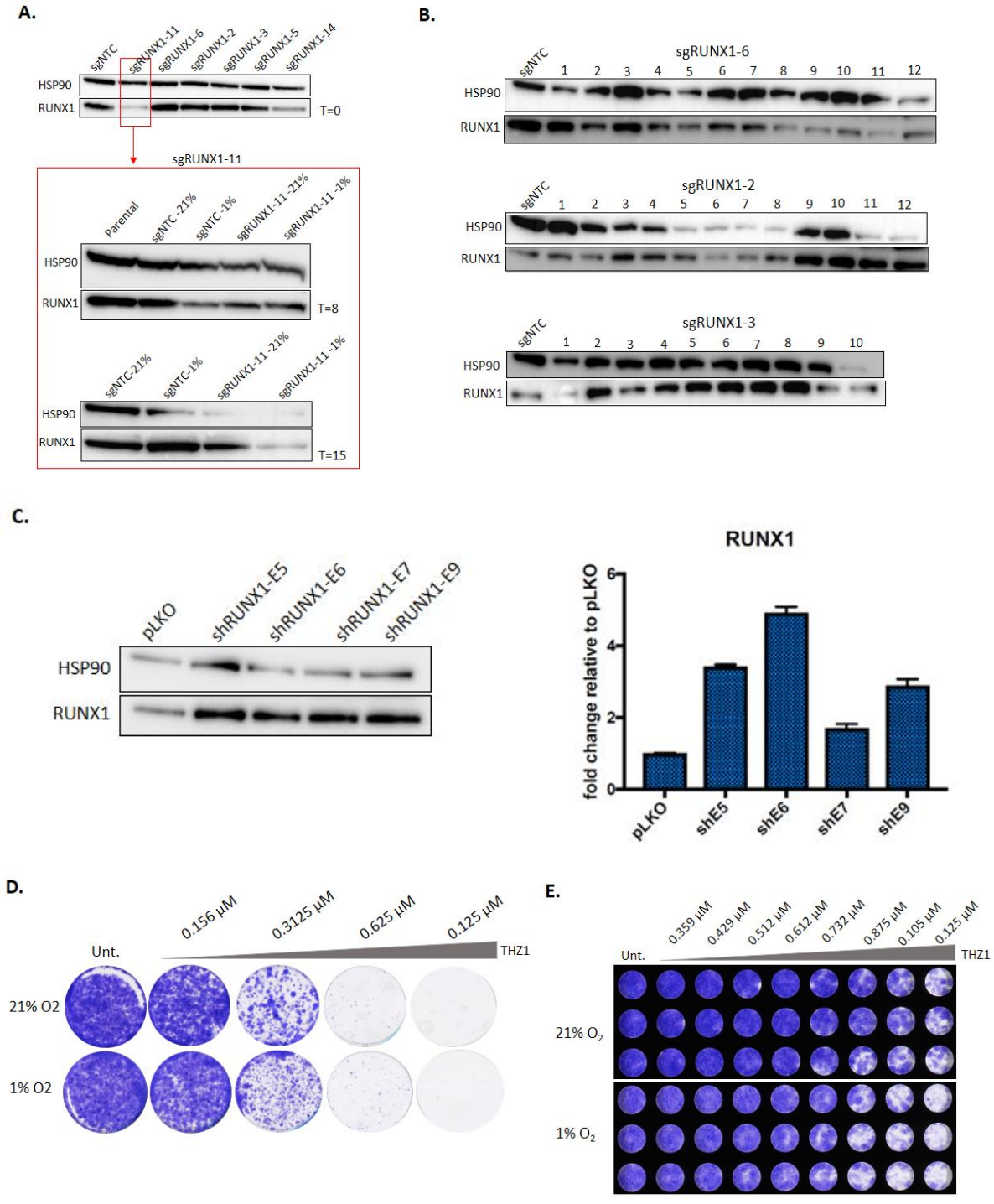

Figure 8: RUNX1 validation.
(A.) SUM159PT cells were transduced with sgRNA-RUNX1 (six different sgRNAs targeting RUNX1 and one non-targeting control gRNA) and seeded at time-point zero (T=0) after puromycin selection. Protein lysates were prepared at the same time and blotted for assessment of RUNX1 levels. Single gRNA-RUNX1-11 displayed the strongest RUNX1 protein level reduction and these cells were followed up for further validation (red box) by being cultured in hypoxia or normoxia. At T=8 and T=15 cells were harvested and blotted for RUNX1. RUNX1 protein levels get enriched over time. HSP90 was used as loading control. **(B.)** Transfected SUM159PT cells were isolated into single cell clones by Fluorescent Activated Cell Sorting after puromycin selection. Cells were harvested and blotted for RUNX1 protein levels. **(C.)** SUM159PT cells were transduced

with shRNA-RUNX1 (four different short hairpins targeting RUNX1 —shE5, shE6, shE7, shE9— and empty vector pLKO as control). Cells were harvested for protein and RNA analyses after puromycin selection. **(D.)** Parental cells were seeded at low-density in 6-well plate setting and allowed to attach overnight both at 21% and 1% O_2 conditions. Cells were then treated with increasing doses of THZ1 (refreshed every 2 days) for a period of 7 days. Cells were fixed, stained and scanned. **(E.)** Parental cells were seeded at low-density (400 cells per well in 96-well plate setting) and allowed to attach overnight both at 21% and 1% O_2 conditions. Cells were then treated with increasing doses of THZ1 (refresh every 2 days) for a period of 5 days after which they were fixed, stained and scanned (three technical replicates per condition). (Unt. = untreated, NTC = non-targeting control).

For these reasons, a logical way to proceed is to validate the hits in monoclonal populations. In order to do so, we isolated single cell clones by Fluorescent Activated Cell Sorting (FACS) from these polyclonal populations. This experiment was performed right after the puromycin selection, in order to start with an enriched population of cells knockout for the targeted gene. Despite the technical difficulties in isolating such populations, we were able to grow some clones but, unfortunately, the results of the western blots never revealed a true RUNX1 knockout level (Figure 8B.).

The fact that we could not achieve any RUNX1 knockout clones when trying to isolate monoclonal populations, nor observe any knockdown levels in the polyclonal setting, led us to think that RUNX1 might be an essential gene and thus its depletion display a straight lethal phenotype. In fact, RUNX1 knockout mice are embryonic lethal due to defects in liver hematopoiesis and central nervous system haemorrhages.[136,137] In cancer this could still be a context-dependent event in SUM159PT cells. If this holds true, the absence of RUNX1 knockout in the western blots could be explained as the result of the outgrowth cells that were edited on a silent mutation (mutation in one nucleotide that does not display phenotype).[135]

In an attempt to rule out or confirm this hypothesis, we next used a short hairpin RNA (shRNA) approach. This method leads to a knockdown of the target gene instead of a knockout. SUM159PT cells were transduced with lentivirus particles carrying five different shRNAs targeting RUNX1. Also with this approach, we did not detect reduced levels of RUNX1 RNA nor protein (Figure 8C). In fact, we even observed an upregulation of RUNX1 transcripts in the shRUNX1 treated cells; this event makes sense if it is the case that this gene controls its own expression through a negative feedback loop and once the protein level decreases, the gene compensates by increasing its transcription resulting also in the upregulation of protein levels. Of relevance to mention that cells transfected with one of the

shRNAs (shRNA-E8) did not survive to the viral transduction and subsequent puromycin selection, which can also support the notion that RUNX1 is indeed a straight lethal gene in this particular cell line.

RUNX1 has been known for its role in the development of several types of leukemia,[136] and more recently it has also been found mutated in breast cancer.[138,139] One study shows a putative correlation between the expression of RUNX1 and poor patient prognosis in TNBC patients with primary operable BC, where authors show that when RUNX1 protein levels are highly expressed the survival rate decreases compared to the low/absent RUNX1 expression.[140] For this reason, in parallel to the genetic validation of the screen, we also pursued a pharmacologic validation of RUNX1 in the context of hypoxia. However, the direct targeting of transcription factors is not something easy to achieve,[141] especially as a cancer therapy due to the many roles that transcription factors have in non-malignant cells. One way to pharmacologically inhibit general transcriptional machinery is through an indirect inhibition, for instance, the targeting of transcription co-factors.

A recent study shows that THZ1, a selective covalent inhibitor of cyclin-dependent kinase 7 (CDK7), negatively impact the transcription activity of RUNX1 in Jurkat T-ALL cell.[142] The same authors also used THZ1 in a TNBC work, where they show that TNBC, but not hormone receptor-positive breast cancer cells, are dependent on CDK7 transcription.[143] For these reasons we decided to use THZ1 to interfere with RUNX1 activity. Furthermore, THZ1 was never explored under hypoxia, and so it would still be novel to test it in this context.

In Figures 8D and 8E are shown the results of THZ1 treatment in colony formation (CF) assays performed in 6 and 96-well plates settings. The CFs on the 6-well setting (Figure 8D) did not show differences when cells were grown in hypoxia versus normoxia conditions upon increasing concentrations of THZ1 treatment. Even so, this experiment was also performed in a 96-well setting (Figure 8E) and here we observed a slight difference in the phenotype, cells cultured at 1% O_2 showed a stronger cell proliferation impairment compared to when cultured at 21% O_2. In order to clarify these discrepancies, the experiments must be repeated with better controls, for instance, take along cells to grow in hypoxia but without THZ1 treatment, because this slight difference in cell proliferation can be the result of hypoxia on itself (Figure 7B). Another point to be addressed is RUNX1 protein levels and

its target genes upon THZ1 treatment, to verify THZ1 effect on RUNX1 in this cell line in the tested oxygen tensions.

Overall the experiments presented in this thesis suggest that RUNX1 does not validate as a screen hit and thus is not synthetic lethal with hypoxia. Using a CRISPR/Cas9 approach, we only managed to obtain knockdown of RUNX1 with one gRNA (sgRNA-RUNX1-11), but that phenotype was rapidly lost in the polyclonal population, preventing a proper conclusion about the theoretical synthetic lethality of RUNX1 with hypoxia. Monoclonal populations of CRISPR-RUNX1 edited cells also failed to produce RUNX1 deficient cells. Additionally, the shRNA approach and qRT-PCR analyses further suggest that, indeed, RUNX1 might be a straight lethal gene in SUM159PT. With regard to the pharmacological approach, the results also did not show a synergy which encouraged us to pursue further experiments.

In resume, due to the above presented technical limitations, we were never able to create $RUNX1^{KO}$ cells and thus, the synergy between $RUNX1^{KO}$ and hypoxia was never truly tested.

2.1.2. HIF1AN validation

HIF1AN gene encodes the hypoxia-inducible protein 1-alpha inhibitor (FIH1), an important protein in the cellular hypoxia adaptation that influences the expression of a large spectrum of genes dependent on the oxygen pressure variations.[144,145]

The significant biological role of HIF1AN associated with the fact that it was the second strongest hit in the CRISPR screen giving synthetic lethality with hypoxia made it an interesting candidate gene for follow up validation.

The same approach was taken to validate HIF1AN hit. Lentivirus particles carrying three sgRNAs targeting HIF1AN gene were produced and used to transduce SUM159PT cells. Efficient knockdown of FIH1 was confirmed by Western blot at different time points in a polyclonal cell population (Figure 9A). However, in a long-term colony formation assay, we did not observe any phenotype difference when cells were cultured at different oxygen tensions (Figure 9B).

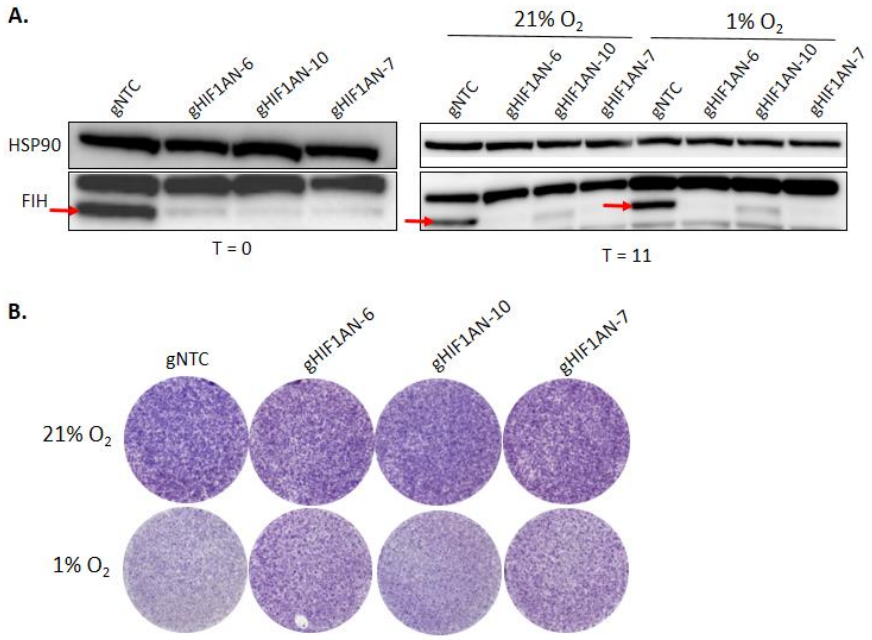

Figure 9: HIF1AN validation.
(A.) SUM159PT cells were transduced with sgRNA-HIF1AN (with three different gRNAs targeting HIF1AN gene and one non-targeting gRNA control) and seeded at time-point zero (T=0) after puromycin selection (left of panel A). Cells were also seeded at the end of CFs experiments, at time-point 11 days after puromycin selection (T=11) in both hypoxia and normoxia conditions (right on panel A). Protein lysates were harvested and prepared at the same time and blotted for assessment of FIH1 protein levels (red arrow). **(B.)** Transduced cells were seeded at low-density in a 6-well plate setting, and cultured for a period of 10 days (medium refresh every 3 days) at 21% and 1% O_2 after which they were fixed, stained and scanned.

Our conclusions are that, either even a residual level of FIH1 still present in the polyclonal population is enough to maintain its function, or either that the loss of HIF1AN is not synthetic lethal with hypoxia. We could also have isolate monoclonal population of complete HIF1AN knockout cells and repeat these experiments, but given the complete absence of phenotype with a polyclonal setting we did not think it was promising enough to move forward with the validation of this gene.

2.1.3. ASXL2 and RBBP7 validation

The same strategy was used in the validation of ASXL2 and RBBP7.

Putative Polycomb group protein ASXL2 encoded by ASXL2 gene shown decent reduced protein levels at time-point zero and thus, those cells were cultured in hypoxia for a

period of 8 days (Figure 10A). ASXL2 impairment did not show any phenotype in combination with hypoxia conditions (Figure 10B).

Histone-binding protein RBBP7 encoded by RBBP7 did not show protein reduction, at time-point zero, strong enough to justify a further evaluation on the synthetic lethality with hypoxia (Figure 10A).

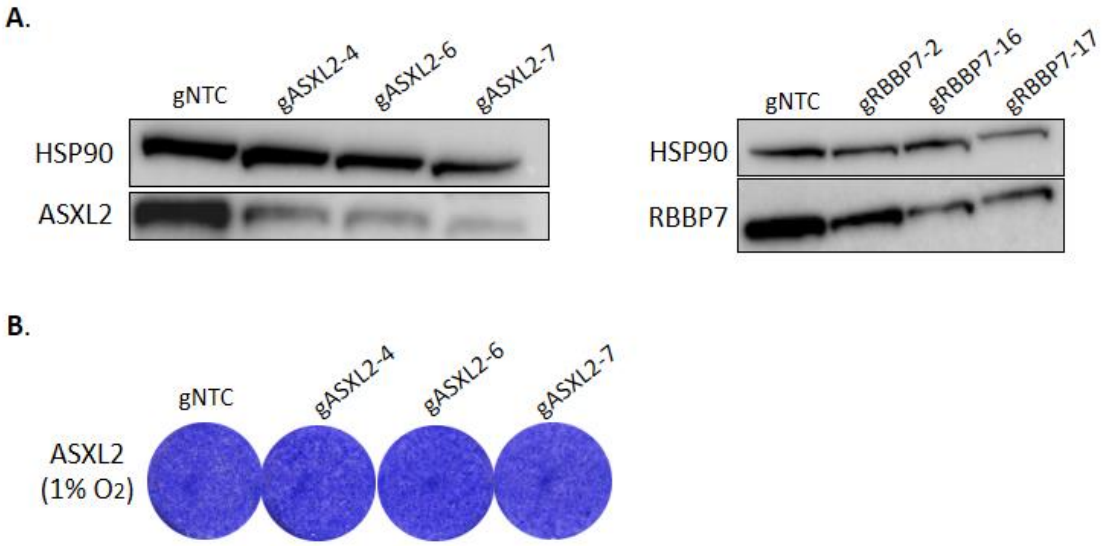

Figure 10: ASXL2 and RBBP7 validation
(A.) SUM159PT cells were transduced with sgRNA-ASXL2 and with sgRNA-RBBP7 (both with three different sgRNAs targeting both genes and one non-targeting sgRNA control) and seeded at time-point zero (T=0) after puromycin selection. Protein lysates were harvested and prepared at the same time and blotted for assessment of ASXL2 and RBBP7 protein levels. **(B.)** Transduced cells were seeded at low-density in a 6-well plate setting, and were cultured for a period of 8 days (medium refresh every 3 days) at 1% O_2 conditions after which they were fixed, stained and scanned.

ASXL2 gene did not validate in a polyclonal setting despite some degree of protein reduction, and as similarly to HIF1AN, the total absence of phenotype in this setting made us not continue any further validation with monoclonal populations.

Regarding the RBBP7 gene, we cannot properly conclude about its validation. The levels of protein were not reduced and, once this was the gene with the weakest score on the hits list, we did not further invest in its validation.

2.1.4. Discussion and conclusions

From the four hits considered for validation, two of them did not validate, namely ASXL2 and HIF1AN. CFs assays where the level of protein was knocked down did not show any phenotype when cultured in hypoxic conditions.

The other two hits, RUNX1 and RBBP7, also did not validate. Although in these two cases we cannot really rule out the possibility of a validation because we never managed to achieve truly knockouts cell populations, despite the several attempts and different methods to create such populations.

Because RUNX1 was the strongest hit and thus the one that held a more promising phenotype, it was the hit in which we dedicated more effort. A pharmacological validation using a CDK7 inhibitor (THZ1) with the ability to interfere with the transcription of RUNX1 was performed but failed in showing convincing results. Furthermore, recent studies report that RUNX1 inhibitors shown efficacy in impairing Triple-negative BCs,[141] and it would be interesting to explore these findings in a context of hypoxia. Even more, recently was the discovery that the target genes of RUNX1 strongly overlapped with those of ASXL2,[146] so the fact that both genes appeared in the top score hits might be related to this common target genes role under hypoxia.

Several attempts in previous genetic screens with the aim to find synthetic lethality interactions with hypoxia have been performed. Screens using shRNA, CRISPR, several cell models, three targeted libraries (kinome, epigenome, hypoxia library) and yet all failed, demonstrating how hard it is to identify such interactions. The validation of this screen also falls within the scope of those results. We did not find any hit that has validated. Nevertheless, this does not mean that we should stop trying. For example, these screens could be performed in cells been treated with anti-angiogenic drugs (e.g. bevacizumab), because the response to genetic setting (in this case by culturing the cells in 1% O_2) is always different when compared to a pharmacological setting, where you have off-target effect events promoted by the drugs that do not occur in the same way with the genetic editing. Another suggestion would be to perform these screens to find essential genes upon AA treatment in an in vivo setting. A recent paper alerts for caution when interpreting in vitro results. The authors report that when comparing the results of in vitro and in vivo screens

experiments with the aim to identify essential genes for the survival of a type of brain cancer have shown almost no overlap,[147] suggesting that the translation from in vitro to in vivo settings can be more complex than what we thought.

2.2. Cellular senescence as a vulnerability used to kill cancer cells.

Aiming at finding synthetic lethal interactions with the CDK4/6 inhibitor Palbociclib, a former Ph.D. in our lab performed a genetic screen in 3 TNBC cell lines (HCC1806, CAL-51 and CAL-120) using a shRNA kinome library. The results from the screen revealed cyclin-dependent kinase 2 (CDK2) as the strongest hit with multiple hairpins in the three cell line models.

Palbociclib is a selective inhibitor of cyclin-dependent kinase 4 and 6 (CDK4/6) already used in the clinic for treatment of ER-positive and HER-negative advanced breast cancer.[148] TNBC are resistant to Palbociclib.[149] We confirmed this by treating a panel of TNBC cell lines with Palbociclib in a long-term CF assay (Figure 11). Table 3 (Materials and Methods section) lists the most important driving mutations in each of the TNBC cell lines used in this study.

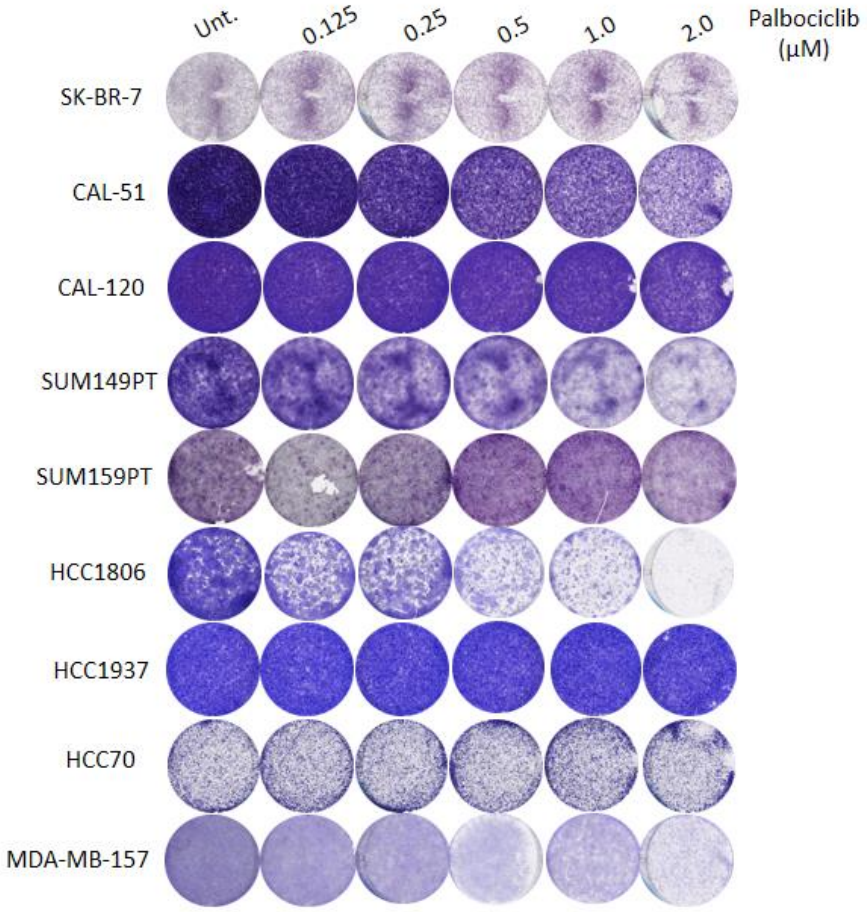

Figure 11: Palbociclib treatment in a panel of TNBC cell lines.

Cell lines were seeded at low-density numbers and allowed to attach overnight before being treated with Palbociclib at increasing concentrations (medium and drug refreshed every 3 days). After 10 days of culture, cells were fixed and stained. (Unt. = untreated control).

In general, we can conclude that all the tested cell lines are, to some extent, resistant to Palbociclib treatment. HCC1806 cell line showed more sensitivity, and CAL-51, along with SUM159PT, displayed milder sensitivity with higher drug concentrations.

Literature and results from our lab showed that drug resistance (acquired or intrinsic) often develops by the reactivation of the inhibited pathway.[150–152] So is not so surprising that we found a hit that is in the same pathway as CDK4/6 (the targets of Palbociclib). Furthermore, the targeting of the cell cycle, through CDKs inhibition, has the advantage of being a process in which cancer cells are more dependent than when compared with most of the cells in the human body, which are mainly in a quiescent state.

However, when trying to validate this finding, $CDK2^{KD}$ in combination with Palbociclib treatment did not prove to be truly synthetic lethal. Instead, a significant number of cells survived upon treatment. Microscopic examination showed a different morphology of the remaining cells: increased size, flattened cytoplasm, and polynucleation. These cells appear not to be dividing, thus suggesting that they could be in a senescent or quiescent state. Further characterization using senescence-associated β-galactosidase (SA-β-gal) staining and biochemical markers of cellular senescence, for example, loss of phosphorylated RB and up-regulation of cyclin-dependent kinase inhibitor 2A ($p16^{Ink4A}$), showed that these cells were indeed in a senescence state. Altogether, this screen revealed a strategy to induce senescence through the combination of Palbociclib treatment and CDK2 knockdown.

Before I arrived in the lab, all the in vitro validation with shRNA had already been performed. The next step then would be to validate this model in vivo, because the ultimate goal of all translational cancer research is to bring the lab knowledge closer to the clinic. To this aim, we tested the model with CRISPR, since classical shRNA is often not a stable system to perform long-term in vivo experiments.

This strategy to induce senescence could then be explored and used in a one-two punch model approach. In a first step, we would induce senescence in cancer cells and in a second step, eliminate them with a senolytic agent.

2.2.1. CRISPR /Cas9 (re)validation of the senescence inducible model in TNBC

In order to generate stable CDK2^{KO} clones to model the senescence phenotype in vivo (treatment with Palbociclib of CDK2^{KO} tumors), we did a revalidation of the screen using a CRISPR/Cas9 approach in three TNBC cell lines. CAL-51 because it was one of the cell lines in which the shRNA screen was performed. SUM159PT because it is a cell line that was previously tested in mouse xenografts. Moreover, SUM159PT cells are cultured in 5% FBS (half of what usually is used for culturing other cell lines), making it a suitable cell line for tumor engraftment once CDK2 is genetically inactivated. Finally, SK-BR-7 in order to have a better representation of TNBC heterogeneity and thus giving us a more robust model in case of validation.

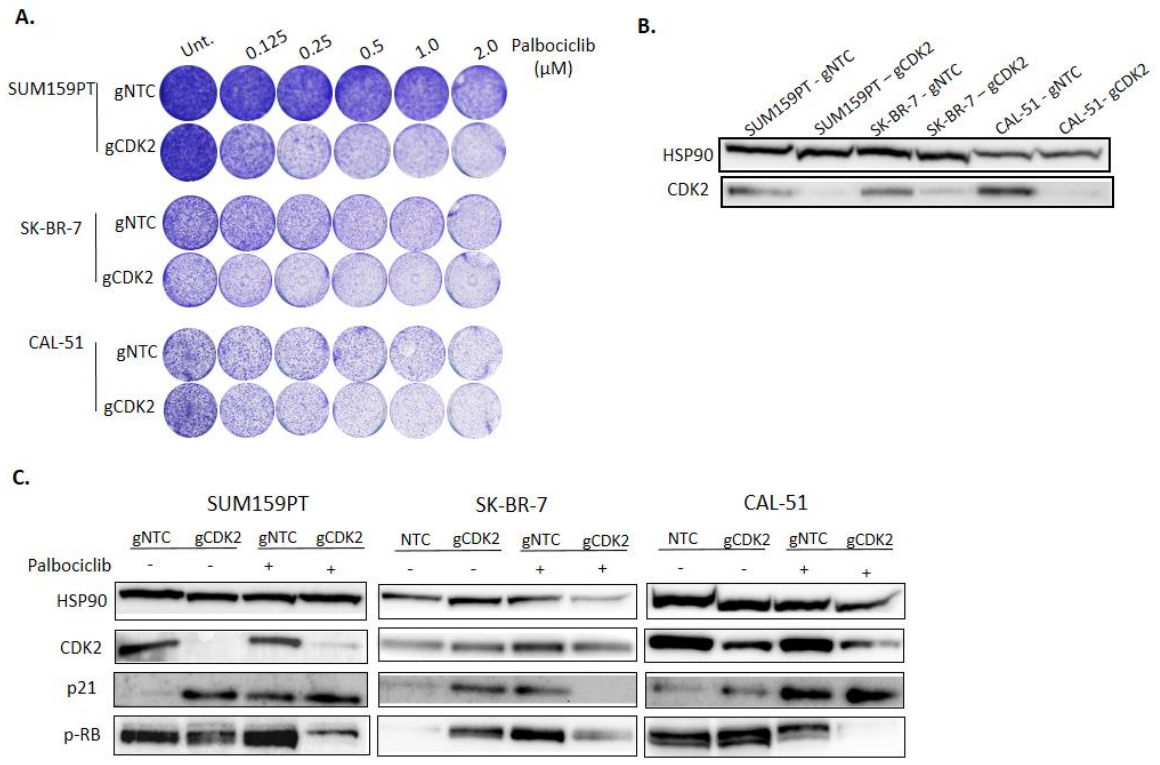

Figure 12: Palbociclib treatment in combination with CRISPR editing of CDK2 induces senescence in TNBC.
(A.) Colony formation assays of three TNBC cell lines (SUM159PT, SK-BR-7 and CAL-51) seeded after complete puromycin selection. Cells were treated with Palbociclib in increasing concentrations for 7 days (drug refreshed every 2-3 days). **(B.)** CDK2 protein levels in all the cell lines 10 days after puromycin selection in polyclonal populations. (HSP90 as a loading control) **(C.)** Biochemical profile of CDK2 knockdown cells treated with Palbociclib (2 µM). SUM159PT cells were harvested 19 days after puromycin selection and with eight days of Palbociclib treatment; CAL-51, SK-BR-7 cells were harvested 26 days after puromycin selection and with 14 days of Palbociclib treatment. (Unt.= untreated control, NTC = non-targeting control).

CDK2^{KO} and NTC cells were treated with Palbociclib (SUM159PT for eight days; CAL-51, SK-BR-7 for 14 days) and blotted for senescence markers (Figure 12C). SUM159PT showed the most pronounced reduction in proliferation upon Palbociclib treatment in combination with CDK2^{KO}. In the other two cell lines, the differences in proliferation were not as pronounced as in SUM159PT. However, the cellular features of senescence previously described were observed after microscopic inspection. This observation is consistent with the fact that SUM159PT was the TNBC line with the most reduced CDK2 levels.

When using CRISPR editing the goal is to get a complete knockout of the targeted protein, but as discussed in the previous section, often cells that are genetically edited have a proliferative disadvantage and overtime are less represented in the bulk population. This is one of the problems in doing these experiments in polyclonal cell population setting, as it was this case. It is notable an enrichment overtime for CDK2 protein level in SK-BR-7 and CAL-51 (Figure 12B-C).

This correlation between the efficiency of CDK2^{KO} levels and a visible phenotype in the CFs upon Palbociclib was also observed in SW1463, a colon cancer cell line, and to a less extent in two lung cancer cell lines (H1944 and H2122) where the phenotype is only observed at higher Palbociclib concentrations (Figure 13).

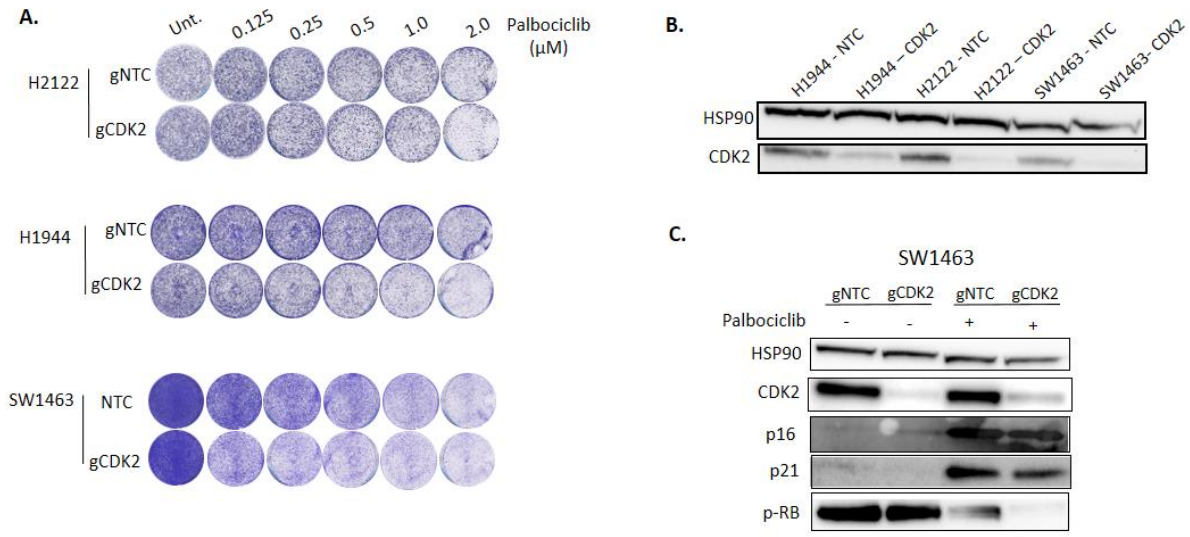

Figure 13: Palbociclib treatment in combination with CRISPR editing of CDK2 induces senescence in other cancer types (lung and colon).

(A.) Colony formation assays of one CRC cell line (SW1463) and two lung cancer cell lines (H2122 and H1944) seeded after complete puromycin selection. Cells were treated with Palbociclib in increasing concentrations for 7 days (drug refreshed every 2-3 days). **(B.)** CDK2 protein levels in all the cell lines 10 days after puromycin selection in polyclonal populations. (HSP90 as a loading control). **(C.)** Biochemical profile of SW1463 CDK2 knockdown cells treated with Palbociclib (2 µM). Cells were harvested 26 days after puromycin selection and with 14 days of Palbociclib treatment. (Unt.= untreated control, NTC = non-targeting control).

Common to the majority of these cell lines is the up-regulation of cyclin-dependent kinase inhibitor 1A (p21) (except in SK-BR-7) and the highly-decreased RB phosphorylation that occurs only in the combination of $CDK2^{KO}$ with Palbociclib treatment. With these expected senescence biomarkers and the observed reduction in proliferation on the CFs assays, we can say that Palbociclib treatment upon $CDK2^{KO}$ impairs cell proliferation via induction of a senescence state. These findings are not only applicable to TNBC but extended to other cancer types as well, as shown here with (one) colorectal and (two) lung cancer cell lines.

Put together, these results suggest that the degree of CDK2 reduction might be correlated with the effectiveness of the induction and maintenance of a senescence phenotype.

In the meantime, we had started in vivo pilot experiments to test the engraftment of CRISPR/Cas9 $CDK2^{KO}$ edited cells. Surprisingly, mice injected with CAL-51 and A549 (lung cancer) $CDK2^{KO}$ cells showed a strong tumor growth impairment when compared to the growth of control tumors (CDK2 wild-type) (data not shown). These were not the circumstances in which we wanted to test our model. To fully recapitulate our in vitro phenotype, both arms —$CDK2^{KO}$ and CDK2 wild type-tumors— should have comparable tumor volume before Palbociclib administration.

The observation that $CDK2^{KO}$ alone already provokes a strong tumor growth impairment made us consider other approaches for the in vivo experiment: to test an inducible shRNA-CDK2 system and the use of a small molecule inhibitor of CDK2.

2.2.2. Using doxycycline-inducible shRNA-CDK2 and Palbociclib treatment to induce senescence in TNBC

The use of an inducible shRNA system that we could activate at any given moment was an option with which we could tackle the problem of using cells edited with CRISPR/Cas9 prior engraftment into the mice, as described in the previous section.

Eight TNBC cell lines were transfected with doxycycline-inducible shRNAs targeting CDK2 (three different shRNA-CDK2, sh40/76/77, were used to minimize off-target effects and to give more confidence to the study). As mentioned previously, CAL-51, CAL-120 and HCC1806 were used because the screen was performed on these cell lines and so the induction of senescence should, in theory, also validate using a shRNA inducible system.

Firstly, we started by assessing the levels of $CDK2^{KD}$ in that panel of eight TNBC lines (Figure 14A) and performed CFs assays to evaluate cell proliferation when this $CDK2^{KD}$ inducible system was used in combination with Palbociclib versus $CDK2^{KD}$ alone (Figure 14B).

$CDK2^{KD}$ levels upon doxycycline treatment were not equally reduced across the cell panel (Figure 14A). This was also verified observing under the microscope the level of expression of a red fluorescent protein (RFP) that functions as a reporter for shRNA induction in the used constructs. In other words, doxycycline activates both the shRNA system and the expression of RFP, which can be used to visually confirm the level of shRNA-CDK2 induction. We observed that not all cells were RPF-positive upon doxycycline, and in some cell lines, such as SK-BR-7 and CAL-120, we observed RFP signal in only 50% of the cells. This issue could be overcome with cell sorting, to isolate monoclonal populations. However, due to time limitations, these experiments were not performed and thus will not be discussed in this thesis.

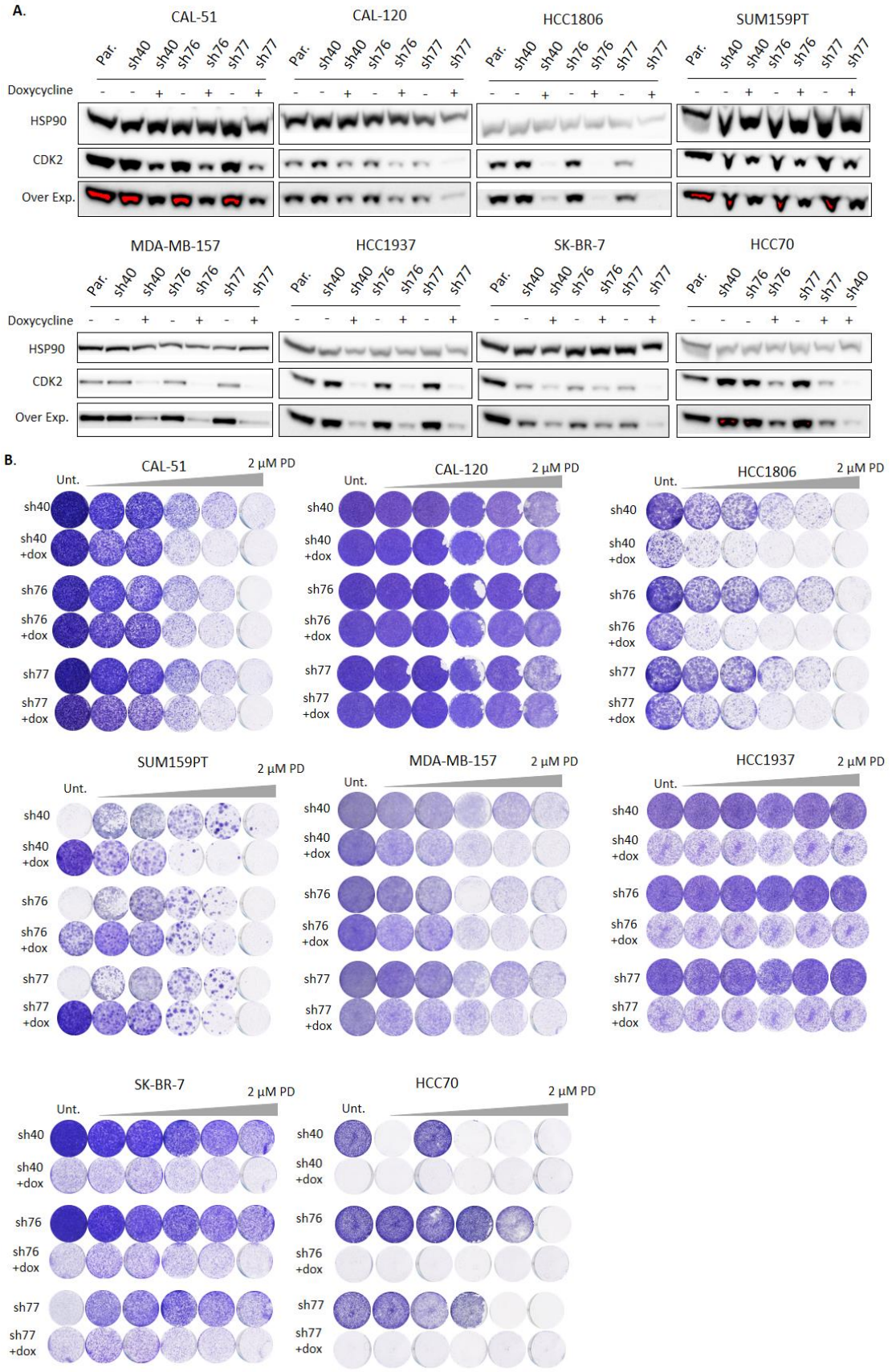

Figure 14: CDK2 knockdown levels and its proliferation effect in a panel of TNBC cell lines.

A panel of TNBC lines was transfected with a doxycycline-inducible shRNA-CDK2 system (three different small hairpins with different sequences were used: shCDK2-40, shCDK2-76, shCDK2-77). **(A.)** Cells were seeded and protein lysates prepared at the same time for western blot assessment of CDK2 levels. The shCDK2 inducible system does not have the same level of efficiency across the panel of cell lines. In the 'Over Exp.' image is shown the CDK2 levels with a longer exposure time to confirm that these are knockdowns and not knockouts. **(B.)** Cells were seeded at low densities in 6-well plate setting and treated with increasing concentration of Palbociclib up to 2 μM (refreshed every 3 days) for a period of 13-15 days, after which all the plates were fixed, stained and scanned. The control wells from SUM159PT are missing because cells detached due to overgrowth. (PD = Palbociclib; Unt. = untreated; dox = doxycycline).

In only half of the cell lines (HCC1806, MDA-MB-157, HCC1937, HCC70) this system conferred a substantial CDK2 reduction on the protein level. In general, we can say that all the cell lines exhibited, within a broad range, sensitivity to Palbociclib treatment upon $CDK2^{KD}$. CAL-120 was an exception since it did not show any visible impairment of proliferation, but it was one of the cell lines where CDK2 protein levels were the least reduced. In truth, to some extent, in the cases where $CDK2^{KD}$ was more efficient is visible a stronger proliferation reduction. For some cell lines (HCC1937, SK-BR-7 and HCC70), $CDK2^{KD}$ alone seemed to have a significant impairment in proliferation regardless of Palbociclib treatment.

Another interesting observation was that, in virtually all cell lines, with a microscope observation it was possible to see cells with two totally distinct morphologies growing in clusters within the same wells. However, we could not find a strong link between these different morphologies with the RPF signal status. We would expect that cells with non-senescence morphology would not show RPF signal, and the other way around for cells with a morphology of senescence, but it was not the case. The RPF signal was "randomly" distributed across these two morphological distinct phenotypes.

Based on these experiments we concluded that $CDK2^{KD}$ confers more sensitivity to Palbociclib treatment, leading cells to a growth impairment that might be the result of entering into a senescence state. Moreover, there is a category of TNBC lines that are sensitive to $CDK2^{KD}$. The results also suggest that the absence of phenotype in proliferation might have to do with the insufficient $CDK2^{KD}$ similarly to what we previously observed using the CRISPR approach.

From the eight cell lines used in the last panel, we selected five of them for further senescence characterization: the original cell lines where the screen was performed, CAL-51, CAL-120 and HCC1806, alongside with SUM159PT and MDA-MB-157. In Figure 15 are depicted the results of the experiments where we assessed for senescence biochemical biomarkers and looked at SA-β-galactosidase activity.

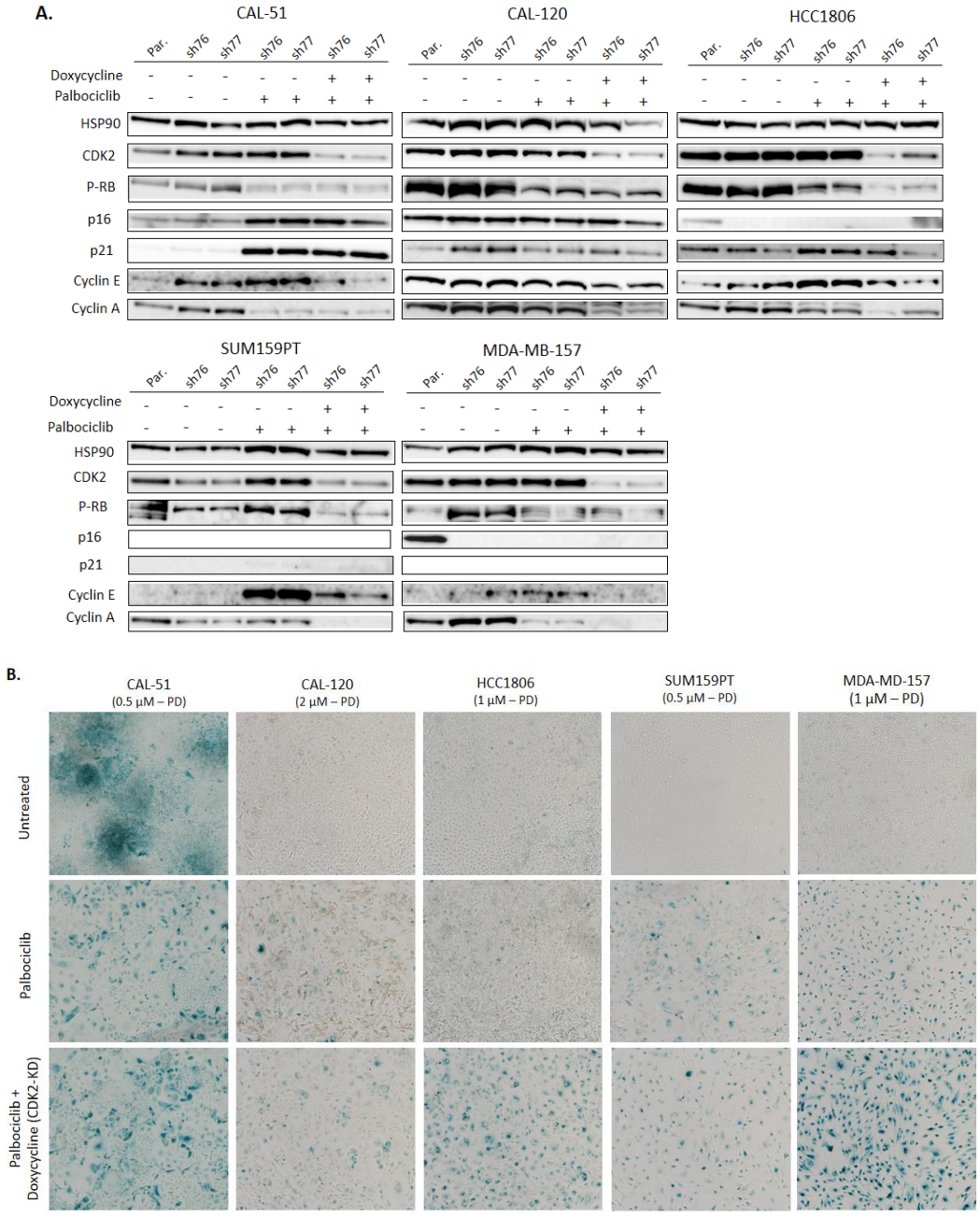

Figure 15: Senescence induction in a panel of TNBC.
A panel of five TNCB cell lines transduced with shRNA-CDK2 (sh76 and sh77) were treated with doxycycline (to induce CDK2 knockdown) in combination with Palbociclib or with Palbociclib alone as WT control, for a period of 10 days (Palbociclib was refreshed every 3 days and used at different concentrations

indicated below each cell line in (B.); new doxycycline was added daily to the medium to keep a constant expression of the shRNA inducible system). (A.) Cells were seeded for Western blot assessment of senescence biomarkers (p21, p16, p-RB), and for the levels of CDK2 and two of its binding partners (cyclin E and cyclin A). (B.) Cells were seeded for CF assay at low densities in 6-well plate setting and treated with Palbociclib (refresh every 3 days) for 6 days after which the shCDK2-76 ABT-263 untreated wells of all the plates were fixed and stained for the senescence biomarker SA-β-galactosidase.

The Western blots show CDK2 levels generally reduced after doxycycline treatment and variable across the panel (Figure 15A). The phosphorylation levels of RB (p-RB) were downregulated in Palbociclib and Palbociclib + $CDK2^{KD}$ conditions, but to a greater extent in the former. Unfortunately, the blots for p16 and p21 did not technically work (proteins could not be detected, and thus are not included). Together, CDKs and Cyclins carefully regulate the cell cycle. CDK2 can bind to multiple cyclins (cyclin types A, B, D and E). We blotted for Cyclin E and A because those two are the preferred pairing cyclins of CDK2. Cyclin E is most active during G1 to S phase transition, while Cyclin A has its peak of activity during S and G2 phase. The results show that upon $CDK2^{KD}$ there is an impairment on the expression of both Cyclins, with Cyclin A being more affected than Cyclin E. Indeed, Cyclin A is equally downregulated with Palbociclib alone and Palbociclib in combination with doxycycline in CAL-51 and MDA-MB-157. Previous studies showed that Palbociclib alone can impair the levels of expression of Cyclin A and induce a quiescent state in primary liver cancer models.[149]

As expected, in general, the untreated cells — no Palbociclib nor doxycycline— did not stain for SA-β-galactosidase activity (Figure 15B) (in CAL-51 we observed background staining due to high cellular confluency). As for the cells treated with Palbociclib alone, there is some staining (mostly CAL-51 and MDA-MB-157) and the levels of p-RB are equally diminished in both conditions indicating that Palbociclib alone already induced some degree of senescence in these cell lines. HCC1806 and MDA-MB-157 were the cell lines with the most pronounced $CDK2^{KD}$, which correlates with a more pronounced SA-β-gal activity staining.

To further strengthen the senescence characterization of these cells, we could also have looked to the senescence-associated secretory phenotype (SASP)[153] which can be detected by mRNA analysis. Nevertheless, with the presented senescence biomarkers we have shown that $CDK2^{KD}$ in combination with Palbociclib treatment induces senescence in these cell lines.

2.2.3. Killing senescence cells with ABT-263 - genetic validation

Naturally, the only good cancer cell is a dead one. At this point the plan was to apply the second punch by treating senescent cells with ABT-263, a senolytic compound that has already demonstrated efficiency in eliminating senescent cells.[154,155]

Using the same panel of TNBC cell lines we tested the effect this drug at two different concentrations.

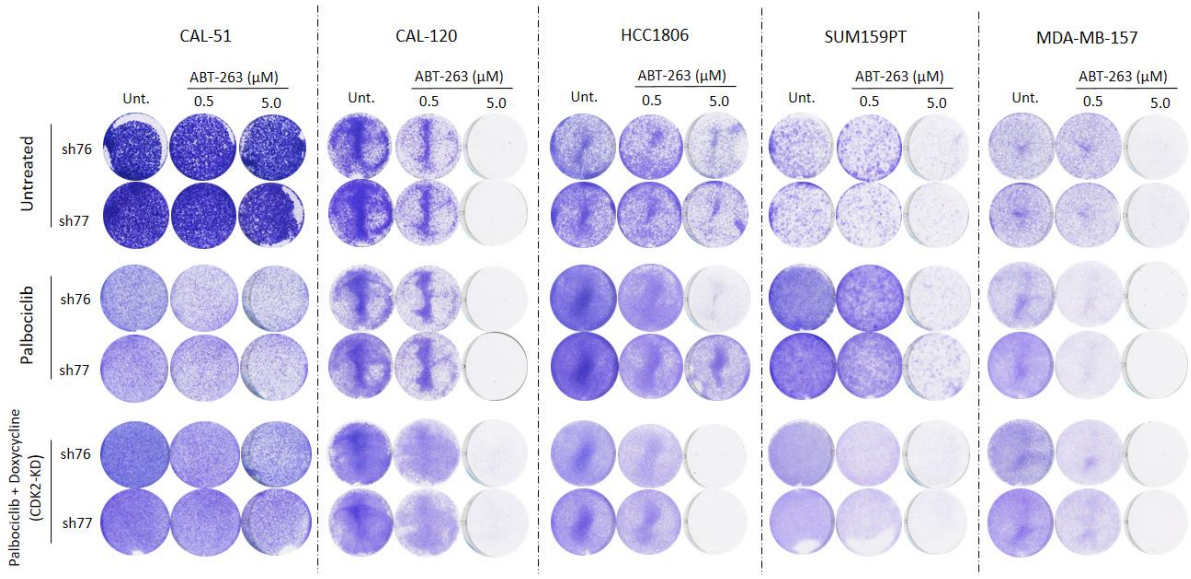

Figure 16: ABT-263 treatment of shCDK2 + Palbociclib senescence cells.
A panel of five shRNA-CDK2 (sh76 and sh77) transduced TNBC cell lines were treated for a period of 10 days with doxycycline (to induce CDK2 knockdown) in combination with Palbociclib or with Palbociclib alone as WT control (Palbociclib was refreshed every 3 days and used at different concentrations indicated in Figure 15; new doxycycline was added daily to the medium to keep a constant expression of the shRNA inducible system), before being seeded for CFs at low densities in 6-well plate setting and treated with Palbociclib and ABT-263 (refresh every 2 days) for a period of 6 days after they were fixed, stained with 0.1% Crystal Violet solution and scanned. (Unt. = untreated).

It is important, first of all, to alert the reader to the fact that in these CFs assays each untreated (no doxycycline) well is the control of the subsequent wells treated with ABT-263 (Figure 16). It would be a mistake to proportionally compare the different condition arms because in each one the original seeding cell numbers were different. The rationale behind it has to do with the senescence state induced by the combination of Palbociclib and $CDK2^{KD}$, making logical to seed cells at higher confluency at the beginning of the experiment. Cells treated with Palbociclib alone or untreated (still proliferating) were seeded

in fewer numbers so that the control wells would reach full confluency at the end of the experiment time.

The cell lines in which ABT-263 treatment killed preferentially senescent cells were HCC1806 and SUM159PT. Although, in HCC1806 cells treated with Palbociclib alone, the two different shRNAs used (sh76 and sh77) show a different phenotype when treated with 5 µM of ABT-263. With respect to CAL-120 and MDA-MB-157, this experiment did not reveal big differences between the effect of ABT-263 on cells treated with Palbociclib alone and cells treated in combination with $CDK2^{KD}$.

Also here these experiments should be repeated after an optimization of the ABT-263 doses. Perhaps with doses lower than 5 µM we can find a better therapeutic window for ABT-263. Most cell lines are already sensitive to 5 µM ABT-263 in the baseline (Figure 16). We used this dose of ABT-263 based on previous experiments with CAL-51 cells. We assumed that 5 µM was a safe concentration to kill only senescent (and not proliferating) cells. A titration of ABT-263 should be performed to assess the sensitivity of the TNBC cell line panel to this senolytic agent.

2.2.4. Killing pharmacological-induced senescence cells with ABT-263

Obviously at the end of the day what really matters is to translate and recapitulate the genetic models to a pharmacological setting. Thus, using the same cell lines, we tested two small molecule inhibitors targeting CDK2: CVT-313 and Indisulam.[156] The two drugs act by two different molecular mechanisms. CVT-313 directly inhibits CDK2 phosphorylation (which Western blots failed to show because of the poor quality of the p-CDK2 antibodies) inhibiting cell proliferation at the G1 to S phase transition.[157] On its turn, Indisulam also arrests cells in G1 phase by suppressing the binding of the Cyclin E/CDK2 complex, resulting in the decrease of CDK2 levels.[158]

In Figure 17A is shown the Western blots of the cells for senescence markers. The CDK2 levels are in general decreased, but cell lines respond differently to the two inhibitors. For instance, Indisulam is more effective in reducing CDK2 levels in CAL-51 and CAL-120 whereas CVT-313 is more efficient in SUM159PT and MDA-MB-157. As for the case of

HCC1806, at a concentration of 2 μM, Indisulam proved to be lethal whereas with 5 μM CVT-313 there was no reduction of CDK2 levels at all. Once that 2 μM of Indisulam in combination with 1 μM of Palbociclib efficiently killed all cells without the application of the second punch with ABT-263 treatment, in the next experiments a lower dose of Indisulam could be tested.

In fact, HCC1806 was the only cell line in which this small molecule inhibitor (CVT-313) does not seem to have any effect in promoting senescence in combination with Palbociclib. These cells were still proliferating, and did not show any reduction of p-RB levels, did not stain for SA-β-galactosidase activity (Figure 17B) neither displayed a typical morphology of senescent cells. This absence of phenotype could be a drug dose-related issue and thus it would be worthy to repeat the experiment with a higher dose of CVT-313, but in general, using doses even higher than this can compromise the use of a drug in vivo. The toxicity conferred by this inhibitor has been related to off-target effects[159,160] thus, the development of better CDK2 inhibitors is still needed.

When treated with Palbociclib alone all cell lines, with the exception of CAL-51, did not stain for SA-β-galactosidase activity. In SUM159PT cells the observed staining was due to the background caused by the over confluency; in addition the morphology of those cells also did not display a senescence phenotype. On the other hand, when cells were treated with the combination of Palbociclib + CDK2 inhibitors the SA-β-galactosidase activity staining was positive and the typical senescence morphology was also visible. Nevertheless, it is also important to mention that for CAL-51 Palbociclib treatment alone also conferred similar morphology and SA-β-galactosidase activity, as already seen in the previous section.

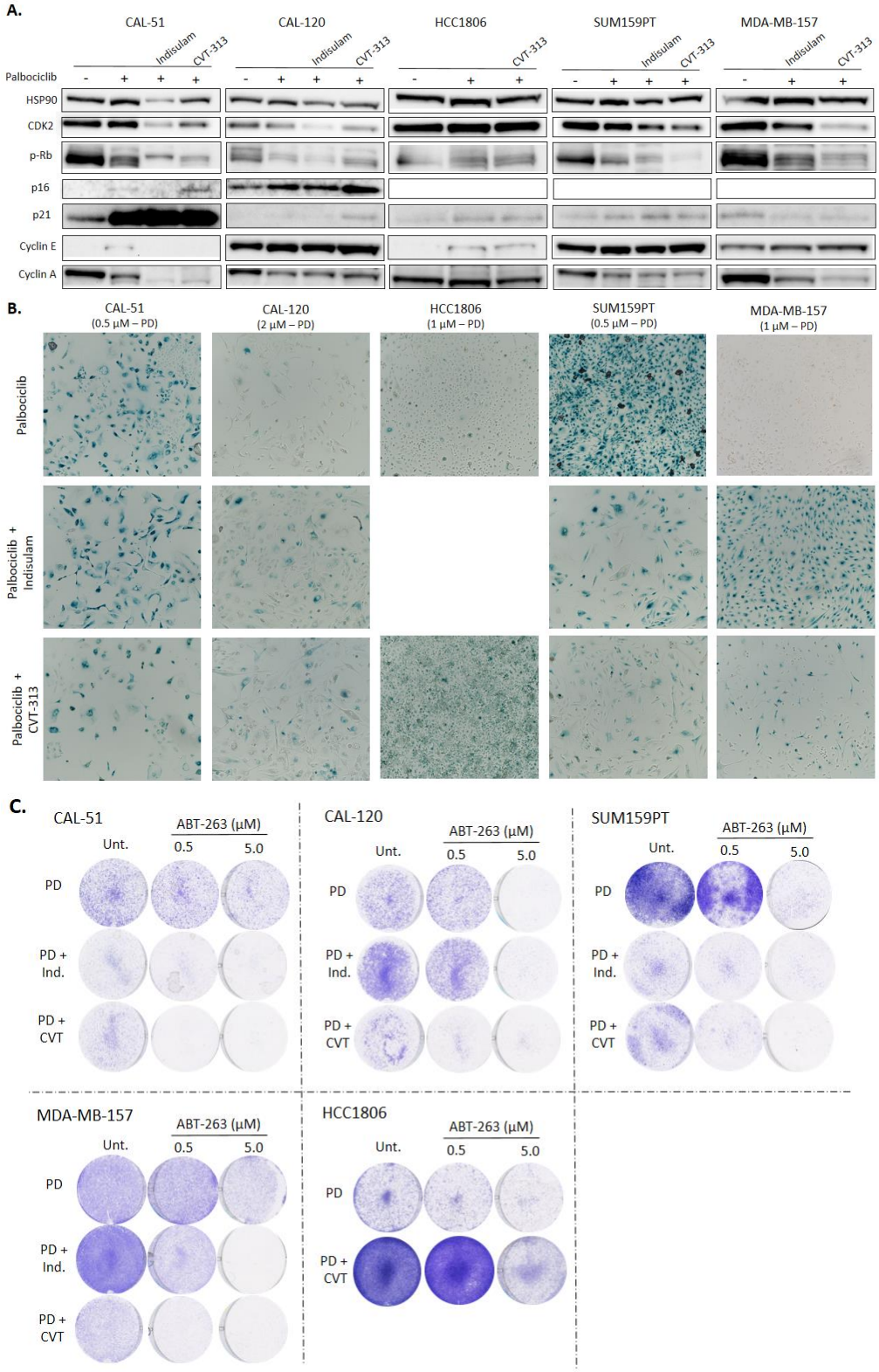

Figure 17: ABT-263 treatment upon CDK2/4/6 pharmacological inhibition.
A panel of 5 TNBC cell lines was treated with two CDK2 inhibitors (Indisulam (2 µM) and CVT-313 (5 µM)) and with Palbociclib for a period of 10 days. Cells were treated with different Palbociclib concentrations (note that these concentrations are different from the ones used in the genetic approach). **(A.)** cells were seeded for biochemistry analysis of senescence biomarkers (p21, p16, p-RB), CDK2 levels and two of its binding partners (cyclin E and cyclin A). HSP90 as a loading control. Simultaneously, cells were seeded at different low

densities in 6-well plate setting and continued to be treated with Palbociclib and the CDK2 inhibitors, and with ABT-263 (drugs refreshed every 2 days) for 8 days after which the wells without ABT-263 treatment (control wells) were fixed and stained for the senescence biomarker SA-β-galactosidase activity **(B)**, and later stained with 0.1% Crystal Violet solution and scanned **(C)**. (Unt. = untreated; PD = Palbociclib).

Biochemically, the senescence markers revealed a similar phenotype with the one observed with the genetic approach described in the previous section. Excluding HCC1806, RB phosphorylation was downregulated both when treated with Palbociclib alone and in combination with the CDK2 inhibitors but to a greater extent in the combination. The same similarities were observed with the levels of Cyclins. In this pharmacological approach, the levels of Cyclin A protein expression were impaired but not the levels of Cyclin E.

All together we can conclude that it is possible to induce senescence with CDK2/4/6 inhibitors in TNBC.

MDA-MB-157 cell line seemed to be the one in which our senescence model better validates, despite the lack of the detection for p21 and p16, p-RB is proportionally reduced with the levels of CDK2 inhibition when using the two different inhibitors. These results were in accordance with the CFs where we could see that when treated with CVT-313 the ABT-263 effect was stronger compared to when cells were treated with Indisulam (Figure 17C). Important to recall that, as in the genetic approach, here each untreated well at different conditions works as a control to the other wells. The same proves to be true for SUM159PT, the CFs show a greater sensitivity to ABT-263 when cells are in a senescence state induced by the combination of CDK2/4/6 inhibitors. In both cases (SUM159PT and MDA-MB-157) there is a parallel between CDK2 reduction levels, after the drugs (CDK2 inhibitors and Palbociclib), and the senolytic effect of ABT-263. That is, a greater impairment in CDK2 levels correlates with a stronger effect of ABT-263.

In general, the induction of senescence state through our proposed model seems to work, although some optimization is still needed, namely proliferation assays to determine which Palbociclib and CDK2 inhibitors concentrations demonstrate better synergy in inducing senescence for each cell line, perform titrations matrices with CellTiter-Blue to quantify cell viability upon treatment with ABT-263, as well as to take more controls into account, for instance, look into the effect of the CDK2 inhibitors alone and in combination with ABT-263 treatment.

2.2.5. General discussion and conclusions

TNBC therapy needs improvement. Researchers all over the world are working on finding new cancer vulnerabilities that can be exploited to improve the current therapies.

Through a genetic screen trying to find synthetic lethal interactions with the CDK4/6 inhibitor Palbociclib in TNBC cell lines, we ended up uncovering a model of induction of senescence. In this model a CDK2 knockdown in combination with Palbociclib treatment induces senescence.

The role of senescence cells has both been linked with tumor suppression as well as a promoter of tumorigenesis, but generally it is fair to say that cellular senescence is an anti-cancer mechanism that is partially subverted by cancer. Interestingly, senescent tumor cells exist spontaneously within the heterogeneous population in a tumor and it is now known that tumor cells undergo senescence in response to chemo and radiotherapy.[161] This is relevant because subpopulations of spontaneous senescent cells and therapy-induced senescence cells might lead to a compensatory resistance to apoptosis via alternative signaling pathways, and although in a senescent state, they remain still to be cancer cells with the ability to promote tumorigenesis.

We validated the induction of senescence both with a genetic and a pharmacological approach. While the main focus of this thesis was TNBC, we also have shown that the model can be extended to other cancer types as well. This strategy could then be used in combination with a second treatment using a senolytic agent able to specifically eliminate senescent cells.

It was challenging to compare the results of the two different approaches experiments since they contradict each other in some cases. Looking at CAL-51, for example, we did not expect such notorious difference in the CFs phenotype where, with the genetic approach it seems that ABT-263 does not have any effect, whereas with the small molecules CDK2 inhibitors we can appreciate the opposite. Making it even more confusing is the fact that the biochemistry and SA-β-galactosidase staining are comparable in both approaches.

The presence or absence of the senescence phenotype cannot be explained by the genetic background of TNBCs. One of those genetic traits that we thought it could give us some explanation to the different phenotype responses was the status of the p53 protein.

Given the role of p53 in senescence, it is very intuitive to think that the CDK2/4/6 inhibition senescence phenotype is p53 dependent. However, because of the several p53 mutant cell lines (Table 3, Materials and Methods) that also display senescence features, this doesn't seem to be a p53 dependent event. As the mechanistic explanation of the phenotype was not in the scope of this thesis, this matter is not going to be discussed further.

Despite some confounding results concerning the effectiveness of ABT-263 in specifically kill senescence cells, we showed that indeed ABT-263 reveals a certain degree of selectivity towards senescent cells. In addition, the fact that studies have been reporting the existence of therapy-induced senescence cells in patient tumors only reinforces further the need for the development of novel senolytic drugs that can eventually be used to target senescent cancer cells that have acquired resistance to chemo and radiotherapy.

The CDK2 inhibitor Indisulam was already been used in the clinic and have shown tolerable toxicity,[158,162] which can be an advantage over others CDK2 inhibitors. However, there was no clear distinction between which of the two CDK2 inhibitors used in this study —Indisulam or CVT-313— is the most efficient in promoting senescence in combination with Palbociclib. Given the lack of more efficient CDK2 inhibitors and their different and complementary mechanisms of action, future experiments could be done to assess if the use of these two CDK2 inhibitors together would prove to be more efficient in inhibiting CDK2. If so, a triple combination with Palbociclib could at the end be even more beneficial.

2.3. Summary and future directions

In the first part of the project, we showed that all the four candidate genes extracted from a functional genetic screen aiming to find synthetic lethal interactions with hypoxia proved to not validate. With this work we were hoping to find lethal interactions with hypoxia that could be then pharmacologically targetable. The goal was to improve the already used AA therapies that so far have not shown clinical relevance.

The failure, up until now, in identifying synthetic lethal interaction with hypoxia despite the many attempts, suggests that hypoxia regulation and adaptation are processes that we still do not fully understand. Regardless, more work should be done to find the weaknesses that hypoxia impose over cancer cells. Perform more in vitro functional genetic screens with different libraries and with different cancer types would be interesting experiments to do, as well as in vivo screens could reveal new insights in finding essential genes with antiangiogenic therapy.

The second part of this project showed more promising results. We were able to validate the finding that CDK2 knockdown/knockout in combination with Palbociclib treatment induces senescence in TNBC and that these senescence induced cells can be targeted with senolytic agents to be specifically eliminated. Although we only tested ABT-263 as senolytic there are more drugs that can be tested in the scope of this one-two punch strategy. As it was not under the objectives of this thesis, the mechanistic explanation behind this induced senescent phenotype was not assessed with more detail. Nevertheless, this phenotype does not appear to be p53 dependent.

The next step would be to implement this strategy in an in vivo setting. Ideally, first, we would engraft CDK2 deficient tumors (and appropriate controls) in mice, treat with Palbociclib and evaluate the subsequent effects of ABT-263. If this first experiment shows positive results, then a similar protocol would be performed using CDK2 pharmacological inhibitors (CVT-313 and Indisulam) instead of the genetic approach.

3. Materials and Methods

3.1. Cell culture

A total of 13 cell lines were used during this project. HEK-293T cell line was used for lentivirus production. In the validation of the hypoxia screen only SUM159PT was used; and for the senescence model validation, to better represent the heterogeneity in TNBC, a panel of nine TNBC cell lines was used: SUM159PT, SUM149PT, CAL-51, CAL-120, MDA-MB-157, SK-BR-7, HCC1806, HCC70 and HCC1937. As well as SW1463, a colorectal cancer cell line, and H2122 and H1944, two lung cancer cell lines. Cells were cultured at 37 ºC at 5% CO_2. At normoxia conditions the oxygen percentage was 21% and in hypoxia around 1%.

Table 2: List of the cell lines used, culture medium composition and cell seeding numbers for colony formations assay.

Cell line	Composition of the culture medium	CF 6-well setting seeding number
CAL-51	DMEM 20% FBS; 1% Pen./Strep.	10.000
CAL-120	DMEM 10% FBS; 1% Pen./Strep.	30.000
SUM159PT	DMEM-F12 5% FBS; 1% Pen./Strep; 5µg/ml Insulin; 1µg/ml 53,5 µl Hydrocortisone	10.000
SUM149PT	DMEM-F12 5% FBS; 1% Pen./Strep; 5µg/ml Insulin; 1µg/ml Hydrocortisone	20.000
SK-BR-7	DMEM-F12 10% FBS; 1% Pen./Strep.	30.000
MDA-MB-157	RPMI 1640 10% FBS; 1% Pen./Strep.	20.000
HCC1806	RPMI 1640 10% FBS; 1% Pen./Strep.	10.000
HCC70	RPMI 1640 10% FBS; 1% Pen./Strep.	50.000

HCC1937	RPMI 1640 10% FBS; 1% Pen./Strep.	50.000
SW1463	RPMI 1640 10% FBS; 1% Pen./Strep.	30.000
HEK293T	DMEM 10% FBS; 1% Pen./Strep.	
H2122	DMEM 10% FBS; 1% Pen./Strep.	30.000
H1944	DMEM 10% FBS; 1% Pen./Strep.	30.000

Fetal Bovine Serum (FBS) (Thermo Scientific); Penicillin/Streptomycin antibiotics (Gibco); Insulin (Sigma Aldrich); Hydrocortisone (ref); DMEM, DMEM-F12 and RPMI 1640 culture mediums (Gibco).

Table 3: List of cell line mutations.

Cell line	p53	p21	p16	kRas
CAL-51	WT	WT	WT	WT
CAL-120	Mut. (homozygous)	WT	WT	WT
SUM159PT	Mut.	WT	Mut. (homozygous)	WT
SUM149PT	Mut.	WT	null	WT
SK-BR-7	WT	WT	WT	Mut. (heterozygous)
MDA-MB-157	Mut. (heterozygous)	WT	WT	WT
HCC1806	Mut. (heterozygous)	WT	WT	WT
HCC1187	Mut. (heterozygous)	WT	WT	WT
HCC1395	Mut. (homozygous)	WT	WT	WT
HCC1937	Mut. (homozygous)	WT	WT	WT
HCC70	Mut. (homozygous)	WT	WT	WT
SW1463 (CRC)	Mut. (homozygous)	WT	WT	Mut. (homozygous)
H1944 (Lung)	WT	WT	WT	Mut. (heterozygous)
H2122 (Lung)	Mut. (heterozygous)	WT	WT	Mut. (homozygous)

3.2. Colony formation assays

Culture medium and drugs were refreshed every 2-3 days. When the control wells, containing cells not subjected to treatment, were confluent the experiment ended and cells were fixed with a 2% formaldehyde in phosphate buffered saline (PBS) for 1 hour followed by a staining step using a 0.1% crystal violet solution (dissolved in water) for about 20 minutes; then this staining solution was removed and the excess was gently rinsed with water, finally the plates were left to dry overnight and scanned the next day. Table 4 shows the list of drugs used during this project.

Table 4: List of drugs used during the project and their targets.

Drug	Target	Stock concentration in DMSO (mM)
THZ1	CDK7	5
Palbociclib	CDK4/6	5
Indisulam	Cyclin E/CDK2 complex	10
CVT-313	CDK2	10
ABT-263	Bcl-XL, Bcl-2, Bcl-w	10

3.3. Cell growth assay

SUM159PT cells were seeded in 6-well setting (10.000 cells per well) under hypoxia and normoxia conditions. Cells were washed with PBS, collected and counted at different time points (triplicates counted twice) and, at the same time, fixed and stained.

3.4. Western Blot

Cells were seeded in 6-well plate wells prior to the day of harvest with high-density numbers (200.000 – 300.000 cells). First the medium was removed and cells washed with cold PBS (plates placed on ice), RIPA buffer (25 mM Tris – HCl pH 7.6, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS) containing phosphatases inhibitors (phosphatase inhibitors cocktail II and III (Sigma)) and complete protease inhibitor (Roche) was then added to the wells which were then scratched with a cell lifter; samples were incubated on

ice for 30 minutes, vortexed every 10 minutes, and then centrifuged at 14000 rpm at 4º C for 10 minutes. The concentration of protein was quantified by Bicinchoninic Acid (BCA) assay (Pierce BCA, Thermo Scientific) and samples were normalized to the same quantity of protein and added with DTT redox agent for the denaturation step that followed by 5 minutes of heating at 95º C; after denatured, samples were loaded into precast 4-12% polyacrylamide gel (Invitrogen) where they were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDA-PAGE) for around 1 hour at 200 volts after which they were then transferred to a polyvinylidene fluoride (PVDF) membrane (at 330 or 660 mA for 60-90 minutes). Next, the membranes were blocked in a 5% milk blocking solution in PBS-0.1% Tween-20 (PBS-T) for 60 minutes followed by the incubation with the primary antibody diluted 1:1000 in blocking buffer, with constant shaking, at 4º C overnight. Membranes were washed 3 times (10 minutes each) in PBS-T, followed by the incubation with the appropriate secondary antibody diluted 1:10,000 in blocking buffer at room temperature and constant shaking for 1 hour; again, membranes were washed with PBS-T 3 times for 10 minutes. Finally, a chemiluminescence substrate (Pierce ECL, Thermo Scientific) was added to the membranes (placed between plastic sheets) and were placed in a ChemiDoc machine (Bio-Rad) that detected the chemiluminescence signals emitted. Table 5 shows the list of antibodies used in this project.

Table 5: List of antibodies used for Western blot during this project.

Target protein	Isotype	Predicted Molecular weight (kDa)	Brand
Hypoxia-inducible factor 1-alpha inhibitor (FIH-1)	Rabbit	40	Novus
Runt-related transcription factor 1 (RUNX1)	Mouse	49	Abcam
Putative Polycomb group protein ASXL2	Rabbit	238	Bethyl
Histone-binding protein RBBP7	Rabbit	52	Bethyl
Cyclin-dependent kinase 2 (CDK2)	Mouse	34	Santa Cruz

Retinoblastoma-associated protein (RB) (phosphorylated)	Rabbit	110	Cell signaling
Cyclin-dependent kinase inhibitor 1 (p21)	Mouse	21	Santa Cruz
Cyclin-dependent kinase inhibitor 2A (p16)	Mouse	16	Santa Cruz
Bcl-2-like protein1 (Bcl-Xl)	Rabbit	30	Cell signaling
Cyclin E	Rabbit	53	Santa Cruz
Cyclin A	Rabbit	54	Santa Cruz
Heat shock protein HSP 90-alpha (HSP90)	Mouse	90	Santa Cruz

3.5. qRT-PCR

To assess RUNX1 knockdown levels, SUM159 cells were collected after transduction with lentiviral particles carrying shRNA sequences targeting RUNX1 (sequences on table Y) and two days of puromycin selection. RNA was isolated using Isolate II Genomic RNA kit (Bioline) according to manufacturer instructions. The yield of RNA extraction was measured using the Nanodrop ND1000 system. cDNA was obtained by reversed transcription using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) for qRT-PCR according to manufacturer's protocol, using 1 µg of RNA per sample. Gene expression was detected by SYBR Green assay using the AB 7500 Fast Real-time PCR system, following the manufacturer's instructions.

3.6. Cloning of the sgRNA's for the hypoxia screen validation

Table 6 shows all the sgRNA's oligonucleotide sequences that were cloned to target and edit each gene (sequences were chosen from the CRISPR epigenetic library and purchased from Invitrogen).

Table 6: List of the sgRNA sequences used to target the top four gene hits of the hypoxia screen.
RUNX1 gene had six sgRNA that come up as a hit followed by HIF1AN, ASXL2 and RBBP7 each one with three single guides.

screen rank	Ordered oligo	ID	gRNA sequence
1	CTTGTGGAAAGGACGAAACACCGGGTAGGTGGCGACTTGCGGTgt t t aagagct agaaat agcaag	RUNX1-11	GGTAGGTGGCGACTTGCGGT
2	CTTGTGGAAAGGACGAAACACCGGCCATCTGGAACATCCCCTAgt t t aagagct agaaat agcaag	RUNX1-6	GCCATCTGGAACATCCCCTA
3	CTTGTGGAAAGGACGAAACACCGCACTTACTTCGAGGTTCTCGgt t t aagagct agaaat agcaag	RUNX1-2	CACTTACTTCGAGGTTCTCG
4	CTTGTGGAAAGGACGAAACACCGCACTTCGACCGACAAACCTGgt t t aagagct agaaat agcaag	RUNX1-3	CACTTCGACCGACAAACCTG
5	CTTGTGGAAAGGACGAAACACCGGCACTTACTTCGAGGTTCTCgt t t aagagct agaaat agcaag	RUNX1-5	GCACTTACTTCGAGGTTCTC
6	CTTGTGGAAAGGACGAAACACCGTACCGCAGCCATGAAGAACCgt t t aagagct agaaat agcaag	RUNX1-14	TACCGCAGCCATGAAGAACC
7	CTTGTGGAAAGGACGAAACACCGGACGCGGAATGGGCCTAGTCgt t t aagagct agaaat agcaag	HIF1AN-6	GACGCGGAATGGGCCTAGTC
8	CTTGTGGAAAGGACGAAACACCGTTCATCCCAGGCGGGGCGGAgt t t aagagct agaaat agcaag	HIF1AN-10	TTCATCCCAGGCGGGGCCGA
9	CTTGTGGAAAGGACGAAACACCGGCAGTTATAGCTTCCCGACTgt t t aagagct agaaat agcaag	HIF1AN-7	GCAGTTATAGCTTCCCGACT
10	CTTGTGGAAAGGACGAAACACCGCGCACCTGTCGATCTACCTCgt t t aagagct agaaat agcaag	ASXL2-4	CGCACCTGTCGATCTACCTC
11	CTTGTGGAAAGGACGAAACACCGGCACCTGTCGATCTACCTCTgt t t aagagct agaaat agcaag	ASXL2-7	GCACCTGTCGATCTACCTCT
12	CTTGTGGAAAGGACGAAACACCGCTGCGATGGATGAAACGGCAgt t t aagagct agaaat agcaag	ASXL2-6	CTGCGATGGATGAAACGGCA
13	CTTGTGGAAAGGACGAAACACCGACCCTTGTCACTGTCACAATgt t t aagagct agaaat agcaag	RBBP7-2	ACCCTTGTCACTGTCACAAT
14	CTTGTGGAAAGGACGAAACACCGTCTGCGGCATGTAACGAGCAgt t t aagagct agaaat agcaag	RBBP7-16	TCTGCGGCATGTAACGAGCA
15	CTTGTGGAAAGGACGAAACACCGTGACCTCTTAATCTGAGATCgt t t aagagct agaaat agcaag	RBBP7-17	TGACCTCTTAATCTGAGATC

Five micrograms of vector pLentiCRISPR2.1 were linearized by digesting with 2 µL of BsmBI enzyme, in 5 µl of Buffer 3.1, and mQ water up to 50 µl; this digestion was incubated for 4 hours at 55 °C. The product of the digestion was run on a 1x TAE (0.6% agarose) gel with SYBR-safe DNA staining, and the DNA (digested) was recovered using a DNA purification kit (Isolate II Genomic DNA kit (Bioline). The oligonucleotide sequences were cloned into the vector in a reaction mixture with 50 ng of the digested DNA backbone, 1 µl from 100 µM from oligonucleotide solution, mQ water up to 10 µl and 10 µl of GIBSON assembly mix (New England BioLabs), and was allowed to incubate for 1 hour at 50 °C. The next step was to electroporate this vector into Endura competent bacteria (Lucigen). In an Eppendorf tube (work on ice) 43 µl of mQ water, 2 µl of the GIBSON assembled product and 5 µl of bacteria were mixed, transferred into a pre-chilled electroporation cuvette and electroporated around 1.8 kV. Immediately after 500 µl of LB (Lysogenic Broth) medium (20 g of LB Broth Lennox in 1L of deionized water followed by an autoclave step) was added and the electroporated bacteria suspension was transferred to an Eppendorf tube and incubated for 1 hour at 37 °C while shaking. Twenty-five microliters of this bacteria suspension were then plated on LB agar supplemented with carbenicillin plates (20 g of LB Broth Lennox, 15 g of Bacto-Agar in 1L of deionized, autoclaved and 1 mL of carbenicillin was added) and let to grow overnight at 37 ºC. Next day, 2-3 colonies from each construct were randomly picked and let to grow up in 5 mL LB + carbenicillin (1000x from 100 mg/mL stock) medium and let grow overnight. Next morning, DNA plasmids were isolated using Invitrogen PureLink Genomic DNA mini Kit, according to manufacturer's protocol,

and its concentration measured using a Nanodrop ND10000 system. To confirm if the oligos were successfully cloned, a solution mixture of 500 ng of plasmid DNA in mQ water up to 15 µl, 1 µl of hU6-343 primer (10 µM) and 4 µl of BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher) was prepared and submitted to PCR (5 minutes at 96 ºC followed by 30 cycles of 30 seconds at 96 ºC, 15 seconds at 50 ºC and 4 minutes at 60 ºC, ending with "infinite" time at 10 ºC); the PCR samples were sent to sequence and the insertions were confirmed using the SnapGene program.

3.7. Lentivirus production

E.coli bacteria (from the whole genome TRC library - stored at -80 °C in glycerol stocks), which contain a pLKO vector with an insert of shRNA targeting RUNX1 and an antibiotic resistance marker were picked. Bacteria were cultured overnight at 37 °C in 2X LB medium (20g of Bacto-Tryptone, 10g of yeast extract, 10g of NaCl in 1L of water) with carbenicillin. Plasmid DNA was isolated from the bacteria using a standard DNA isolation protocol (from Roche), after which the DNA concentration was measured (using a Nanodrop ND1000 system). Lentivirus particles were prepared by transfecting HEK293T cells with plasmid DNA from the shRNA library. To do that, we mixed 1 µg of plasmid DNA of our library, 1 µg of lentivirus packaging mix (pMD2.G envelope plasmid and pMDLg/pRRE packaging plasmid) and 6 µL of polyethylenimine (PEI) in 100 µL DMEM medium (quantities used in a 6-well plate setting). The mix was vortexed briefly, incubated for 15 minutes at room temperature and added to one million HEK293T cells seeded the day before in 2 mL of DMEM medium. Cells were incubated overnight; the next day the medium was refreshed; after 48 hours of incubation, the medium containing the lentivirus was filtered (by 20-µm filters), collected and stored at -80°C.

3.8. Using shRNA-CDK2 doxycycline-inducible system for the cellular senescence induction validation

Eight TNBC cell lines (CAL-51, CAL-120, SUM159PT, MDA-MB-157, HCC19937, HCC1806, HCC70 and SK-BR-7) were transduced with three different doxycycline-inducible shRNAs targeting CDK2 (sh40/76/77, from Dharmacon). Twenty-four hours after the transduction cells were selected with puromycin (1 μL/mL) for 48h. When the selection was finished, cells were trypsinized and reseeded for experiments. Doxycycline was added daily to the medium to keep a constant expression of the shRNA inducible system. Doxycycline activates both the shRNA system and the expression of an RFP reporter, which can be used to visually confirm the level of shRNA-CDK2 induction (a control without doxycycline addition was used for each cell line).

3.9. Fluorescence-activated cell sorting

Cells were sorted into single cells at the Flow Cytometry Facility of the Institute. A cell suspension of 10 million cells/ml were handed out to the FACS facility that performed the sorting.

Isolate single cells using FACS sorting presents some hurdles. Many of the isolated single cells end up not surviving since it is hard for cells to divide when they do not have the presence of neighboring cells. Another issue is that this technique oftentimes cannot isolate exactly just one cell, putting in the same well two or more cells, which is then visible at the microscope as the growth multiple colonies in different parts of the well, so when that happened those cells were discarded

3.10. β-Galactosidase staining

Cells were washed twice with PBS and then stained for β-Galactosidase using a Senescence β-Galactosidase Staining Kit (Sigma) according to manufacturer's protocol. Cells were left to stain overnight until a maximum period of 48h (the time required for the staining to be visible varies among cell lines).

4. References

1. What Is Cancer? - National Cancer Institute. Available at: https://www.cancer.gov/about-cancer/understanding/what-is-cancer. (Accessed: 12th January 2018)
2. What Is Cancer? Available at: https://www.cancer.org/cancer/cancer-basics/what-is-cancer.html. (Accessed: 26th October 2017)
3. Weinberg, R. A. *The Biology of Cancer*. (2013).
4. Ashkenazi, A. Death Receptors: Signaling and Modulation. *Science (80-.).* **281,** 1305–1308 (1998).
5. Evan, G. I. & Vousden, K. H. Proliferation, cell cycle and apoptosis in cancer. *Nature* **411,** 342–348 (2001).
6. Alberts, B. *et al. Molecular Biology of the Cell*. **1,** (2014).
7. Luo, J., Solimini, N. L. & Elledge, S. J. Principles of Cancer Therapy: Oncogene and Non-oncogene Addiction. *Cell* **136,** 823–837 (2009).
8. Lengauer, C., Kinzler, K. W. & Vogelstein, B. Genetic instabilities in human cancers. *Nature* **396,** 643–649 (1998).
9. Vogelstein, B. *et al.* Cancer Genome Landscapes. *Science (80-.).* **339,** 1546–1558 (2013).
10. Ichim, G. & Tait, S. W. G. A fate worse than death: Apoptosis as an oncogenic process. *Nat. Rev. Cancer* **16,** 539–548 (2016).
11. Oncogenes and tumor suppressor genes | American Cancer Society. Available at: https://www.cancer.org/cancer/cancer-causes/genetics/genes-and-cancer/oncogenes-tumor-suppressor-genes.html. (Accessed: 26th October 2017)
12. Weir, H. K., Thompson, T. D., Soman, A., Moller, B. & Leadbetter, S. The Past, Present, and Future of Cancer Incidence in the United States from 1975 Through 2020. *HHS Public Access* **33,** 395–401 (2015).
13. Jemal, A., Bray, F. & Ferlay, J. Global Cancer Statistics: 2011. *CA Cancer J Clin* **49,** 1,33-64 (1999).
14. Ames, B. N. Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science (80-.).* **221,** 1256–1263 (1983).
15. Calle, E. E. & Kaaks, R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat. Rev. Cancer* **4,** 579–591 (2004).
16. Hoeijmakers, J. H. J. DNA damage, aging, and cancer. *N. Engl. J. Med.* **361,** 1475–85 (2009).
17. Jones, P. a & Baylin, S. B. The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.* **3,** 415–28 (2002).
18. Jones, P. A. & Baylin, S. B. The Epigenomics of Cancer. *Cell* **128,** 683–692 (2007).
19. Nowell, P. C. The Clonal Evolution of Tumor Cell Populations. *Science (80-.).* **194,** 23–28 (1976).
20. Foulds, L. The Experimental Study of Tumor Progression : A Review. *Am. Assoc. Cancer Res.* **14,** (1954).
21. Greaves, M. & Maley, C. C. Clonal evolution in cancer. *Nature* **481,** 306–13 (2012).
22. Caldas, C. Cancer sequencing unravels clonal evolution. *Nat. Biotechnol.* **30,** 408–410 (2012).

23. Podlaha, O., Riester, M., De, S. & Michor, F. Evolution of the cancer genome. *Trends Genet.* **28,** 155–163 (2012).
24. Hanahan, D. & Weinberg, R. A. The Hallmarks of Cancer. *Cell* **100,** 57–70 (2000).
25. Christoph A. Klein. The Metastasis Cascade. *Science (80-.).* **321,** 1785–1787 (2008).
26. Chaffer, C. L. & Weinberg, R. A. A Perspective on Cancer Cell Metastasis. *Science (80-.).* **331,** (2011).
27. Cavallo, F., De Giovanni, C., Nanni, P., Forni, G. & Lollini, P. L. 2011: The immune hallmarks of cancer. *Cancer Immunol. Immunother.* **60,** 319–326 (2011).
28. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144,** 646–674 (2011).
29. Croce, C. M. Oncogenes and Cancer. *N. Engl. J. Med.* **358,** 502–511 (2008).
30. Felsher, D. W. & Bishop, J. M. Reversible tumorigenesis by MYC in hematopoietic lineages. *Mol. Cell* **4,** 199–207 (1999).
31. Jain, M. Sustained Loss of a Neoplastic Phenotype by Brief Inactivation of MYC. *Science (80-.).* **297,** 102–104 (2002).
32. Shlush, L. I. & Hershkovitz, D. Clonal Evolution Models of Tumor Heterogeneity. *ASCO Educ. B.* 662–665 (2015).
33. Types of Cancer Treatment | American Cancer Society. Available at: https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types.html. (Accessed: 26th October 2017)
34. Treatment for Cancer - National Cancer Institute. Available at: https://www.cancer.gov/about-cancer/treatment. (Accessed: 24th July 2017)
35. Urruticoechea, A. *et al.* Recent Advances in Cancer Therapy: An Overview. *Curr. Pharm. Des.* **16,** 3–10 (2010).
36. About radiotherapy | Cancer in general | Cancer Research UK. Available at: https://www.cancerresearchuk.org/about-cancer/cancer-in-general/treatment/radiotherapy/about. (Accessed: 24th July 2017)
37. Chabner, B. a & Roberts, T. G. Timeline: Chemotherapy and the war on cancer. *Nat. Rev. Cancer* **5,** 65–72 (2005).
38. Allan, J. M. & Travis, L. B. Mechanisms of Therapy-Related Carcinogenesis. *Nat. Rev. Cancer* **5,** 943–955 (2005).
39. Snyder, L. C., Astsaturov, I. & Weiner, L. M. Overview of monoclonal antibodies and small molecules targeting the epidermal growth factor receptor pathway in colorectal cancer. *Clin. Colorectal Cancer* **5 Suppl 2,** S71–S80 (2005).
40. What Is Targeted Cancer Therapy? Available at: https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/targeted-therapy/what-is.html. (Accessed: 24th July 2017)
41. Huang, M., Shen, A., Ding, J. & Geng, M. Molecularly targeted cancer therapy: Some lessons from the past decade. *Trends Pharmacol. Sci.* **35,** 41–50 (2014).
42. Tortora, G. *et al.* Overcoming resistance to molecularly targeted anticancer therapies: Rational drug combinations based on EGFR and MAPK inhibition for solid tumours and haematologic malignancies. *Drug Resist. Updat.* **10,** 81–100 (2007).

43. Holohan, C., Van Schaeybroeck, S., Longley, D. B. & Johnston, P. G. Cancer drug resistance: an evolving paradigm. *Nat. Rev. Cancer* **13,** 714–726 (2013).
44. Kaelin, W. G. The Concept of Synthetic Lethality in the Context of Anticancer Therapy. *Nat. Rev. Cancer* **5,** 689–698 (2005).
45. Sharma, S. V., Bell, D. W., Settleman, J. & Haber, D. A. Epidermal growth factor receptor mutations in lung cancer. *Nat. Rev. Cancer* **7,** 169–181 (2007).
46. Farmer, H. *et al.* Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* **434,** 917–921 (2005).
47. Lord, C. J., Tutt, A. N. J. & Ashworth, A. Synthetic Lethality and Cancer Therapy: Lessons Learned from the Development of PARP Inhibitors. *Annu. Rev. Med.* **66,** 455–470 (2015).
48. Khalil, D. N., Smith, E. L., Brentjens, R. J. & Wolchok, J. D. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nat. Rev. Clin. Oncol.* **13,** 394–394 (2016).
49. Finn, O. J. Immuno-oncology: Understanding the function and dysfunction of the immune system in cancer. *Ann. Oncol.* **23,** 8–11 (2012).
50. Sideras, K. *et al.* Role of the immune system in pancreatic cancer progression and immune modulating treatment strategies. *Cancer Treat. Rev.* **40,** 513–522 (2014).
51. Sakuishi, K. *et al.* Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J. Exp. Med.* **207,** 2187–2194 (2010).
52. Mellman, I., Coukos, G. & Dranoff, G. Cancer immunotherapy comes of age. *Nature* **480,** 480–489 (2011).
53. Lo, J. A. & Fisher, D. E. The melanoma revolution: From UV carcinogenesis to a new era in therapeutics. *Science (80-.).* **346,** 945–949 (2014).
54. Jiang, T. & Zhou, C. The past, present and future of immunotherapy against tumor. *Transl. lung cancer Res.* **4,** 253–64 (2015).
55. Curigliano, G. & Criscitiello, C. Successes and limitations of targeted cancer therapy in breast cancer. *Prog. tumor Res.* **41,** 15–35 (2014).
56. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer Statistics , 2017. *CA Cancer J Clin* **67,** 7–30 (2017).
57. IDC — Invasive Ductal Carcinoma. Available at: http://www.breastcancer.org/symptoms/types/idc. (Accessed: 24th July 2017)
58. Weigelt, B. & Reis-Filho, J. S. Histological and molecular types of breast cancer: is there a unifying taxonomy? *Nat. Rev. Clin. Oncol.* **6,** 718–730 (2009).
59. Weigelt, B., Geyer, F. C. & Reis-Filho, J. S. Histological types of breast cancer: How special are they? *Mol. Oncol.* **4,** 192–208 (2010).
60. Haydaroglu, A. & Ozyigit, G. *Principles and practice of modern radiotherapy techniques in breast cancer*. (2013).
61. Anderson, W. F., Rosenberg, P. S., Prat, A., Perou, C. M. & Sherman, M. E. How many etiological subtypes of breast cancer: Two, three, four, or more? *J. Natl. Cancer Inst.* **106,** 1–11 (2014).
62. Barcenas, C. H. Annual Report to the Nation on the Status of Cancer, 1975-2011, Featuring Incidence

of Breast Cancer Subtypes by Race/Ethnicity, Poverty, and State. *Breast Dis.* **27,** 36–38 (2016).

63. Rakha, E. A., Reis-Filho, J. S. & Ellis, I. O. Basal-like breast cancer: A critical review. *J. Clin. Oncol.* **26,** 2568–2581 (2008).
64. Badve, S. *et al.* Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. *Mod. Pathol.* **24,** 157–167 (2011).
65. Hussain, H. a & Harvey, A. J. Evolution of breast cancer therapeutics: Breast tumour kinase's role in breast cancer and hope for breast tumour kinase targeted therapy. *World J. Clin. Oncol.* **5,** 299–310 (2014).
66. Bianchini, G., Balko, J. M., Mayer, I. A., Sanders, M. E. & Gianni, L. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nat. Rev. Clin. Oncol.* **13,** 674–690 (2016).
67. Foulkes, W. D., Smith, I. E. & Reis-Filho, J. S. Triple-Negative Breast Cancer. *N. Engl. J. Med.* **363,** 1938–1948 (2010).
68. Masoud, V. & Pagès, G. Targeted therapies in breast cancer: New challenges to fight against resistance. *World J. Clin. Oncol.* **8,** 120 (2017).
69. Carey, L., Winer, E., Viale, G., Cameron, D. & Gianni, L. Triple-negative breast cancer: Disease entity or title of convenience? *Nat. Rev. Clin. Oncol.* **7,** 683–692 (2010).
70. Hudis, C. A. & Gianni, L. Triple-Negative Breast Cancer: An Unmet Medical Need. *Oncologist* **16,** 1–11 (2011).
71. Hudis, C. A. Trastuzumab — Mechanism of Action and Use in Clinical Practice Clifford. *new Engl. J. o f Med.* 39–51 (2007).
72. Lehmann, B. D. B. *et al.* Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J. Clin. Invest.* **121,** 2750–2767 (2011).
73. Pareja, F. *et al.* Triple-negative breast cancer: the importance of molecular and histologic subtyping, and recognition of low-grade variants. *npj Breast Cancer* **2,** 16036 (2016).
74. Carey, L. A. *et al.* The triple negative paradox: Primary tumor chemosensitivity of breast cancer subtypes. *Clin. Cancer Res.* **13,** 2329–2334 (2007).
75. Kalimutho, M. *et al.* Targeted Therapies for Triple-Negative Breast Cancer: Combating a Stubborn Disease. *Trends Pharmacol. Sci.* **36,** 822–846 (2015).
76. Prat, A. *et al.* Clinical implications of the intrinsic molecular subtypes of breast cancer. *Breast* **24,** S26–S35 (2015).
77. Folkman, J. & Shing, Y. Angiogenesis. *J. Biol. Chem.* **267,** 10931–10934 (1992).
78. Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86,** 353–364 (1996).
79. Tonini, T., Rossi, F. & Claudio, P. P. Molecular basis of angiogenesis and cancer. *Oncogene* **22,** 6549–6556 (2003).
80. Weidner, N., Semple, J. P., Welch, W. R. & Folkman, J. Tumor angiogenesis and metastasis - Correlation in Invasive Breast Carcinoma. *N. Engl. J. Med.* **324,** (1991).
81. Wicki, A. & Christofori, G. The angiogenic switch in tumorigenesis. *Tumor Angiogenes. Basic Mech. Cancer Ther.* **19,** 67–88 (2008).

82. Leite De Oliveira, R., Hamm, A. & Mazzone, M. Growing tumor vessels: More than one way to skin a cat - Implications for angiogenesis targeted cancer therapies. *Mol. Aspects Med.* **32,** 71–87 (2011).
83. Kerbel, R. S. Tumor Angiogenesis. *N. Engl. J. Med.* **358,** 2039–2049 (2008).
84. Simon, M. C. The Hypoxia Response Pathways — Hats Off! *N. Engl. J. Med.* **375,** 1687–1689 (2016).
85. Greer, S. N., Metcalf, J. L., Wang, Y. & Ohh, M. The updated biology of hypoxia-inducible factor. *EMBO J.* **31,** 2448–2460 (2012).
86. Brown, J. M. & Wilson, W. R. Exploiting tumour hypoxia in cancer treatment. *Nat. Rev. Cancer* **4,** 437–447 (2004).
87. Vaupel, P., Höckel, M. & Mayer, A. Detection and Characterization of Tumor Hypoxia Using pO $_2$ Histography. *Antioxid. Redox Signal.* **9,** 1221–1236 (2007).
88. Tan, C. *et al.* Identification of a novel small-molecule inhibitor of the hypoxia-inducible factor 1 pathway. *Cancer Res.* **65,** 605–612 (2005).
89. Bristow, R. G. & Hill, R. P. Hypoxia and metabolism: Hypoxia, DNA repair and genetic instability. *Nat. Rev. Cancer* **8,** 180–192 (2008).
90. Keith, B., Johnson, R. S. & Simon, M. C. HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression. *Nat. Rev. Cancer* **12,** 9–22 (2011).
91. Ferrara, N., Gerber, H.-P. & LeCouter, J. The biology of VEGF and its receptors. *Nat. Med.* **9,** 669–676 (2003).
92. Folkman, J. Tumor angiogenesis. *Adv. Cancer Res.* **43,** 175–203 (1950).
93. Norden, A. D., Drappatz, J. & Wen, P. Y. Antiangiogenic therapies for high-grade glioma. *Nat. Rev. Neurol.* **5,** 610–620 (2009).
94. Campisi, J. & D'Adda Di Fagagna, F. Cellular senescence: When bad things happen to good cells. *Nature Reviews Molecular Cell Biology* **8,** 729–740 (2007).
95. Hayflick, L. The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res.* **37,** 614–636 (1965).
96. Bodnar, A. G. *et al.* Extension of life-span by introduction of telomerase into normal human cells. *Science (80-.).* **279,** 349–352 (1998).
97. Di Leonardo, A., Linke, S. P., Clarkin, K. & Wahl, G. M. DNA damage triggers a prolonged p53-dependent G1arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev.* **8,** 2540–2551 (1994).
98. Robles, S. J. & Adami, G. R. Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts. *Oncogene* **16,** 1113–1123 (1998).
99. Wang, C. *et al.* DNA damage response and cellular senescence in tissues of aging mice. *Aging Cell* **8,** 311–323 (2009).
100. Coppé, J.-P. *et al.* Senescence-Associated Secretory Phenotypes Reveal Cell-Nonautonomous Functions of Oncogenic RAS and the p53 Tumor Suppressor. *PLoS Biol.* **6,** e301 (2008).
101. Blagosklonny, M. V. Cell senescence and hypermitogenic arrest. *EMBO Reports* **4,** 358–362 (2003).
102. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16(INK4a). *Cell* **88,** 593–602

(1997).

103. Campisi, J. Suppressing cancer: The importance of being senescent. *Science* **309,** 886–887 (2005).
104. Braig, M. & Schmitt, C. A. Oncogene-induced senescence: Putting the brakes on tumor development. *Cancer Research* **66,** 2881–2884 (2006).
105. Trost, T. M. *et al.* Premature senescence is a primary fail-safe mechanism of ERBB2-driven tumorigenesis in breast carcinoma cells. *Cancer Res.* **65,** 840–849 (2005).
106. Moiseeva, O., Mallette, F. A., Mukhopadhyay, U. K., Moores, A. & Ferbeyre, G. DNA damage signaling and p53-dependent senescence after prolonged beta-interferon stimulation. *Mol. Biol. Cell* **17,** 1583–92 (2006).
107. Demidenko, Z. N. & Blagosklonny, M. V. Growth stimulation leads to cellular senescence when the cell cycle is blocked. *Cell Cycle* **7,** 3355–3361 (2008).
108. Campisi, J. Aging, Cellular Senescence, and Cancer. *Annu Rev Physiol* 685–705 (2014).
109. Beauséjour, C. M. *et al.* Reversal of human cellular senescence: Roles of the p53 and p16 pathways. *EMBO J.* **22,** 4212–4222 (2003).
110. Yamakoshi, K. *et al.* Real-time in vivo imaging of p16Ink4a reveals cross talk with p53. *J. Cell Biol.* **186,** 393–407 (2009).
111. Zhang, J., Pickering, C. R., Holst, C. R., Gauthier, M. L. & Tlsty, T. D. p16INK4a modulates p53 in primary human mammary epithelial cells. *Cancer Res.* **66,** 10325–10331 (2006).
112. Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell* **88,** 323–331 (1997).
113. Li, T. *et al.* Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. *Cell* **149,** 1269–1283 (2012).
114. Muñoz-Espín, D. & Serrano, M. Cellular senescence: From physiology to pathology. *Nature Reviews Molecular Cell Biology* **15,** 482–496 (2014).
115. Cho, K. A. *et al.* Morphological adjustment of senescent cells by modulating caveolin-1 status. *J. Biol. Chem.* **279,** 42270–42278 (2004).
116. Kurz, D. J., Decary, S., Hong, Y. & Erusalimsky, J. D. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J. Cell Sci.* **113 (Pt 2,** 3613–22 (2000).
117. Lee, B. Y. *et al.* Senescence-associated β-galactosidase is lysosomal β-galactosidase. *Aging Cell* **5,** 187–195 (2006).
118. Kosar, M. *et al.* Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- And insult-dependent manner, and follow expression of p16ink4a. *Cell Cycle* **10,** 457–468 (2011).
119. Narita, M. *et al.* Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113,** 703–716 (2003).
120. Coppé, J.-P., Desprez, P., Krtolica, A. & Campisi, J. The Senescence-Associated Secretory Phenotype: The Dark Side of Tumor Suppression. *Annu. Rev. Pathol. Mech. Dis.* **5,** 99–118 (2010).
121. Freund, A., Orjalo, A. V, Desprez, P. & Campisi, J. Inflammatory networks during cellular senescence : causes and consequences. *Trends Mol. Med.* **16,** 238–246 (2010).

122. Coppé, J. P. *et al.* A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen. *PLoS One* **5,** (2010).
123. Coppé, J.-P., Kauser, K., Campisi, J. & Beauséjour, C. M. Secretion of Vascular Endothelial Growth Factor by Primary Human Fibroblasts at Senescence. *J. Biol. Chem.* **281,** 29568–29574 (2006).
124. Adams, P. D. Healing and Hurting: Molecular Mechanisms, Functions, and Pathologies of Cellular Senescence. *Molecular Cell* **36,** 2–14 (2009).
125. Coppe, J. P. *et al.* Tumor suppressor and aging biomarker p16 INK4a induces cellular senescence without the associated inflammatory secretory phenotype. *J. Biol. Chem.* **286,** 36396–36403 (2011).
126. Campisi, J. Cellular senescence: Putting the paradoxes in perspective. *Curr. Opin. Genet. Dev.* **21,** 107–112 (2011).
127. McElhaney, J. E. & Effros, R. B. Immunosenescence: what does it mean to health outcomes in older adults? *Curr. Opin. Immunol.* **21,** 418–424 (2009).
128. Kang, T. W. *et al.* Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* **479,** 547–551 (2011).
129. Xue, W. *et al.* Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* **445,** 656–660 (2007).
130. Nardella, C., Clohessy, J. G., Alimonti, A. & Pandolfi, P. P. Pro-senescence therapy for cancer treatment. *Nat. Rev. Cancer* **11,** 503–511 (2011).
131. Eales, K. L., Hollinshead, K. E. R. & Tennant, D. A. Hypoxia and metabolic adaptation of cancer cells. *Oncogenesis* **5,** e190 (2016).
132. Chen, X. *et al.* XBP1 promotes triple-negative breast cancer by controlling the HIF1α pathway. *Nature* 103–107 (2014).
133. Evers, B. *et al.* CRISPR knockout screening outperforms shRNA and CRISPRi in identifying essential genes. *Nat. Biotechnol.* **34,** 11–14 (2016).
134. Ichikawa, M. *et al.* AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. *Nat. Med.* **10,** 299–304 (2004).
135. Peng, R., Lin, G. & Li, J. Potential pitfalls of CRISPR/Cas9-mediated genome editing. *FEBS J.* **283,** 1218–1231 (2016).
136. Okuda, T. *et al.* AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84,** 321–30 (1996).
137. Wang, Q. *et al.* Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 3444–9 (1996).
138. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumors. *Nature* **490,** 61–70 (2012).
139. Banerji, S. *et al.* Sequence analysis of mutations and translocations across breast cancer subtypes. *Nat Cell Biol* **486,** 405–409 (2012).
140. Ferrari, N. *et al.* Expression of RUNX1 correlates with poor patient prognosis in triple negative breast cancer. *PLoS One* **9,** (2014).

141. Illendula, A. *et al.* Small Molecule Inhibitor of CBFβ-RUNX Binding for RUNX Transcription Factor Driven Cancers. *EBioMedicine* **8,** 117–131 (2016).
142. Kwiatkowski, N. *et al.* Targeting transcription regulation in cancer with a covalent CDK7 inhibitor. *Nature* **511,** 616–20 (2014).
143. Wang, Y. *et al.* CDK7-Dependent Transcriptional Addiction in Triple-Negative Breast Cancer. *Cell* **163,** 174–186 (2015).
144. Mazure, N. M. & Pouysségur, M. C. B.-H. and J. Protein kinases and the Hypoxia-Inducible Factor-1, two switches in angiogenesis. *Curr. Pharm. Des.* **9,** 531–541 (2003).
145. Semenza, G. L. Hypoxia-Inducible Factor 1 (HIF-1) Pathway. *Sci. STKE* **2007,** (2007).
146. Micol, J.-B. *et al.* ASXL2 is essential for haematopoiesis and acts as a haploinsufficient tumour suppressor in leukemia. *Nat. Commun.* **8,** 15429 (2017).
147. Miller, T. E. *et al.* Transcription elongation factors represent in vivo cancer dependencies in glioblastoma. *Nature* **547,** 355–359 (2017).
148. Boér, K. Impact of palbociclib combinations on treatment of advanced estrogen receptor-positive/human epidermal growth factor 2-negative breast cancer. *Onco. Targets. Ther.* **9,** 6119–6125 (2016).
149. Finn, R. S. *et al.* PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. *Breast Cancer Res.* **11,** R77 (2009).
150. Prahallad, A. *et al.* Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature* **483,** 100–103 (2012).
151. Sun, C. & Bernards, R. Feedback and redundancy in receptor tyrosine kinase signaling: relevance to cancer therapies. *Trends Biochem. Sci.* **39,** 465–474 (2017).
152. Sun, C. *et al.* Intrinsic resistance to MEK inhibition in kras mutant lung and colon cancer through transcriptional induction of ERBB3. *Cell Rep.* **7,** 86–93 (2014).
153. Coppe, J. P., Desprez, P. Y., Krtolica, A. & Campisi, J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* **5,** 99–118 (2010).
154. Chang, J. *et al.* Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. *Nat Med* **22,** 78–83 (2016).
155. Zhu, Y. *et al.* Identification of a novel senolytic agent, navitoclax, targeting the Bcl-2 family of anti-apoptotic factors. *Aging Cell* **15,** 428–435 (2016).
156. Rufini, a, Tucci, P., Celardo, I. & Melino, G. Senescence and aging: the critical roles of p53. *Oncogene* **32,** 5129–43 (2013).
157. Brooks, E. E. *et al.* CVT-313, a specific and potent inhibitor of CDK2 that prevents neointimal proliferation. *J. Biol. Chem.* **272,** 29207–29211 (1997).
158. Supuran, C. T. Indisulam: an anticancer sulfonamide in clinical development. *Expert Opin. Investig. Drugs* **12,** 283–287 (2003).
159. Merrick, K. a *et al.* Switching Cdk2 On or Off with Small Molecules to Reveal Requirements in Human Cell Proliferation. *Mol Cell* **42,** 624–636 (2012).

160. Lu, Y. *et al.* Identify on- and off- target effects of targeted therapeutics on signaling networks by reverse phase protein microarrays. *Cancer Res.* **67,** 2466 LP-2466 (2014).
161. Shay, J. W. & Roninson, I. B. Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene* **23,** 2919–2933 (2004).
162. Midgley, R. S. *et al.* A phase I and pharmacokinetic study of lapatinib in combination with infusional 5-fluorouracil, leucovorin and irinotecan. *Ann. Oncol.* **18,** 2025–2029 (2007).