Understanding PLK4's role in Cancer

Irina Suheila Martins Leal Fonseca

Dissertation presented to obtain the Ph.D degree in Molecular Biosciences

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
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Cover: Representative figure of the centrosome; MCF10A-Plk4 p53 Knock-out cells, where the DNA is marked with DAPI (in blue) and centrosomes are marked with Centrin-1 (in green) and with CP110 (in red); MCF10A-Plk4 p53 Knock-out mammosphere image.

Graphics Author: Irina Fonseca

UNDERSTANDING PLK4'S ROLE IN CANCER

Irina Suheila Fonseca

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Declaration

I hereby declare that this dissertation, and the data herein contained, is the result of my own work conducted between August 2016 to November 2022 in the laboratories of Doctor Mónica Bettencourt-Dias, at the Instituto Gulbenkian de Ciência in Oeiras, Portugal, and Doctor Joana Paredes, at i3S - Instituto de Investigação e Inovação em Saúde in Porto, Portugal.

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The work developed during this period resulted in the following publications:

1. **Fonseca, I.**, Horta, C., Ribeiro, A.S. *et al.* Polo-like kinase 4 (Plk4) potentiates *anoikis*-resistance of p53KO mammary epithelial cells by inducing a hybrid EMT phenotype. *Cell Death Dis* **14**, 133 (2023). https://doi.org/10.1038/s41419-023-05618-1

2. **Fonseca, I.S.**, Bettencourt-Dias, M. (2019). The Cell Cycle, Cytoskeleton and Cancer. In: Fior, R., Zilhão, R. (eds) Molecular and Cell Biology of Cancer. Learning Materials in Biosciences. Springer, Cham. https://doi.org/10.1007/978-3-030-11812-9_4

3. **Marteil G†,** Guerrero A, Vieira AF, Almeida BP, Machado P, Mendonça S, Mesquita M, Villarreal B, **Fonseca I**, Francia ME, Dores K, Martins NP, Jana SC, Tranfield S, Barbosa-Morais NL, Paredes J, David Pellman, Godinho SA, Bettencourt-Dias M†. Over-elongation of centriole in cancer promotes centriole amplification and chromosome missegregation. *Nat Commun* **9**, 1258 (2018). https://doi.org/10.1038/s41467-018-03641-x
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“Science may set limits to knowledge, but should not set limits to imagination.”
– Bertrand Russel

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Summary

Centrioles are essential organelles within eukaryotic cells, as centrosomes forming the main microtubule organizing center of cycling animal cells, and also as basal bodies templating cilia and flagella assembly. Due to their essential functions in cells, centriole biogenesis is tightly regulated in space, time and number. However, several diseases have been associated to deregulation of those structures, including cancer.

In multiple human cancers, increased centrosome number (hereafter described as centrosome amplification, CA) and structure abnormalities have been widely observed, as well as increased expression of Polo-like kinase 4 (Plk4), a trigger for centriole biogenesis. Both CA and Plk4 have been linked to tumorigenesis, invasion, metastasis, and worse patient prognosis. Still, whether CA or Plk4 has a role in potentiating cancer stem-like features, such as *anoikis* resistance and Epithelial to Mesenchymal Transition (EMT) is still unexplored.

During my Ph.D., I aimed at understanding what is the impact of centrosome amplification and Plk4 overexpression in the acquisition of stem cell characteristics. I took advantage of a previously described mammary non-tumorigenic cell line, the MCF10A-Plk4 cell line, where Plk4 is transiently overexpressed by doxycycline treatment. Then, I created a stable p53 knock-out (KO) mammary epithelial cell line model through the CRISPR/Cas9 technique, in order to have viable and cycling cells upon CA induced via Plk4 overexpression, and to mimic what occurs in cancer (where the tumor suppressor p53 is often mutated). The newly stablished p53KO cell line was characterized regarding its p53 and p21 protein expression, centrosome number, cell cycle profile, cell viability and tumorigenic capacity (Chapter 3). Such cell line can be used as a tool to further study other important roles of CA and Plk4 in pre-tumorigenic conditions, in a p53KO background.

Then, I used the MCF10A-Plk4 and the MCF10A-Plk4^{p53KO} cell lines to study the direct or indirect role of CA and Plk4 in inducing stem-like properties, namely the *anoikis*-resistance capacity of epithelial non-tumorigenic cells (Chapter4). I show for the first time that, in the absence of p53, Plk4 overexpression significantly potentiates resistance to cell death by *anoikis* of non-tumorigenic p53KO mammary epithelial cells. Importantly, this effect is independent of Plk4's role in centrosome biogenesis, implying Plk4's additional cellular functions. Moreover, the *anoikis* resistance capacity is associated with the induction of a hybrid epithelial-mesenchymal phenotype, and upregulation of the cell adhesion protein P-cadherin. P-cadherin is partially required for Plk4-induced *anoikis* resistance. Kaplan-Meier plots show a significant correlation between tumors with high Plk4 and P-cadherin expression and a worse disease-free survival (DFS) and overall survival (OS) in breast cancer patients. As it has been shown that CA induced by Plk4 overexpression induces invasive capacities of other cells through the secretion of pro-invasive factors, I also evaluated Plk4's role in inducing *anoikis* resistance of breast cancer cells through paracrine signaling. Interestingly, the conditioned media of Plk4-induced p53KO mammary epithelial cells also potentiates *anoikis* resistance of breast cancer cells, being also partially dependent on soluble P-cadherin secretion.
Finally (Chapter 5), I discuss how this work provides new insights into Plk4's role as an oncogene through autocrine and paracrine signaling to promote malignancy, independently of its role in centriole biogenesis. My work also highlights the relevance in exploring further Plk4 role in cancer progression besides its role as centriole biogenesis regulator, particularly as new discoveries are being made in the cancer field.

Sumário

Os centríolos são organelos com um papel essencial em células eucarióticas, uma vez que são os principais organizadores de microtúbulos em células animais (na forma de *centrossomas*), e também como corpos basais para a biogénese de cílios e flagelos. Devido às suas funções celulares, a biogénese dos centríolos é rigorosamente regulada no espaço, no tempo e no número. No entanto, várias doenças têm sido associadas a alterações nestes organelos, incluindo o cancro.

Em múltiplos cancros humanos, tem sido observado um aumento do número de centrossomas (posteriormente referido como amplificação de centrossomas, AC) e de anomalias estruturais, bem como o aumento da expressão do seu principal regulador, a cinase Plk4. Tanto a AC, como a elevada expressão de Plk4, têm sido associadas aos processos de tumorigénese, invasão, e metastização, assim como correlacionados com um mau prognostico dos doentes oncológicos. Ainda assim, se a AC ou a Plk4 têm um papel em potenciar características estaminais em células transformadas, tais como a resistência à *anoikis* ou a transição epitelial-mesenquimal (EMT), ainda está por explorar.

Durante o meu doutoramento, tive como objetivo compreender qual o impacto da AC e/ou da sobre expressão da Plk4 na aquisição de características estaminais. Para isso usei uma linha celular mamária não tumorigénica previamente descrita, a MCF10A-Plk4, onde a Plk4 é sobre expressa transitoriamente com o tratamento com doxiciclina. Para garantir a sobrevivência das células após a manipulação dos centríolos, e de forma a mimetizar o que ocorre no cancro (onde o gene supressor tumoral p53 é frequentemente mutado), o p53 foi silenciado neste modelo (p53KO), através da técnica de CRISPR/Cas9. A linha celular p53KO foi caracterizada em relação à sua expressão das proteínas p53 e p21, o número de centrossomas, o perfil do ciclo celular, a viabilidade celular e sua capacidade tumorigénica (Capítulo 3). Esta linha celular pode ser utilizada como ferramenta para estudar de forma mais aprofundada outras funções importantes mediadas

pela AC e da expressão de Plk4 em condições pré-tumorigénicas, na ausência do p53.

Posteriormente, usei as linhas MCF10A-Plk4 e MCF10A-Plk4^{p53KO} para estudar o papel direto ou indireto da AC e da Plk4 na indução de propriedades estaminais, nomeadamente a resistência a *anoikis* de células epiteliais não tumorigénicas (Capítulo 4). Assim, demonstro pela primeira vez que, na ausência do p53, a sobre expressão da Plk4 potencia significativamente a resistência à morte celular por *anoikis* de células epiteliais mamárias não tumorigénicas. É importante realçar que este efeito é independente do papel da Plk4 na biogénese dos centrossomas, sugerindo que esta cinase possui funções celulares adicionais. Curiosamente, a resistência a *anoikis* induzida por Plk4 está associada à indução de um fenótipo epitelial-mesenquimal híbrido, sendo que o aumento da expressão da P-caderina, uma proteína de adesão celular, é parcialmente necessária para a resistência a *anoikis* induzida pela Plk4. Análises de Kaplan-Meier mostram ainda uma correlação significativa entre tumores com elevada expressão de Plk4 e P-caderina e uma pior sobrevida total e tempo livre de doença em doentes com cancro de mama. Visto que foi demonstrado que a AC induzida pela sobre expressão da Plk4 induz capacidades invasivas a outras células através da secreção de fatores pró-invasivos, avaliei também o papel da Plk4 na indução de resistência a *anoikis* de células tumorais de cancro da mama através de sinalização parácrina. Curiosamente, na ausência de p53, o meio condicionado das células epiteliais mamárias induzidas pela Plk4, também induz a resistência a *anoikis* das células neoplásicas, sendo também parcialmente dependente da secreção da fração solúvel da P-caderina.

Por último (Capítulo 5), discuto como este trabalho proporciona uma nova visão do papel da Plk4 como um oncogene, através da sinalização autócrina e parácrina, para promover a tumorigénese, independentemente do seu papel na biogénese do centríolo. O meu trabalho também destaca a relevância de explorar melhor o papel da Plk4 na progressão do cancro para além do seu papel principal como

regulador do centríolo, particularmente à medida que novas descobertas estão a ser feitas na área da oncologia.

List of Abbreviations

%	Percentage
µg	micrograms
µL	microliters
µm	micrometers
µM	micromolar
µg	micrograms
2D	Two dimensional, *xy*
3D	Three dimensional, *xyz*
ANOVA	Analysis of variance
APC	Anaphase promoting complex
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine triphosphate
ATR	Ataxia Telangiectasia and Rad3-related
Bad	Bcl-2-associated death promoter
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma-2
BER	Base excision repair
Bid	Bcl-2-interacting domain
Bim	Bcl-2-interacting mediator of cell death
CA	Centrosome amplification
CAM	Chicken Chorioallantoic Membrane
Cas9	CRISPR associated protein 9
CCL5	Chemokine (C-C motif) Ligand 5
CDH3	P-cadherin gene
cDNA	complementary Deoxyribonucleic Acid
CDKs	Cyclin-Dependent Kinases
CO_2	Carbon dioxide

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSCs	Cancer Stem Cells
CTBP1	C-terminal-binding protein 1
CTC	Circulating Tumor Cells
DAPI	4’,6-diamidino-2-phenylindole
DCFDA	2’,7’-dichlorofluorescin diacetate
DFS	Disease-free survival
DIABLO	Direct inhibitor of apoptosis binding protein with low pI
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic Acid
Dox	Doxycycline
Doxo	Doxorubicin
DSB	Double Strand Brake
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
EDD	Embryonic development day
EGF	Epidermal Growth Factor
EMT	Epithelial to mesenchymal transition
EMT-TFs	Epithelial to mesenchymal transition transcription factors
FACS	Fluorescence-Activated Cell Sorting
FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
GADD45	Growth Arrest and DNA Damage
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDF15	Growth/differentiation factor 15
gRNA	Guide Ribonucleic acid
HDR	Homologous Recombination

HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HMF	Human Mammary Fibroblasts
Hsp70	Heat shock protein 70
IC_{50}	Half maximal inhibitory concentration
ILK	Integrin-linked kinase
IGF	Insulin-Like Growth Factor
IL-6	Interleukin 6
IL-8	Interleukin 8
KO	Knock-out
LOH	Loss of heterozygosity
ml	milliliters
mM	millimolar
MDM2	Murine double minus 2
MEF	Mouse embryonic fibroblasts
MET	Mesenchymal to epithelial transition
MFE	Mammosphere-forming Efficiency
MMP1	Metalloproteinase 1
MMP2	Metalloproteinase 2
mRNA	messenger Ribonucleic acid
MT	Microtubule
MTOC	Microtubule Organizing Center
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa B
ng	nanograms
NHEJ	Non-Homologous End Joining
NI	Non induced
NSCC	Non-Stem Cancer Cells
OE	Overexpression

OS	Overall survival
P53R2	P53 inducible ribonucleotide reductase
PARP	Poly-ADP ribose polymerase
PBS	Phosphate-Buffered Saline
PCM	Pericentriolar material
PCR	Polymerase chain reaction
PDGF	Platelet-Derived Growth Factor
PIG3	P53-Inducible Gene 3
PLK4	Polo-Like-Kinase 4
PTEN	Phosphatase and tensin homolog
PUMA	P53 upregulated modulator of apoptosis
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Rb	Retinoblastoma
ROS	Reactive oxygen species
RNA	Ribonucleic acid
Rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinases
SAC	Spindle Assembly Checkpoint
SAS6	protein Spindle Assembly Abnormal protein 6
SCF	Skp1-Cullin-F-box-protein
SD	Standard Deviation
Slug	Snail family transcription repressor 2
SMAC	Second mitochondria-derived activator of caspases
Snail	Snail family transcription repressor 1
sP-cad	Soluble P-cadherin
siRNA	Small interfering Ribonucleic acid
SRB	Sulforhodamine B
Src	Proto-oncogene tyrosine-protein kinase

TBHP	Tert-Butyl hydroperoxide
TGFβ1	Transforming growth factor beta 1
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TP53	Tumor protein 53
TRAIL	TNF- related-apoptosis-inducing ligand
UV	Ultra violet
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WT	Wild Type
Zeb2	Zinc finger E-box binding homebox 2

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Chapter 1.

General Introduction

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1.1 The Cell Cycle

The first illustration of cells was made in 1665 by Robert Hook(Gest, 2004). This illustration led to the first observation that cells are the structural base of life and, over the subsequent centuries, cells were discovered in every living organism. In 1958, Rudolf Virchow stated that cells are not only the basic structure unit of whole life, but also the physiologic unit of life, arising from pre-existing cells – *Omnis cellula e cellula* – "All cells come from cells"(Byers, 1989; Virchow, 1859). This occurs by the process of cell division, in which one cell gives rise to two identical daughter cells. Cell division is essential for growth and replenishment of old cells, but also underlies the understanding of cancer. Ever since the 19th century (1858), scientists have observed cells dividing, but little was known about how they were able to do it. The machinery that regulates cell cycle progression only started to be elucidated in the 80s.
In 2001, the Nobel Foundation awarded three cell biologists with the Nobel Prize in Physiology or Medicine for their outstanding contributions to the study of the cell cycle in eukaryotic cells. Sir Paul M. Nurse identified, cloned and characterized cyclin-dependent kinases (CDKs) in yeast and showed that CDK's function is conserved in higher eukaryotes(Hochegger et al., 2008; Simanis & Nurse, 1986). CDKs are the major kinases regulating cell cycle progression. Richard Timothy Hunt also received the same award for his discovery of cyclins and for showing that cyclins are degraded during the cell cycle(Evans et al., 1983). Cyclins are the

partners of CDKs that regulate their activity along the cell cycle. Cyclins levels oscillate during the cell cycle, being synthesized only at specific stages in response to various molecular cues, and then degraded by ubiquitin-mediated proteolysis(Machetel & Weber, 1991), while CDKs are constitutively expressed in cells. Finally, Leland Hartwell identified a particular class of genes that control the cell cycle, introducing the concept of checkpoints, i.e., regulatory control points during cell cycle progression(Hartwell et al., 1970).

In order to divide, a cell must complete several important tasks: grow, copy its genetic material (DNA) and physically split into two identical daughter cells. The cell cycle in eukaryotic cells consists of 3 phases (**Figure 1.1**): Interphase (G1+S+G2); mitotic phase and cytokinesis. In interphase, most somatic cells have two gap phases, G1 and G2, that precede and follow DNA replication (S phase), respectively.

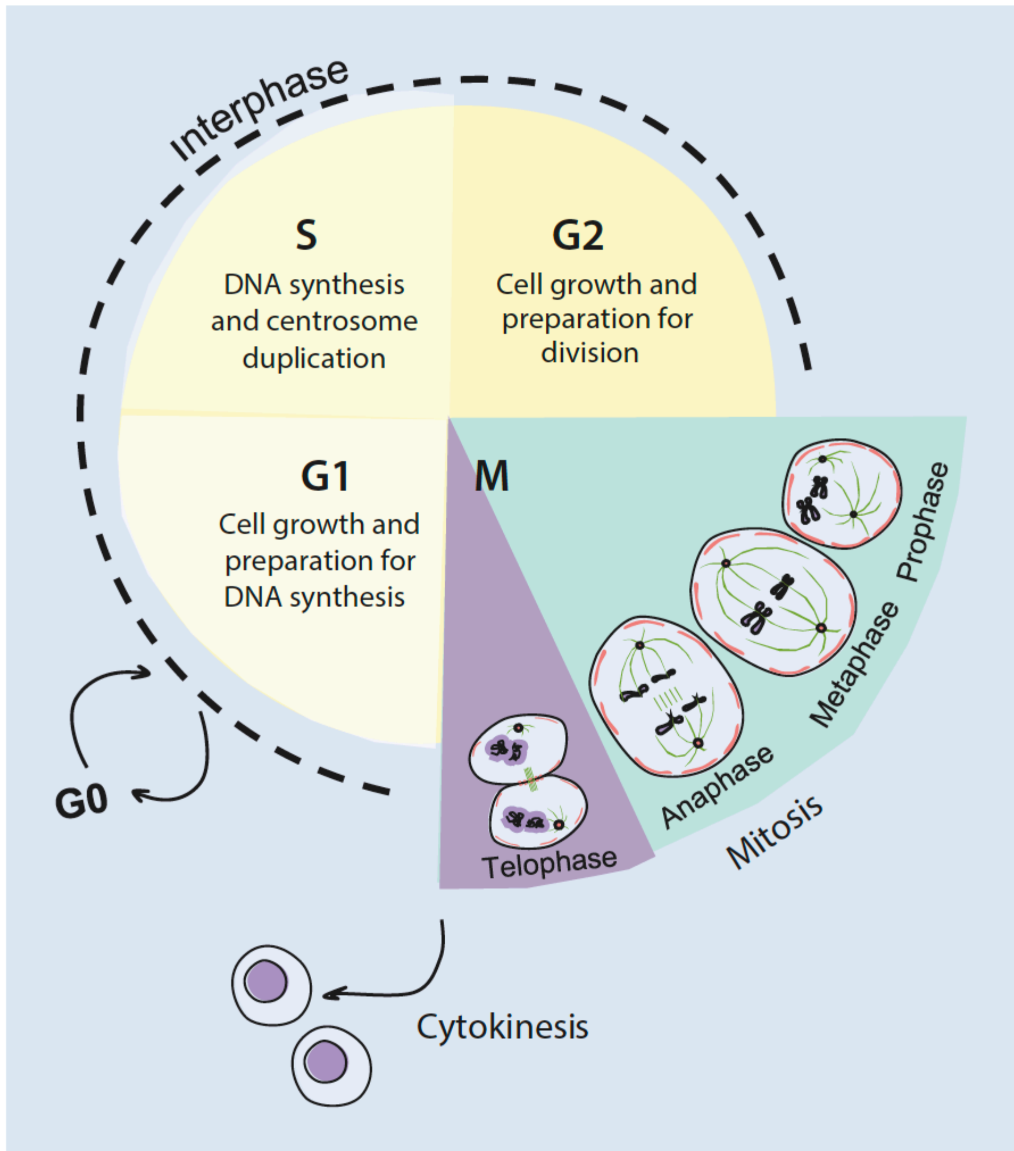

Figure 1.1. The eukaryotic cell cycle. The cell cycle consists of Interphase, Mitosis and Cytokinesis. In the interphase, most somatic cells have two gap phases, G1 and G2, that precede and follow DNA replication (S phase), respectively. During the G1 phase, there is growth and preparation of the cell for DNA replication. In S phase, DNA synthesis and centrosome duplication occur. In the G2 phase the cell prepares to enter mitosis, organizing the microtubules to form a mitotic spindle at the transition with mitosis. The mitotic phase is highly regulated and the sequence of events has been traditionally

divided into phases mostly based on the state of chromatin and position of chromosomes, known as prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, the chromatin condenses, the nucleolus disappears and the mitotic spindle begins to form, with the migration of centrosomes to the both poles of the cell. In prometaphase, the nuclear membrane breaks down and the kinetochore microtubules attach to kinetochores. In metaphase, all the chromosomes align in the "metaphase plate" and the mitotic spindle is formed. During anaphase, the sister chromatids separate from each other and are pulled towards the opposite ends of the cell. Finally, in telophase, the mitotic spindle is broken down, and two new nuclei forms, one for each set of chromosomes. The nuclear membrane and nucleoli reappear, and then, the chromosomes begin to decondense. Subsequently there is physical separation of cell membrane to form two new cells - Cytokinesis. The G0 phase represents a resting phase when cells leave the cell cycle temporarily or permanently. *Adapted from Heng, Y. W. et al., 2010.*

1.1.1 Cell cycle phases

G1 Phase

In the G1 phase, the cell synthetizes proteins and organelles (such as mitochondria and ribosomes), growing in size, guaranteeing that both daughter cells will inherit sufficient amounts (or number) of proteins/organelles **(Figure 1.1).** Because of this, a G1 or G1/S checkpoint is established by the cell (**Figure 1.1 and 1.3A**). The G1/S checkpoint is the main decision point for a cell. It represents a control mechanism to ensure that everything is ready for DNA synthesis in the following phase (S phase). At this point, the cell fate, to commit to divide or not, is determined. If the cell passes the G1 checkpoint, it enters the S phase, becoming irreversibly committed to divide. There are several internal and external conditions that the cell checks at this stage, in order to proceed for DNA replication. Some of these conditions are: the cell size (to ensure that the cell is large enough to divide), the existence of nutrients (if the cell has enough energy reserves or available nutrients to divide), and molecular signals (such as growth factors or other positive

cues from neighboring cells), as well as the DNA integrity (to ensure that there is no DNA damage, before proceeding to next phase).
If the cell passes all these criteria, it moves to S phase. If not, it leaves the cell cycle, entering quiescence, also called G0 phase.

S Phase

In S phase, DNA replication occurs. The DNA is duplicated, along with the major microtubule-organizing structure, the centrosome. The centrosome is important for many processes, including cell signaling, polarity and nucleation of microtubules to organize the mitotic spindle, and we devote a later section of this chapter to it (**Figure 1.1**).

G2 Phase

The G2 phase (or second gap phase) starts after DNA synthesis. During this phase, the cell continues to grow and prepares for mitosis, organizing the microtubules to form a mitotic spindle at the transition with mitosis (**Figure 1.1**). The second checkpoint, the G2 or G2/M checkpoint, ensures that the DNA was completely copied during S phase and with no damage, so that the cell can divide sister chromatids in two cells. If errors are detected, cells will be stuck in G2 until the errors are repaired; otherwise, the cell may undergo programmed cell death (apoptosis) (**Figure 1.1 and 1.3A**).

Mitotic Phase

In the mitotic phase (M phase), the cell stops growing and divides into two daughter cells. During mitosis, DNA condenses and is pulled apart by the mitotic spindle (a specialized structure made of microtubules). M phase is highly regulated and the sequence of events has been traditionally divided into phases mostly based on the

condensation state and position of chromosomes in relation to the spindle, known as prophase, prometaphase, metaphase, anaphase, and telophase (**Figure 1.1**). During mitosis, there is a spindle assembly checkpoint (SAC) or M checkpoint (**Figure 1.1 and 1.3A**). Here, the cell assesses whether the spindle has formed and all the sister chromatids are correctly attached to the spindle microtubules before anaphase begins. Overlapping with the end of mitosis, the cytokinesis process occurs, where the cytoplasm, organelles and cytoplasmic membrane split, forming two new cells, often with equal shares of cellular components.

Cell division must be tightly controlled in order to prevent the development of disorders, such as cancer. Each of the phases discussed above is regulated by different cyclin/CDK complexes in eukaryotic cells (**Figure 1.2A**): G1 phase by CDK4/6 complexed with cyclin D; S phase by CDK2 complexed with cyclins E and A; G2 phase by CDK1 complexed with cyclin A; and mitosis by CDK1 complexed with cyclins B(Malumbres, 2014; Malumbres & Barbacid, 2005; Murray, 2004).

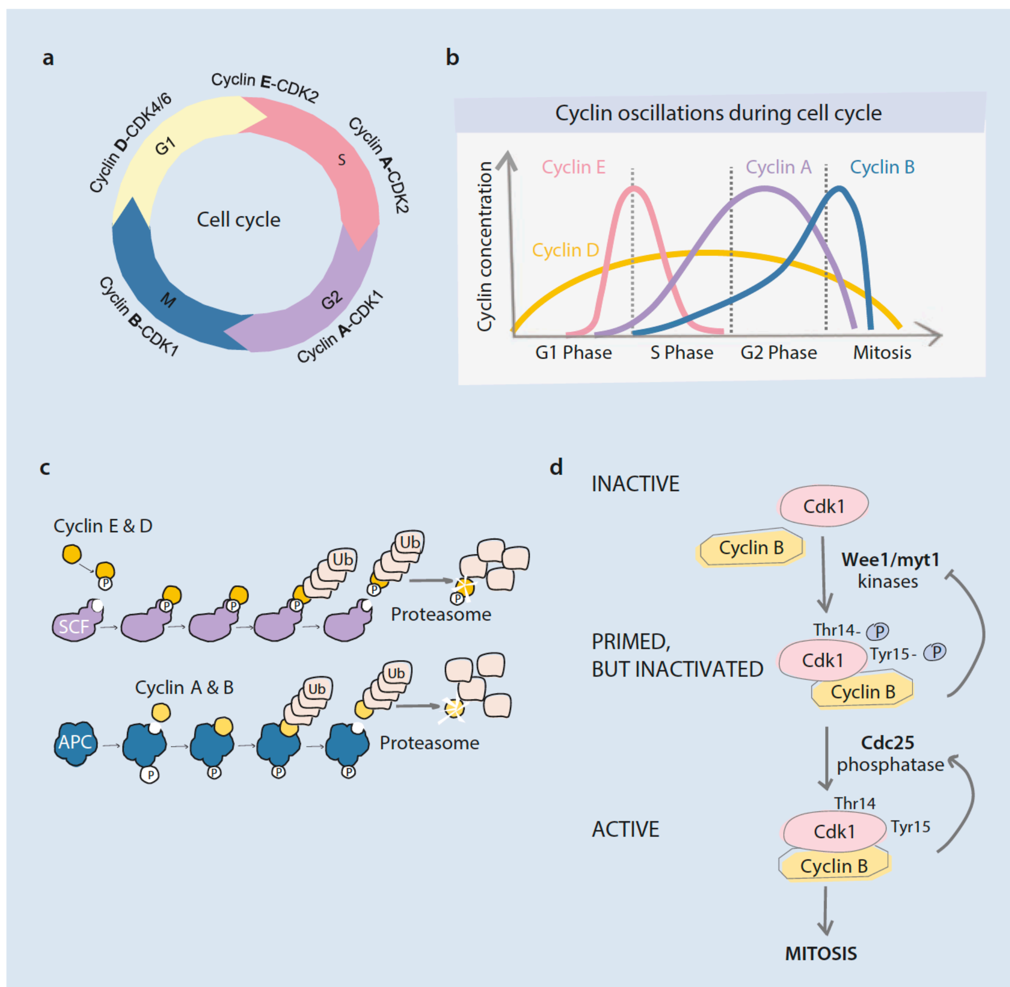

Figure 1.2. The regulation of cell cycle through CDK/Cyclin complex. (A) In eukaryotic cells, there are multiple CDK/cyclin complexes that play specific roles at various phases in the cell cycle. The complex CDK4/6-Cyclin D stimulate the initiation of G1 phase. The progression towards the end of G1 phase is characterized by increasing levels of CDK2-Cyclin E, which in turn triggers the beginning of S phase. CDK2-Cyclin E levels are then degraded, and CDK2 -Cyclin A completes the S-phase and entry to G2 phase. In G2, cyclin A complexes with CDK1 until the beginning of M phase. At the end of G2 Cyclin B couples with CDK1 and their activity increases during mitosis and diminishes at the end of M phase. **(B)** Cyclin levels oscillate during the cell cycle, being synthesized only at specific stages in response to various molecular cues, and then degraded by ubiquitin-mediated proteolysis. **(C)** Cyclin degradation during cell cycle*.* Cyclins must be synthesized for the

progression of the cell cycle, and then degraded immediately after. Two ubiquitin-ligase complexes are responsible for the degradation of cyclins: the skp1-cullin-F-box-protein (SCF) complex that recognizes phosphorylated G1 cyclins (cyclin E and D) and targets it for degradation, and the anaphase promoting complex (APC) that degrades mitotic cyclins (cyclin A and cyclin B) during mitotic progression. The SCF complex is active throughout the cell cycle and the degradation of its substrates depends on their phosphorylation status. On the other hand, the APC is activated at the onset of anaphase and degrades its substrates as cells exit mitosis. **(D)** Regulation of transitions in mitosis often operates through a series of positive and negative feedback loops. CDK-Cyclin B activity to drive G2/M transition and is regulated through kinases and phosphatases, such as Wee1 and cdc25, respectively. With low levels of cyclin B, Wee1 and Myt1 (Wee1/MYT1) kinases inactivate CDK1 by phosphorylating residues T14 and Y15, thus blocking ATP binding and hydrolysis. Once the concentration of Cyclin B increases exceeding a threshold, CDK1 becomes active. Cyclin B-CDK1 phosphorylates and activates the cdc25 phosphatase, allowing cdc25 to remove the inhibitory Thr14 and Tyr15 phosphorylations on CKD1 and thus, allowing the entry into mitosis. Furthermore, Cyclin B-CDK1 is also a negative regulator of wee1 and Myt1, as both kinases are inactivated upon Cyclin B-CDK1 phosphorylation. *Adapted from Suryadinata, R. et al., 2010; Murray, A. W. et al., 2004 and Deibler, R. W. et al., 2010.*

In a cell, each of the CDK/cyclin complex modifies a specific group of protein substrates. The proper phosphorylation of these substrates must also occur at particular times in order for the cell cycle to continue. Depending on the phase of the cell cycle, CDK/cyclin complexes can recognize multiple substrates and coordinate multiple events during each phase. For example, during G1 phase and beginning of S phase, G1-CDKs will catalyze the phosphorylation of proteins that prepare for DNA replication. Later on, during mitosis, M phase CDKs phosphorylate a wide range of other proteins. These include condensing proteins, which are essential for the condensation of mitotic chromosomes, and also laminin proteins, which will form a stabilizing network under the nuclear membrane that dissembles during mitosis. By phosphorylating the proteins that regulate

microtubule dynamic and centrosome maturation, the M-CDKs also influence the assembly of the mitotic spindle.

Actually, CDK’s activity is regulated by many conditions, such as cyclin availability, which is mostly regulated by their transcription and translation and then degradation (**Figure 1.2B**). Two major ubiquitin ligases are involved in cyclin degradation: the skp1-cullin-F-box-protein (SCF) complex that recognizes phosphorylated G1 cyclins (cyclin E and D) and targets it for degradation, and the anaphase promoting complex (APC) that degrades mitotic cyclins (cyclin A and cyclin B) during mitotic progression(Machetel & Weber, 1991; Murray, 2004) (**Figure 1.2C**). The Failure of cyclin degradation leads to cell cycle arrest.

CDK’s activity can also be regulated by activating and deactivating phosphorylation. CDK1/Cyclin B activity, which drives G2/M transition, is regulated through kinases (for example Wee1), and phosphatases (as cdc25)(Coleman & Dunphy, 1994) (**Figure 1.2D)**. During interphase, CDK1/Cyclin B complex is inactivated by phosphorylation of CDK1 at two residues, Threonine 14 (Thr14) and Tyrosine 15 (Tyr15), by Wee1 and Myt1 (Wee1/MYT1) kinases, thus blocking ATP binding and hydrolysis. During the G2/M transition, Myt1 and Wee1 are inactivated, while the phosphatase Cdc25 is activated. Cdc25 dephosphorylates Thr14 and Tyr15, allowing for activation of the CDK1/Cyclin B complex and entry into mitosis(Lew & Kornbluth, 1996; Perry & Kornbluth, 2007). Furthermore, CDK1/Cyclin B is also a negative regulator of Wee1 and Myt1, as both kinases are inactivated upon CDK1/Cyclin B phosphorylation.

CDK’s activity is further regulated by two families of CDK inhibitors: INK4 proteins (composed of INK4A, INK4C and INK4D), and Cip/Kip family (including p21, p27 and p57)(Malumbres & Barbacid, 2005; Satyanarayana & Kaldis, 2009). Deregulation of CDK activity, for example through mutations or inactivation of CDK inhibitors or deregulation of cyclin levels is often observed in cancer(Deshpande et

al., 2005; Malumbres & Barbacid, 2001, 2009; Stamatakos et al., 2010). These can drive cells prematurely into S phase and mitosis and can cause genomic instability.

1.1.2 Cell Cycle Checkpoints, DNA Repair and Spindle Assembly

To avoid transmission of an altered genome to daughter cells, there are elaborate checkpoint pathways which arrest cell cycle progression and promote repair or, in case the damage is unrepairable, stimulate programmed cell death - apoptosis. As described above, there are three known checkpoints (**Figure 1.3A**): the G1 checkpoint (the major checkpoint), the G2/M checkpoint and the spindle assembly checkpoint (SAC) or M checkpoint.

Along the cell cycle, each checkpoint ensures that the conditions of the cell are favorable to move from one phase to the next one. Sensor proteins detect and signal DNA damage to downstream effectors that, in turn, arrest cell cycle progression and promote repair.

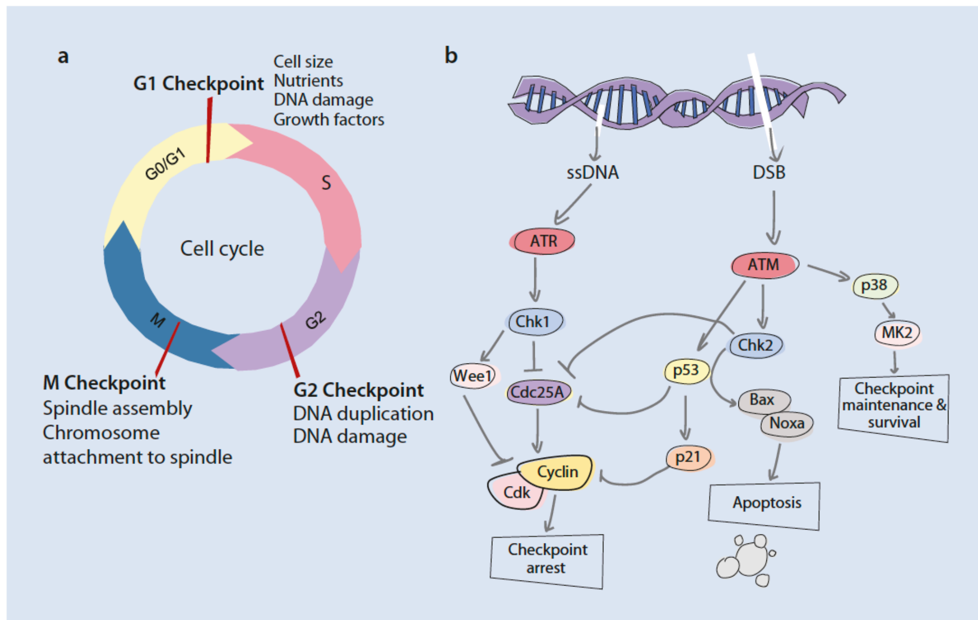

Figure 1.3. Cell cycle checkpoints. (A) The cell cycle checkpoints: The G1/S checkpoint is the main decision point for a cell. It represents a control mechanism to ensure that everything is ready for DNA synthesis in the following phase (S phase). At this point, the cell fate to either divide or not is determined. If the cell passes all the criteria stablished, such as: cell size, nutrients, molecular signals, and DNA integrity, it moves into S phase. If not, it leaves the cell cycle, entering quiescence, also called G0 phase. The second checkpoint, the G2/M checkpoint ensures that the DNA was completely copied during S phase and with no damage, so that the cell can divide. If errors are detected, cells will be stuck in G2 until errors are repaired; otherwise, the cell may undergo programmed cell death (apoptosis). In the M checkpoint, also known as spindle checkpoint, the cell assesses whether the spindle has formed, and all the sister chromatids are correctly attached to the spindle microtubules before anaphase begins. At this point, the Anaphase–Promoting Complex (APC) triggers the transition from metaphase to anaphase, by tagging specific proteins, such as securin, and M-cyclins (Cyclin A and B) for degradation. **(B)** Checkpoint activation signaling cascade related to DNA damage: DNA damage is sensed by diverse sensor and adaptor proteins, which in turn leads to activation of ATM and ATR kinases, allowing the establishment of a DNA-damage checkpoint. Chk1 and p53 are the major components activated during DNA damage response. Chk1 phosphorilates Cdc25 targeting it for degradation and activates Wee. Loss of Cdc25 prevents CDK-Cyclin activation (represented in red box) leading to a cell cycle arrest. P53 is stabilized in the cell by multiple post-translational modifications (represented with colorful dots), and increased the levels of p21, a CDK inhibitor, repressing the transcription of Cyclin B, contributing to CDK/Cyclin inhibition. Furthermore, cells also can activate Chk2 and p38/MK2 pathways, which are involved in apoptosis and checkpoint maintenance, respectively. *Adapted from Suryadinata, R. et al., 2010; Murray, A. W. et al., 2004 and Deibler, R. W. et al., 2010.*

G1/S Checkpoint

For example, in the presence of a DNA double strand brake (DSB), the Ataxia Telangiectasia Mutated (ATM) kinase is activated and triggers the G1 checkpoint by phosphorylating and activating the Checkpoint Kinase 2 (Chk2). In turn, Chk2 inhibits Cdc25A (a phosphatase that removes inhibitory phosphorylation of CDK2/cyclin A and CDK2/cyclin E complexes), preventing cells to proceed to the S phase(Medema & MacŮrek, 2012). In addition, ATM induces the phosphorylation of p53, leading to p53 stabilization. Stabilized p53 induces

p21$^{Cip/Waf1}$, which binds and inhibits CDK2/cyclin A and CDK2/Cyclin E complexes, DNA repair proteins and, if necessary, apoptotic cell death promoters(El-Deiry, 1998; El-Deiry et al., 1993).

G2/M checkpoint

On the other hand, when the DNA damage occurs in S and G2 phase, arising from a broader spectrum of DNA damaging lesions that generate single-stranded DNA (including nucleotide excision/repair process, stalled replication forks or as intermediates of DSB resolutions), the damage is sensed by Ataxia Telangiectasia and Rad3-related (ATR) kinases. ATR activates Checkpoint Kinase 1 (Chk1), which induces Cdc25A proteasomal degradation, blocking further progression through S phase (**Figure 1.3B**). Moreover, ATR and Chk1 also trigger the G2/M checkpoint, phosphorylating p53 and preventing cells with damaged DNA to enter mitosis by inhibiting the CDK1/cyclin B activation(Medema & MacŮrek, 2012).

In order to have an appropriate checkpoint, the response needs to be fast enough to prevent transition to the next phase with damaged DNA, and also durable enough to allow time for efficient DNA repair. The most well studied checkpoint protein is p53 and its transcriptional target p21. And in fact, both the G1 and G2 DNA damage control points are governed by the tumor suppressor p53(Kastan & Bartek, 2004; Laiho & Latonen, 2003; Medema & MacŮrek, 2012). The *TP53* gene is the most frequently mutated gene in human cancers(Rivlin et al., 2011). When the DNA damage checkpoint is triggered, the response will be according to the type of damage(Clancy & Education, 2008; Sancar et al., 2004; B. B. S. Zhou & Elledge, 2000):

- **Single strand brakes**

If the damage induces a single strand brake, the other strand will serve as a template to restore the sequence to the damaged strand. Two mechanisms exist

to remove the damaged nucleotides and replace them with undamaged ones, complementary to that found in the undamaged DNA Strand:

- Base excision repair (BER) - repairs **small, non-bulky DNA lesions** (nucleotides that have suffered relatively minor damage). The modified or damage base is removed by a DNA glycosylase, creating an apurinic or apyrimidic (AP) site. Then, enzymes called AP endonuclease nick the damaged DNA backbone at the AP site. DNA polymerases remove the damaged region and correctly synthesize the new strand using the complementary strand as a template.

- Nucleotide excision repair (NER)(Clancy & Education, 2008) - repairs **bulky, helix-distorting damage**, such as the ones caused by UV light. The damaged site is recognized, then there is an excision of the damaged DNA both upstream and downstream of the damage by endonucleases, and then there is a re-synthesis of removed DNA region.

- **Double strand breaks**

When both strands of the double helix are broken, thus leaving no strand template for repair, the cell can use one of two distinct mechanisms(Hefferin & Tomkinson, 2005):

- The Non-Homologous End Joining (NHEJ) repair, both strands are brought together and joined, however, this usually results in the loss of one or two nucleotides at the site of joining. NHEJ is active through the cell cycle, but it **occurs mostly in G1 phase**, before DNA replication, since there is no DNA template.

- Homologous Recombination DNA repair (HDR), requires an identical or nearly identical sequence to be used as a template for the repair. Therefore, in this pathway, a sister chromatid or a homologous chromosome is used as a template. This repair **usually happens in G2 phase**, after DNA replication. In this case, the repair machinery is able to transfer nucleotide sequence information from the intact double helix to the broken one(Clancy & Education, 2008; Hakem, 2008).

Because the detection of errors and repair mechanisms are crucial for cells, defects in the checkpoint mechanisms, either in the sensing or repairing of the DNA damage or in triggering apoptosis, can favor accumulation of mutations in genes essential for normal proliferation, leading to loss of proliferation control. Indeed, cancer cells are often defective in these checkpoint mechanisms, facilitating tumorigenesis. We can take as an example the SAC checkpoint, also referred as 'mitotic checkpoint' or 'M-phase checkpoint'. SAC prevents chromosome missegregation and aneuploidy by delaying cell division until accurate chromosome segregation is guaranteed. During mitosis, chromosomes must be correctly attached to the microtubule spindle apparatus via their kinetochores. If this attachment is not done properly, the kinetochores activate the SAC network, which blocks cell cycle progression to anaphase(Lara-Gonzalez et al., 2012).

The downstream target of the SAC is the anaphase promoting complex (APC), an E3 ubiquitin ligase that, as described above, targets several proteins for proteolytic degradation, including mitotic cyclins. Once all kinetochores are stably attached to the spindle, SAC is inactivated, alleviating the cell cycle block and allowing chromosome segregation and cell division(Lara-Gonzalez et al., 2012; Musacchio & Salmon, 2007). Dysfunction of SAC network has been implicated in aneuploidy and tumorigenesis(Bharadwaj & Yu, 2004), and in fact, there are therapies

targeting SAC molecular mechanisms for some cancer treatments(Marques et al., 2015).

The excess of proliferation seen in cancer is often associated with a vicious cycle, with a reduction in sensing signals that normally inform a cell to adhere, differentiate, or die. While normal tissues require signals before they can grow and divide, and the production and release of these signals is highly controlled in order to ensure a homeostasis in cell number, cancer cells, however, deregulate these signals, becoming self-sufficient in growth signals.

1.1.2 The Cell Cycle in Cancer

Control of the cell cycle is important to promote tissue expansion and differentiation in a regulated fashion. As discussed above, hundreds of genes intricately control the process of cell division, thus requiring a balance between the activity of genes that promote cell proliferation and those that suppress it. The most common mutations seen in cancer are mutations that generate either oncogenes with dominant gain of function, or tumor suppressor genes, with recessive loss of function. When a normal gene is mutated, which function is to encode proteins that stimulate cell division, it may become an oncogene, contributing to cancer progression(Adamson, 1987; Weinstein & Joe, 2006). These oncogenes exhibit increased activity, thus leading to increased cell division, decreased cell differentiation and inhibition of cell death.

The first oncogene discovered was *src* (proto-oncogene tyrosine-protein kinase), in the 70s, by Michael Bishop and Harold Varmus, who were later on awarded with a Nobel prize in Physiology or Medicine. The super-activation of this oncogene leads to survival, angiogenesis, proliferation and invasion of cancer cells. Another example of these growth-promoting genes, that become oncogenes, include RAS,

which acts to stimulate cell growth and division. Therefore, instead of stopping to divide at the proper time, mutations on these genes lead a cell to continuously progress through further divisions, accumulating genetic errors. Additionally, mutations in genes codifying cyclins D and E are also often seen in cancer(Deshpande et al., 2005; Stamatakos et al., 2010). Uncontrolled CDK's activity, where they are constitutively activated, induces unscheduled proliferation, as well as genomic instability.

In contrast to oncogenes, other cancer-related mutations inactivate genes that suppress cell proliferation or that trigger apoptosis. These genes are known as tumor suppressor genes, as they normally function as a brake on proliferation/cell division or are involved in the maintenance of cell cycle checkpoints or even promoting apoptosis. Once mutated, they no longer exert their normal role in the cell. The first tumor suppressor gene identified was *RB1,* in 1986(Dryja et al., 1986; Goodrich et al., 1991; Murphree & Benedict, 1984; Nevins, 2001)*,* which encodes the retinoblastoma protein (pRb). Its function relies on inhibiting the expression of genes required for the progression into S phase of the cell cycle. Therefore, inactivation of Rb will allow uncontrolled cell division. Rb protein dysfunction has been seen in several cancers, including retinoblastoma(Lohmann & Gallie, 2004), sarcomas(Cance et al., 1990; Reissmann et al., 1989; Wunder et al., 1991), glioblastomas(Venter et al., 1991), bladder(Ishikawa et al., 1991; Miyamoto et al., 1995) and breast(Varley et al., 1989) cancers. Another example of a common mutation in a tumor suppressor gene often seen in cancer refers to mutations in the *TP53* gene(Vogelstein & Kinzler, 2004). p53 is a multifunctional protein that normally senses different cellular stresses, such as DNA damage, inappropriate proliferation, oxidative stress or hypoxia, and also acts as a transcription factor for the expression of checkpoint control genes(Green & Kroemer, 2009; Riley et al., 2008; Yee & Vousden, 2005). As previously mentioned, p53 governs G1/S and

G2/M damage controls. So, genetic alterations that inactivate p53 will inhibit the DNA damage response that prevents cell cycle progression.

For the inactivation of tumor suppressors, both copies of the gene must be mutated in order for tumorigenesis to occur. If one copy of the gene is not mutated, it may provide sufficient activity for the cell to maintain proper growth and division. However, the mutated allele can also antagonize the WT allele, leading to a dominant negative function of the mutant protein(Milner et al., 1991; Milner & Medcalf, 1991). Despite this, such a heterozygous state is often transient as, for example, *TP53* mutations in cancer are frequently followed by loss of heterozygosity of the wild-type allele (LOH). This LOH is often seen when the other allele is either mutated (point mutations) or deleted(Rivlin et al., 2011). By losing p53, normal cells acquire advantages, such as dedifferentiation, epithelial to mesenchymal transition (EMT), and stem-like cell properties, that enables their survival, drug resistance and invasive capacities(Olivos & Mayo, 2016).

1.2 Epithelial to Mesenchymal Transition (EMT)

Cancer research has generated a rich and complex knowledge in the past decades showing that tumors are not an isolated cell mass. Rather, they are a complex tissue composed of multiple distinct cell types that participate in heterotypic interactions with one another. Therefore, the biology of tumors needs to acknowledge and embrace the contributions of the tumor microenvironment to tumorigenesis. Recently, it has become clear that some, or perhaps all types of cancer cells, acquire molecular, biochemical and cellular traits in order to have growth advantages. These traits were described as “The six Hallmarks of cancer” by Hanahan and Weinberg in 2000(Cavallo et al., 2011), and later on, in 2011, updated with four new hallmarks and enabling characteristics(Hanahan &

Weinberg, 2011). According to these authors, the complexity of cancer can be reduced into 10 principles: sustaining proliferative signals; evading growth suppressors; resisting cell death; enabling replicative immortality; inducing angiogenesis; activating invasion and metastasis; genome instability and mutation; tumor promoting inflammation; reprogramming cellular metabolism and, evading immune destruction. Lately, in 2021, four new characteristics have emerged to help understanding the complexities, mechanisms and manifestations of this disease. Besides non-mutational epigenetic reprogramming, polymorphic microbiomes, and senescence cells, **cellular plasticity** has emerged as an important trait to comprehend tumor growth and progression(Hanahan, 2022). Under normal conditions, such as embryonic development or tissue injuries, cellular plasticity serves as a mechanism of tissue adaptation or regeneration. However, it can also predispose tissues to tumor transformation(Brooks et al., 2015; S. Yuan et al., 2019).

Along tumor progression, microenvironment cues or stochastic genetic and epigenetic alterations, may trigger molecular and phenotypic changes, resulting in cellular plasticity, causing tumor heterogeneity, and consequently therapy resistance(Aggarwal et al., 2021; Jing et al., 2011; Quail & Joyce, 2013).

The most well-known case of tumor cell plasticity is the epithelial to mesenchymal transition (EMT), first described by Elizabeth Hay in the 1980s(Hay, 1995, 2005). EMT is the process by which cells switch from an epithelial phenotype towards a mesenchymal phenotype, undergoing several molecular, biochemical and morphological changes(Hay, 2005; Jing et al., 2011; Mani et al., 2008; Pastushenko & Blanpain, 2019). Epithelial cells are polarized, meaning that the apical and basal surfaces are likely to be visually different, with different functions, and adhering to different substrates. They are organized in a regular pattern next to one another, held tightly by cell-cell junctions and adhesion junctions, preventing cells from moving away of the epithelial monolayer. On the other hand,

mesenchymal cells do not exhibit intracellular adhesion, creating structures that are not homogeneous in composition, with irregular shapes, which provide them with increased migratory capacity(J. M. Lee et al., 2006). Moreover, mesenchymal cells possess a more extended and elongated shape, with a front-to-back leading-edge polarity (**Figure 1.4**). Regarding migration, mesenchymal and epithelial cells are mechanistically different. While mesenchymal cells move individually and in a more dynamic way, leaving part of the trailing region behind, epithelial cells move as a sheet, with the maintenance of adhesion between cells(J. M. Lee et al., 2006). Throughout development, cells may alternate between epithelial and mesenchymal stages multiple times. Gastrulation is one of the most remarkable examples of EMT, when epithelial cells of the epiblast lose their characteristics (apical-basal polarity and cell-cell junctions) and migrate along the primitive streak to form the three embryonic germ layers (endoderm, ectoderm, and mesoderm). Moreover, EMT is also essential for the development of the neural crest and of some organs, such as liver, kidney, and other tissues(S. Yuan et al., 2019). In adult tissues and homeostasis conditions, EMT is rarely observed, but it can emerge upon wound healing, tissue inflammation, and organ fibrosis, being also a common feature of malignancy(Boyer et al., 2000; Kalluri & Neilson, 2003). In cancer, EMT is well known for conferring stem-like properties and cell plasticity, leading to the acquisition of a migratory and invasive phenotype by weakening cell-cell adhesion, facilitating metastatic capacity, as well as resistance to chemo and radiotherapy(Jing et al., 2011; Mani et al., 2008; Pastushenko & Blanpain, 2019).

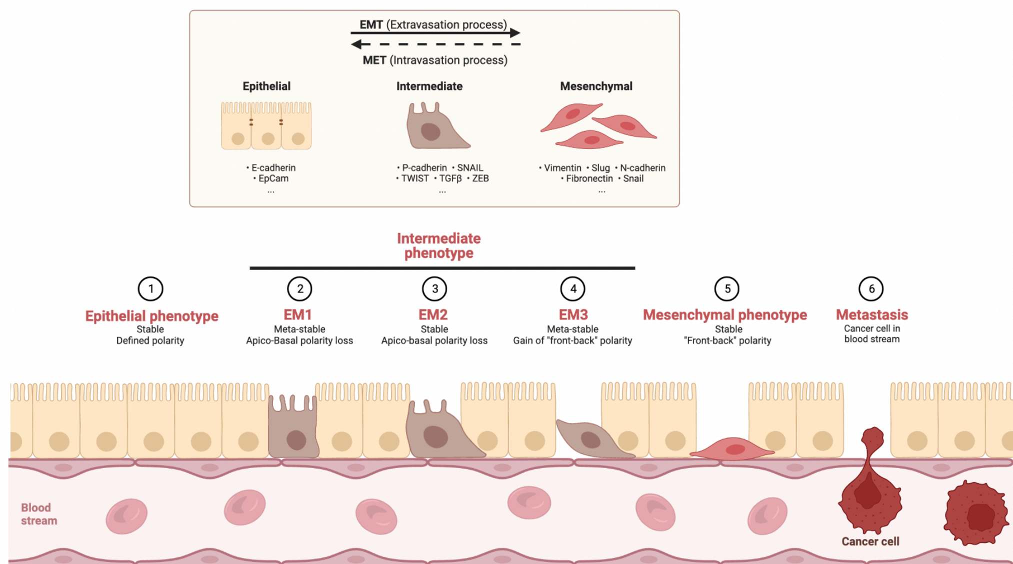

Figure 1.4. The Epithelial to mesenchymal Transition (EMT). Epithelial cells (Apical-Basal polarized cells with organized morphology) undergo phenotypic changes, by losing cell-cell adhesion and epithelial markers, acquiring mesenchymal characteristics, with an elongated shape with a front-to-back leading-edge polarity, with migratory and invasive capacity. Image created with BioRender online Software.

As discussed above, during the EMT process, cells change their morphology from an epithelial phenotype towards a mesenchymal phenotype. This transition leads to changes in cell membrane proteins responsible to maintain cell-cell adhesion and an organized epithelium. Cadherins are transmembrane glycoproteins that mediate cell-cell adhesion, maintaining a normal tissue architecture(Kaszak et al., 2020; Maître & Heisenberg, 2013; Peinado et al., 2004). At the cell membrane, cadherins are present as homodimers tethered to the actin cytoskeleton via multiprotein complex, including α-, β-, and p120-catenin, forming a cadherin-catenin complex. Classical cadherins include epithelial-cadherin (E-cadherin), placental-cadherin (P-cadherin) and neural-cadherin (N-cadherin)(Kaszak et al.,

2020). EMT is known for inducing E-cadherin loss, a crucial component of the epithelial adherens junctions and, in parallel, a concomitant induction of genes associated with the mesenchymal state, such as N-cadherin(Gheldof & Berx, 2013; Lamouille et al., 2014; Loh et al., 2019), known as the “cadherin switch”(Nieman et al., 1999; Onder et al., 2008). The increase in E-cadherin is described as a mechanism of tumor suppression, preventing tumor cells from dissociating from one another, and consequently, avoiding cell migration.(Gheldof & Berx, 2013; Loh et al., 2019). Moreover, E-cadherin can also antagonize the nuclear signaling function of the proto-oncogene β-catenin, altering its ability to regulate target genes that support tumor invasion(A. S. T. Wong & Gumbiner, 2003). In addition, NF-κB activity can be downregulated by the E-cadherin-catenin complex, which promotes inflammation-associated carcinogenesis(Cowell et al., 2009). The loss of E-cadherin, is associated with increase in P-cadherin and N-cadherin, causing cytosolic translocation of p120-catenin, which leads to the induction of tumor cell migration, tumor anchorage-independence (*anoikis* resistance) and increased tumor growth *in vivo*(Gheldof & Berx, 2013; Kaszak et al., 2020; Loh et al., 2019; Ribeiro & Paredes, 2015; Taniuchi et al., 2005).

Tumors contain many of the factors shown to induce EMT during embryogenesis, including growth factors, matrix components, hypoxia, inflammation and, increased tissue stiffness. Transforming growth factor beta (TGFβ), epidermal growth factor (EGF), hepatocyte growth factor (HGF), NOTCH, fibroblast growth factor (FGF), and WNT ligands are some few examples of the pleiotropic and evolutionarily conserved signaling factors that can induce EMT(Puisieux et al., 2014; S. Yuan et al., 2019) . These signaling factors set off a chain of events that results in the expression of one or more so-called “EMT transcription factors” (EMT-TFs). The primary role of EMT-TFs, which include SNAIL1/2, TWIST, ZEB1/2 and PRRX family members (among others), is the suppression of essential genes responsible for maintaining the epithelial state (i.e., proteins comprising junctional complexes

and epithelial intermediate filaments)(Lamouille et al., 2014; S. Yuan et al., 2019). In fact, the main cause of cadherin switch during EMT is due to transcriptional regulation of cadherin expression through the several factors mentioned above. These EMT-TFs acts by directly binding and repressing E-cadherin promoter(Gheldof & Berx, 2013; Kaszak et al., 2020; Peinado et al., 2004). Importantly, EMT does not represent a binary process. Instead, it encompasses a phenotypic spectrum characterized with different degrees of epithelial and mesenchymal features and involves a range of molecular mechanisms. Mesenchymal to Epithelial Transition (MET) is the reverse process of EMT, and can also occur during cancer progression. While EMT seems to be important to facilitate tumor invasion, MET may facilitate tumor growth at the distant site (i.e., metastatic colonization)(S. Yuan et al., 2019). In a spontaneous model of squamous cell carcinoma, the ectopic expression of the EMT-TF TWIST1 promoted EMT and invasion, but inhibited colonization. However, only when TWIST1 was repressed could metastatic growth occur(Tsai et al., 2012). Similarly, knockdown of the EMT-TF PRRX1 in a model of breast cancer metastasis resulted in MET and efficient lung metastasis(Ocaña et al., 2012). Moreover, *in vitro* overexpression of SNAIL1, TWIST1 and Zeb1 in epithelial carcinoma cells has been shown to promote loss of E-cadherin, acquisition of a spindle-like mesenchymal phenotype, and enhanced migratory and invasive behavior(Batlle et al., 2000; Cano et al., 2000; Krebs et al., 2017; Ohkubo & Ozawa, 2004; Peinado et al., 2007; Y. Wang et al., 2014; J. Yang et al., 2004). Loss-of-function studies, on the other hand, have been more difficult to interpret, implying that tissue- and context-dependent differences dictate the molecular mechanisms underlying EMT in a given tumor. In a spontaneous mouse model of pancreatic ductal adenocarcinoma, depleting either SNAIL1 or TWIST1, has little effect on metastasis(Zheng et al., 2015), whereas deleting ZEB1 in a similar model drastically reduced colonization, invasion, and metastasis(Krebs et al., 2017).

Taken together, these studies demonstrates that epithelial-mesenchymal plasticity is crucial for cancer progression and metastasis: while mesenchymal phenotypes favor invasive behavior at the primary site, epithelial phenotypes favor tumor growth at metastatic sites(Lamouille et al., 2014). Moreover, distinct and overlapping EMT inducers, including transcriptional factors, play distinct roles in metastatic spread. This conceptual flexibility has led to the recognition that cells can also encounter in an "intermediate" or "hybrid" epithelial-mesenchymal phenotype – described as partial-EMT, having properties that distinguish them from cells with purely epithelial or mesenchymal phenotypes(Aggarwal et al., 2021; Saxena et al., 2020; S. Yuan et al., 2019) **(Figure 1.4)**. Cells in such intermediate EMT state may express both epithelial and mesenchymal characteristics, or may also have lost epithelial traits without acquiring mesenchymal characteristics(Kröger et al., 2019; Pastushenko & Blanpain, 2019). Studies have shown that the intermediate EMT state promotes tumor collective cell migration(Aiello et al., 2018; Armstrong et al., 2011; Jolly et al., 2016; Lecharpentier et al., 2011), resulting in the formation of highly metastatic circulating tumor cell clusters (CTCs)(Aceto et al., 2014; Armstrong et al., 2011; Lecharpentier et al., 2011; Maddipati & Stanger, 2015). For this reason, and because cells with partial-EMT possess greater plasticity, the intermediate EMT state is thought to confer cancer cells a higher metastatic ability as compared to complete EMT cells(Aggarwal et al., 2021; Aiello et al., 2018; Jolly et al., 2015; Saxena et al., 2020) . Recently, P-cadherin expression has been widely described in human cancers, as well as in animal models as a potential biomarker for the intermediate-EMT state(Ribeiro & Paredes, 2015; Sousa et al., 2020). P-cadherin overexpression has been shown to be significantly associated with collective cell invasion, stem-cell properties and tumorigenesis, and also statistically correlated to high-grade tumors, tumor aggressiveness, and poor patient prognosis(Albergaria et al., 2011; Paredes et al., 2002, 2005, 2007; Ribeiro et al.,

2010; Vieira & Paredes, 2015). One of the functional markers used to identify cells in the intermediate EMT state is their capacity to resist to *anoikis*, a specific mode of apoptosis(Hosein Mohimani, Alexey Gurevich, Alla Mikheenko, Neha Garg, Louis-Felix Nothias, Akihiro Ninomiya, Kentaro Takada, Pieter C. Dorrestein3, 2017; Paoli et al., 2013) (discussed below). Interestingly, P-cadherin overexpression has been shown to induce *anoikis*-resistance of breast cancer cells, by disturbing epithelial cell-cell adhesion, promoting the acquisition of a more undifferentiated cell phenotype, resulting in a phenotypic state where cells stay between epithelial and mesenchymal morphologies(Sousa et al., 2020) .

Cancer cells live in a complex tumor microenvironment (TME), with different cellular and non-cellular components, such as, fibroblasts, endothelial cells, leukocytes, and extracellular matrix (ECM). While these TME components are known to have a strong influence on the phenotype and function of cancer cells, cancer cell signaling can also have a strong influence on the TME. As a result, changes in cancer cell phenotype (due to cellular plasticity) can have a significant impact on surrounding non-cancer cells. These non-cell autonomous effects of cellular plasticity have received much attention, as the mesenchymal state is associate with a different secretome than the epithelial state. EMT in Madin-Darby Canine Kidney (MDCK) cells, for example, results in increased secretion of extracellular proteases, such as MMPs, as well as ECM constituents, like collagens, fibulins and SPARC(Mathias et al., 2010). These secreted factors, in turn, have effects on stromal cells in the TME, influencing fibroblast migration and blood vessel branching. Similarly, studies demonstrated that when cells with high E-cadherin expression are compared to those with low E-cadherin expression, the secretome of these cells are markedly different(Rasanen et al., 2013). Therefore, cells that have undergone EMT may also secrete different factors, allowing for long-distance modifications of the TME or other tumor or non-tumor cells. In fact,

it has been shown that P-cadherin overexpression in breast cancer cell models promotes secretion of MMP-1 and MMP-2, which then lead to P-cadherin ectodomain cleavage(Ribeiro et al., 2010). The soluble P-cadherin (sP-cad) present in the conditioned media of these cells is able to induce *in vitro* invasion of breast cancer cells, demonstrating the impact of the secretome underlying the invasive behavior of P-cadherin expressing cells.

In conclusion, normal development and tissue regeneration rely on the phenotypic adaptability embodied by cellular plasticity. However, cancer cells use this malleability to gain a selective advantage. By using cellular plasticity programs, such as EMT, cancer cells adapt to an unfavorable metabolic environment, avoiding immune attack, spreading from a primary to a metastatic site, and avoiding the toxicity of anti-cancer drugs. Thus, many obstacles preventing cancer treatment are embodied in cell plasticity/EMT programs. As EMT occurs in a variety of circumstances and rarely involves stable genetic alterations, it is still challenging to understand it in terms of molecular mechanisms. Therefore, mechanistic investigations of cell plasticity have been receiving a greater emphasis. The combination of *in vivo*, *in vitro* and clinical studies, has demonstrated that tumor initiation is frequently linked to plasticity and that therapeutic resistance can develop through plasticity(J. M. Lee et al., 2006; Peinado et al., 2004; Zheng et al., 2015). Additionally, is it now known that any cell type can give rise to a carcinoma in a normal tissue, suggesting that the mechanisms that cause cells plasticity, and undergo EMT or MET, are also plastic, depending on the genetic perturbation(Brooks et al., 2015; Nakajima, 2018; Saitoh, 2018b; S. Yuan et al., 2019). These findings provide a rationale for further investigation into the genetic, epigenetic, transcriptional and, post-transcriptional pathways governing cellular plasticity. A better understanding on how cancer cells adapt, and achieve such extensive changes in cell phenotype, will allow the development of new therapeutic strategies for what is likely to be the most difficult aspect of human tumors.

1.3 Cell death by *Anoikis*

As described above, in order for cells to undergo EMT, there is a loss of epithelial markers, where cells detach from other cells and from the ECM, gaining an elongated cell shape with migratory capacities, and resistance to *anoikis*. *Anoikis* is described as the apoptotic cell death caused by anchorage-free/cell-matrix disrupting conditions(Frisch & Screaton, 2001; Grossmann, 2002; Paoli et al., 2013). In normal conditions, when there are insufficient cell-matrix interactions, cells are cell cycle arrested and targeted to die by *anoikis* in order to avoid dysplastic growth. However, some cancer cells (like cancer stem cells) overcome *anoikis*, in order to achieve dissemination and distant organ colonization **(Figure 1.5).**

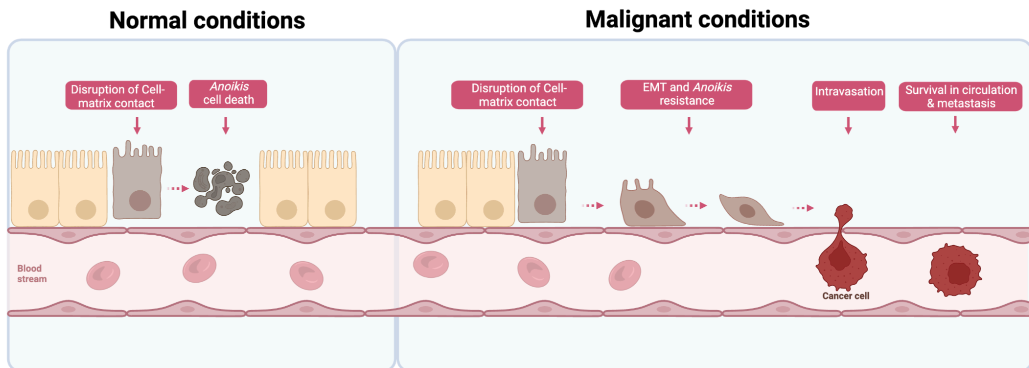

Figure 1.5. *Anoikis* cell death in normal and in malignant conditions. Normal cells die by *anoikis* once they detach from other cells and from the extracellular matrix. However, cancer cells adapt, and are able to survive to *anoikis*, enhancing their metastatic potential.EMT, epithelial to mesenchymal transition. Image created with BioRender online Software.

The ability for most cells to proliferate depends on two major signals: 1) proper positioning within the tissue and, 2) growth factors and cytokines for proliferation(Guadamillas et al., 2011). Integrins are cell surface receptors responsible to sense the ECM, connecting it to the cytoskeleton, activating several cellular signaling pathways, influencing this way cell responses to stimuli(Jean et al., 2011; J. W. Lee & Juliano, 2004; Shattil et al., 2010). Integrins and their downstream signaling mediators, such as the non-receptor tyrosine kinase Src, focal adhesion kinase (FAK), and integrin-linked kinase (ILK), are activated specifically to protect adherent cells against *anoikis*(Frisch & Screaton, 2001; Guadamillas et al., 2011; Meredith et al., 1993). This occurs during physiological developmental processes, including the hollowing of glands and involution processes, where *anoikis* is crucial(Chiarugi & Giannoni, 2008).

There are two distinct mechanisms by which *anoikis* can be mediated: the intrinsic or the extrinsic apoptotic pathways(Guadamillas et al., 2011) **(Figure 1.6).** The intrinsic pathway involves the participation of the pro-apoptotic members of the Bcl-2 (B cell lymphoma-2) protein family, such as Bad (Bcl-2-associated death promoter), Bax (Bcl-2-associated X protein), Bid (Bcl-2-interacting domain) and Bim (Bcl-2-interacting mediator of cell death). The Bcl-2 protein family members permeabilize the mitochondrial membrane, resulting in the release of pro-apoptotic factors, such as cytochrome c and SMAC/DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI) into the cytosol, leading to the activation of caspases(Chiarugi & Giannoni, 2008; Frisch & Screaton, 2001; Grossmann, 2002). The extrinsic pathway comprises the stimulation of death receptors, such as Apo1/Fas or TRAIL [tumor necrosis factor (TNF)-related-apoptosis-inducing ligand] receptor(Frisch & Screaton, 2001; Grossmann, 2002). As a result, a death-inducing signaling complex (DISC) is formed, activating caspase-8. Caspase 8 can directly induce apoptosis, or it can also activate Bid, triggering the intrinsic pathway(Chiarugi & Giannoni, 2008).

Caspases are sequentially activated by both intrinsic and extrinsic apoptotic pathways, which leads to DNA degradation and cell death(Frisch & Screaton, 2001; Grossmann, 2002; Guadamillas et al., 2011; Paoli et al., 2013). Numerous strategies have been developed by tumor cells in order to avoid *anoikis* and outcome cell ability to grow and metastasize(Guadamillas et al., 2011). Some of them involve cellular adaptation that enable cancer cells to act as if they were normal cells, such as producing ECM components although not attached to them, and thus preventing the induction of *anoikis.* Other approaches aim to hyperactivate survival and proliferative cascades, in order to counterbalance the consequences of *anoikis* induction. More recently, autophagy (or catabolic self-degradation) and entosis (cell-in-cell invasion) has been shown to contribute for *anoikis* circumvention, by inducing cells into a dormant state until they receive a signal initiated at the ECM(Guadamillas et al., 2011).

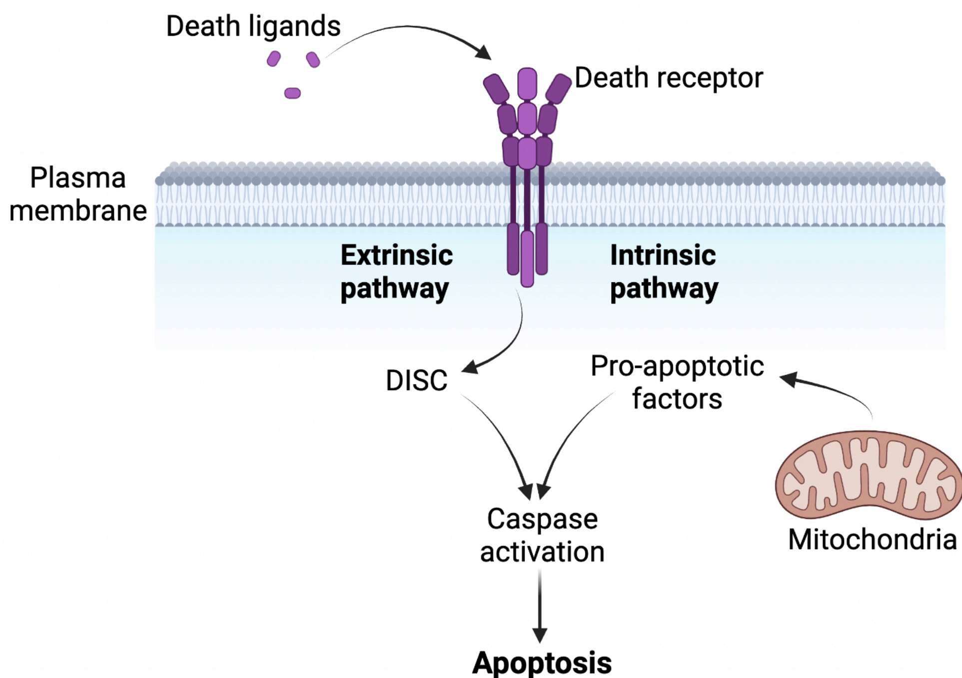

Figure 1.6. Intrinsic and extrinsic *anoikis* pathways. Pro-apoptotic factors are released from the mitochondria in the intrinsic *anoikis* pathway, activating caspase signaling. In the extrinsic pathway, the binding of extracellular apoptotic "death" ligands to their specific receptors at the plasma membrane is required for the initiation of the pathway. Both pathways can occur independently until the final step of DNA degradation, or they can crosstalk earlier and act simultaneously. DISC, death-inducing signaling complex. *Adapted from Guadamilas et al., 2011*. Image created with BioRender online Software.

Anoikis resistance is a crucial step in the acquisition of malignancy(Chiarugi & Giannoni, 2008; Freedman & Shin, 1974). Cells that are able to circumvent *anoikis* have the potential to disseminate through the body, colonize other tissues and grow to form metastasis(Gassmann & Haier, 2008; Guadamillas et al., 2011). The relationship between EMT and *anoikis* resistance is considered as an adaptive strategy during cancer progression. By acquiring an EMT-like phenotype, cells adapt to the new environment (detached from ECM and other cells), by de-differentiating and regaining the ability to proliferate and migrate, or by altering their surface integrins repertoire(Guadamillas et al., 2011).

EMT and *anoikis* resistance share common regulators that can modulate both the increased expression of cell survival genes and the coordinated balance of expression of epithelial and mesenchymal genes(Guadamillas et al., 2011). For instance, the tuberous sclerosis complex 2 (TSC2), also known as tuberin in epithelial cells, whose absence results in E-cadherin downregulation and resistance to *anoikis;* and the guanine-nucleotide-exchange factor Tiam1 in colon tumor cells, which also leads to resistance to *anoikis* through E-cadherin downregulation(Guadamillas et al., 2011). Additionally, and as previously mentioned, the expression of some EMT master transcriptional regulators, is linked to the acquisition of *anoikis* resistance, such as NF-κB, which confers *anoikis* resistance in a PI3K-Akt-pathways-dependent manner(Cowell et al., 2009; Madrid et al., 2001; Ozes et al., 1999). The Snail EMT-TF, which induces EMT by

repressing E-cadherin transcription, suppresses cell death by inhibiting caspase-3 and by activating the pro-survival PI3K-Akt pathway(Barrallo-Gimeno & Nieto, 2005; Vega et al., 2004) , while the transcription factor Twist, which regulates EMT by increasing the expression of N-cadherin and fibronectin, promotes *anoikis* resistance by modulating the levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax(Kwok et al., 2005). The Twist-Snail signaling axis, which is involved in EMT, *anoikis* resistance and metastasis, is triggered by the neurotropic tyrosine receptor kinase (TrkB), which has also been associated with tumor malignancy and metastasis(Smit et al., 2009). Furthermore, the coordinated regulation of EMT and *anoikis* is demonstrated by the fact that the polarity regulator Scribble alters the normal three-dimensional structure of the acinar lumen by impairing the establishment of the apical-basal polarity, which results in EMT-like de-differentiation, and by promoting *anoikis* resistance via pathways involving Rac and JNK(Zhan et al., 2008).

Another mechanism that cancer cells use to circumvent *anoikis* is by "ignoring" signals that would normally lead to cell cycle arrest and *anoikis*, by continuously activate the pro-survival signals, such as PI3K, Ras-Erk, NF-κB and Rho GTPase(Tsuji et al., 2009). Autocrine secretion of growth factors, such as HGF, interleukin (IL)-8 and platelet derived growth factor AA (PDGF-AA) in melanoma cells, resulted in an activation loop that trigger proliferation, survival and migration pathways(G. Li et al., 2003). Furthermore, it has been shown that, in the MCF10A cell line, the overexpression of receptor tyrosine kinases (RTKs), such as EGF receptor (EGFR)(Reginato et al., 2003) and TrkB, or the HGF receptor MET in ovarian cancer cells, leads to *anoikis* resistance(Douma et al., 2004; Tang et al., 2010). Moreover, *anoikis* resistance in mammary epithelial cells can also be induced by the overexpression of the RTK ErbB2, through inhibition of the pro-apoptotic protein Bim(Reginato et al., 2003), by increasing the expression of integrin $\alpha 5$ subunit and subsequent Src activation(Haenssen et al., 2010).

Several apoptotic factors, and other signaling molecules, can also be associated to the cytoskeleton, acting therefore as cytoskeletal changer sensors, in a way to prevent *anoikis*(Chiarugi & Giannoni, 2008; Frisch & Ruoslahti, 1997). As an example, the pro-apoptotic proteins Bim and Bcl-2-modifying factor (Bmf) are sequestered by myosin motor complexes when cells are attached to the ECM, and released when there is loss of cell adhesion, and consequently triggering *anoikis*(Puthalakath et al., 2001; Strasser et al., 2000). Additionally, the Src family member p66Shc (focal-adhesion-associated 66kDa isoform of the Src homology and collagen) can also sense attachment created by focal-adhesion-like sites through a RhoA-dependent tension(Ma et al., 2007). In accordance, absence of p66Shc in lung tumors, leads to this mechanosensitive failure, suppressing *anoikis* through Ras hyperactivation and RhoA inactivation(Ma et al., 2007).
Metabolic and oxidative stress can also be triggered by loss of cell adhesion. The production of reactive oxygen species (ROS) following detachment is correlated with *anoikis*(A. E. Li et al., 1999); however, oxidative stress can have opposing effects on cell survival. Overexpression of oncoproteins, such as ErbB2, rescues the ATP deficiency by restoring glucose uptake in an EGFR- and PI3K-pathway dependent maner(Schafer et al., 2009), avoiding in this way a metabolic stress. On the other hand, increased ROS has been detected during metastasis, probably due to hypoxia, which could have a protective effect associated with ROS's ability to elicit pro-survival signals(Giannoni et al., 2008). This suggests that the role of ROS in *anoikis* resistance is probably dependent on several factors and could also be influenced by the crosstalk between integrins and growth factor signaling.

As previously mentioned, two new cellular processes, autophagy and entosis, were described as a potential mechanism for cell survival upon detachment (Guadamillas et al., 2011). Autophagy is a catabolic process in which organelles, proteins and cytosol are destroyed through the lysosomal self-digestion

mechanism. In starvation conditions, autophagy is induced in response to stress, enabling cells to obtain energy and to eliminate damaged organelles/proteins generated due to oxidative damage(Mizushima & Levine, 2010). In short term, autophagy can act as a survival mechanism but, in other situations, it can also cause cell death, suggesting an important balance regulation(Debnath et al., 2005). The same is observed during cancer progression: autophagy can act both as tumor promoter or tumor-suppressor. Autophagy can be induced in less-vascularized areas of the tumor in response to starvation and hypoxia, limiting primary tumor growth(Guadamillas et al., 2011). Furthermore, necrotic cell death tumors and the subsequent inflammatory response can promote tumor growth and metastasis. This situation can be avoided if cells undergo autophagy rather than necrosis, allowing the elimination of cells without causing inflammation (Degenhardt et al., 2006; Mathew et al., 2007). On the other hand, autophagy can induce tumor cell dormancy, allowing their survival in unfavorable conditions, and reactivating their metabolism and cell cycle when conditions improve. Moreover, when autophagy is unable to provide enough energy for the cell to survive, excessive cell consumption can promote tumor necrosis, and the resulting chronic necrosis and inflammation, which is similar to a wound healing process, can stimulate angiogenesis and thus, become an additional contribution of autophagy to tumor growth(Mathew et al., 2007; Yun et al., 2021).

To avoid *anoikis*, autophagy can also provide a mechanism for pre-metastatic tumor cells to migrate. In fibroblasts, for example, the reduced integrin signaling after detachment from the ECM can cause autophagy, delaying the onset of apoptosis and giving cells the ability to survive and reactivate once they attach to the ECM(Fung et al., 2008). Moreover, detachment-induced autophagy contributed to glycolysis in cells expressing the oncogene Ras, promoting cell proliferation(Lock et al., 2011). The increased glycolysis is one crucial feature that enables the support of cancer cells transformation and high proliferation

rate(Heiden et al., 2009). Taking all these factors into account, the specific conditions of each tumor cell determine whether autophagy will act to limit tumor growth or favor resistance to *anoikis* and metastasis.

Recently, a non-apoptotic cell death process has been reported in matrix-detached cells. Entosis is described as a process that is initiated when a cell actively invades a homotypic cell(Guadamillas et al., 2011). After invasion, the invading cell enters an intermediate state, remaining alive within the host cell, until is either degraded by lysosomal mechanism or released(Overholtzer et al., 2007). This phenomenon, also called "cell-in-cell", was first described in 1925 by Lewis Warren(Lewis, 1925), as a non-phagocytic process in which immune cells "eat" other cells. Later one, the same mechanism (described as cell cannibalism) was observed in metastatic tumors, being considered a survival strategy by which tumor cells kill the immune cells, avoiding their elimination(Lugini et al., 2006). Cell cannibalism can also represent a mechanism that ensures sufficient nutrient supply in response to metabolic stress and starvation(Fais, 2007). However, while cell cannibalism is a non-selective mechanism, meaning that cells are "eated" regardless of whether they are dead or alive, entosis is an active invasion of one living homotypic cell by another. The formation of adherens junctions, which involve cadherins, appears to be required for the initiation of entosis following ECM detachment, since the unbalanced myosin II contractile forces associated with adherens junctions push one cell into another. Adhesion to the ECM balances these forces, preventing cell internalization in a β1-integrin-dependent manner. Despite the internalizing cell actively participates in a Rho-ROCK pathway-dependent manner, the activation of this pathway in host cells is not required for the invading cell to be internalized(Overholtzer et al., 2007). However, the exact mechanism by which the invading and recipient detached cells coordinately regulate these processes is still unknown.

Entosis usually results in non-apoptotic, lysosomal cell death, but the internalized cell can occasionally be released. Furthermore, a small proportion of internalized cells is able to divide(Overholtzer et al., 2007), demonstrating that entotic cells can survive within their hosts. Entosis has been described in tumors where ECM deposition was undetectable, regardless of whether the tumor was primary or metastatic, suggesting that this process may be triggered by the loss of ECM attachment(Overholtzer et al., 2007). Entosis may be a secondary mechanism to *anoikis* that aids in the clearance of detached and misplaced cells; however, its biological significance is still unknown. Yet, entosis can have a tumor suppression and/or pro-survival role: while inhibiting entosis in soft agar increases anchorage-independent growth, implying that entosis plays a role in tumor suppression(Overholtzer et al., 2007), the survival and later release of entotic cells, suggests a pro-survival role what determines which fate an internalized cell takes still need further studies.
Upon loss of adhesion, the decision whether a cell initiates entosis or *anoikis*, may give the cell more time to determine whether the deleterious conditions are transient or not. Thus, entosis can be a strategy for removing detached cells without killing them until it is clear if cells must die or not. However, a study has shown that this can be harmful to the organism, since entosis can disrupt cytokinesis and induce cell aneuploidy, promoting thus tumor formation(Krajcovic et al., 2011). Entosis, like autophagy, is a cellular mechanism with anti-tumor effects, in which cancer cells can, in certain conditions, take advantage off and scape *anoikis*, becoming anchorage independent.

The self-defense mechanism *anoikis* plays a critical role in preventing tumor dissemination and metastatic growth, by preventing cells from leaving their natural niche and grow somewhere else. Tumor cells however, use a variety of strategies to avoid this process, losing the anchorage dependency **(Figure 1.7)**. They can

either partially revert to their undifferentiated phenotype by changing the pattern of integrin and other adhesion molecule expression (EMT), or they can develop strategies to overcome detachment-induced cell cycle arrest of cell death (*anoikis*). Despite tumors releasing a large number of cells into vessels, only cells with anchorage-independent growth capacities are able to survive, grow and colonize different tissues. Due to the plasticity derived from their poorly differentiated state, cells can better readapt to their new surroundings and form metastasis.

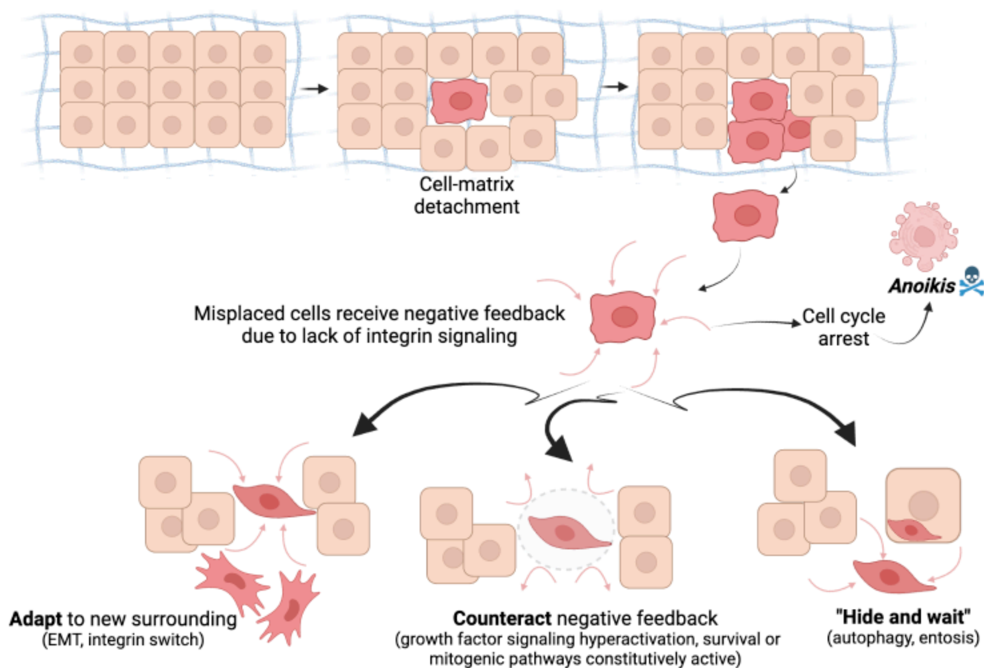

Figure 1.7. Transformed cells use different strategies to circumvent or avoid *anoikis,* becoming thus anchorage-independent. These strategies follow three distinct patterns: adapting to the new environment, through EMT or integrin switching; counteracting negative signaling by hyperactivating survival or mitogenic pathways; or by "hiding and wait", entering a dormant state via autophagy or entosis, and then reactivating the cell cycle once conditions are favorable. *Adapted from Guadamilas et al., 2011.* Image created with BioRender online Software.

1.4 The Centrosome

The centrosome was first discovered in 1880s by Teodor Boveri(Boveri, 1887) and Edouard Van Beneden(van Beneden, E., and Neyt, 1887) and was described as a vital organelle required for animal cells to divide. Centrosomes are the main microtubule (MT) organizing center in cycling animal cells, being formed by a pair of orthogonally positioned barrel-shaped centrioles, called the mother and daughter centrioles, embedded in a proteinaceous matrix called the pericentriolar material (PCM), which confers the MT nucleation capacity(Gould & Borisy, 1977) (**Figure 1.8**). Besides the difference in age between the mother and daughter centriole (the mother centriole was built one cell cycle earlier than the daughter), they also differ structurally at their distal ends, with the older mother centriole, also called grandmother, containing distal and subdistal appendages required for MT anchorage and ciliogenesis, serving as the basal body for cilia and flagella formation (**Figure1.8**). Cilia and flagella are important structures involved in many processes, including sensing extracellular signals, moving fluid and cell motility. Centrioles and cilia are found in most eukaryotic cells with the exception of higher fungi, amoebas and higher plants(Carvalho-Santos et al., 2011).

In addition to its role in cilia and flagella formation, the centrosome also participates in several other processes such as cell polarity and intracellular traffic in interphase, spindle pole organization during mitosis, and also in cell migration(Bettencourt-Dias & Glover, 2007; Stevens et al., 2007; Vinogradova et al., 2012; X. Wang et al., 2009). Abnormalities in centrosome number and structure have been seen in many diseases, including cancer. Centrosomes must thus be generated with high structural fidelity and rigorous number control.

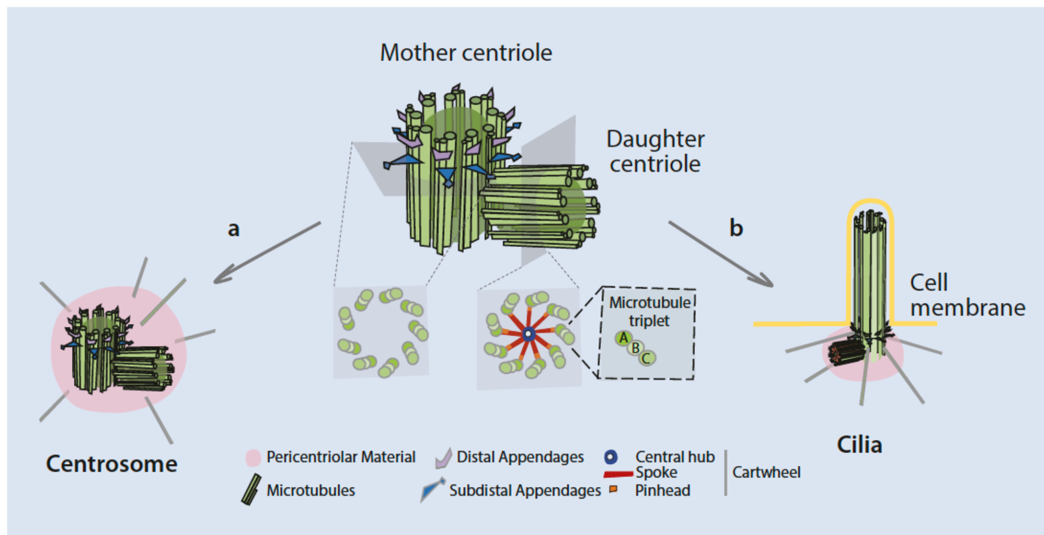

Figure 1.8. Centriole forms centrosomes and cilia. The centrosome is the major microtubule-organizing center in cycling animal cells. The centrosome comprises two cylinder-shaped microtubule-based structures, the centrioles. Each centrosome is composed of a mother and a daughter centriole. The mother centriole possesses distal (purple stick) and subdistal (blue circle) appendages, necessary to anchor at the plasma membrane, serving also as cilia/flagella basal body. **(A)** Schematic representation of a pair of centrioles, which forms the centrosome, surrounded by an organized protein matrix cloud, the pericentriolar material (PCM- pink cloud). As observed, the mother centriole can be distinguished by the distal (purple stick) and subdistal (blue) appendages, and the daughter centriole by the possession of the cartwheel structure. The PCM nucleates and organizes microtubules, ensuring that the centrosome functions as the major microtubule-organizing center in many cells. During G1, the centrosome accumulates small amounts of PCM. At the end of G2, with the preparation to enter in Mitosis, the centrosome accumulates additional PCM, allowing them to organize many more microtubules during mitosis. **(B)** In some non-dividing cells, the centrosome migrates to the cell membrane and assemble cilia from the mother centriole, enabling cell movement and/or chemoreception.

1.4.1 Centrosome Biogenesis

Centrosomes can be formed *de novo* or through a centriole-guided mode. In the *de novo* mode, centriole biogenesis occurs in the absence of a pre-existing one, and occurs mostly in species that only form centrioles at certain phases of their life cycle (e.g., to form motile sperm in mosses(Hodges et al., 2012)). In the centriole-guided mode, procentrioles form exclusively in association with a pre-existing centriole (new daughter centriole forms orthogonally to the existing 'mother' one). This is the major route of assembly in cycling cells and occurs in what is called the canonical centrosome duplication cycle.

Centrosome number and shape are tightly controlled through the canonical centrosome duplication cycle, which is coupled to the chromosome cycle. As cells enter G1 phase, they possess a single centrosome. During G1 the centrioles disengage but remain connected by a fibrous structure, known as interconnecting fibers. In late G1 and beginning of S phase, the centrioles start duplicating in a semi-conservative manner, forming new centrioles perpendicularly to the existing ones. The daughter procentrioles then elongate and maturate, recruiting PCM. Following maturation of the centrosome at the G2 phase and entry into mitosis, the two centrosomes separate to opposite poles of the cell to help forming the mitotic spindle, contributing to appropriate chromosome segregation (**Figure 1.9**). In this cycle the centrosome is duplicated once and only once per cell cycle, and upon cell division, each daughter cell inherits a single centrosome.

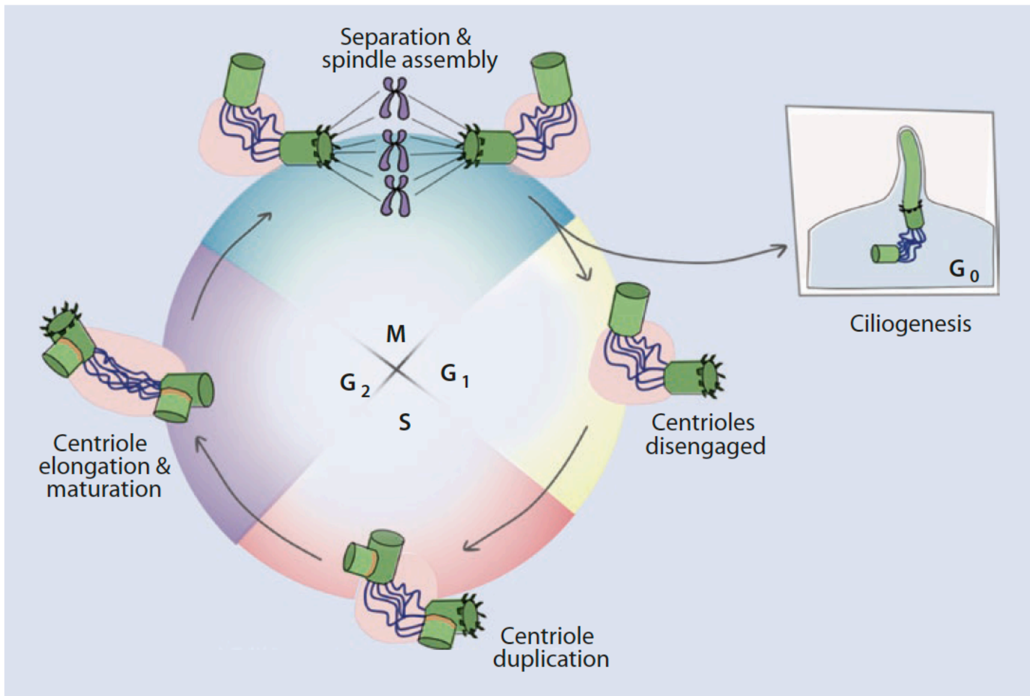

Figure 1.9. Centriole biogenesis. Schematic representation of centrioles duplication cycle**.** The mother centriole can be distinguished by the presence of appendages. The centriole cycle can be described in four steps: 1) Centriole disengagement: During late mitosis and G1 phase the centrioles disengages, but keep tethered. 2) Centriole duplication: In late G1/S, centrioles start duplicating in a semi-conservative manner, forming new centrioles (procentriole) perpendicularly to each mother centriole. 3) Centrosome elongation and maturation: In late S/G2, the procentriole elongates and mature, by recruiting PCM (yellow cloud). 4) Centrosome separation and spindle assembly: At the onset on G2/M the two centrosomes separate, move to opposite poles of the cell and establish the mitotic spindle, contributing to appropriate chromosome segregation. When the cell exits the cell cycle and enters G0, centrioles can move to the plasma membrane, becoming basal bodies and assembling cilia. In mitosis, only the grand-mother centriole possesses the appendages, as the appendages in the newly mother centriole are reabsorbed during mitosis.

1.4.1.1 Polo-like kinase 4 (Plk4)

A key component for centriole biogenesis is the protein kinase Polo-like-kinase 4 (Plk4), which, when in excess, triggers the formation of supernumerary centrosomes, whereas its depletion causes a reduction in centriole number(Bettencourt-Dias et al., 2005; Habedanck et al., 2005a).

Polo-like kinases (Plks) in mammals are essential regulators of the cell cycle, centriole duplication, mitosis and cytokinesis(Lowery et al., 2005; Zitouni et al., 2014). The Plk family in mammals consists in five members: Plk1, Plk2, Plk3, Plk4 and Plk5, differing in their functions and structure. Structurally, all Plks have two polo-box domains (PBD) at the C-terminal (except Plk4), and a catalytically active kinase domain (KD) at the N-terminal (except for Plk5) **(Figure 1.10)**. While the KD determines the kinase activity, the PBD is required for substrate binding through protein-protein interaction and regulation of the kinase activity(Lowery et al., 2005; Vaid et al., 2016; Zitouni et al., 2014). The KD contains an ATP-binding site that ATP-competitive Plks inhibitors can target to exert inhibitory effects(Rudolph et al., 2009; Valsasina et al., 2012; X. Zhang et al., 2021). Functionally, the Plk members have all been described in mammalian cells(Lowery et al., 2005; Vaid et al., 2016; Zitouni et al., 2014). Plk1 is required for centrosome separation and maturation, mitotic entry, spindle assembly, chromosome segregation and cytokinesis; Plk2 and Plk4 are required for centriole duplication; Plk3 is required for DNA replication and mediates cellular stress; and Plk2 and Plk5 are required for neuron differentiation(Lowery et al., 2005; X. Zhang et al., 2021; Zitouni et al., 2014). Plk4 is the most structurally divergent member of the Plk family. While the other members have two PBD, Plk4 contains three PBDs (PB1, PB2, PB3), two of which (PB1 and PB2) are noncanonical and together form a cryptic polo-box (CPB)(Garvey et al., 2021) for target binding and centrosome localization, and only PB3 is located at the C-terminal, critical for Plk4 autoinhibition. Derived from Plk1(Carvalho-Santos et al., 2010), Plk4 is a master regulator of centrosome

amplification and impacts mitotic progression(Habedanck et al., 2005a; Kleylein-Sohn et al., 2007), receiving therefore a priority concern as it has been identified as a link between centrosomes and cancer.

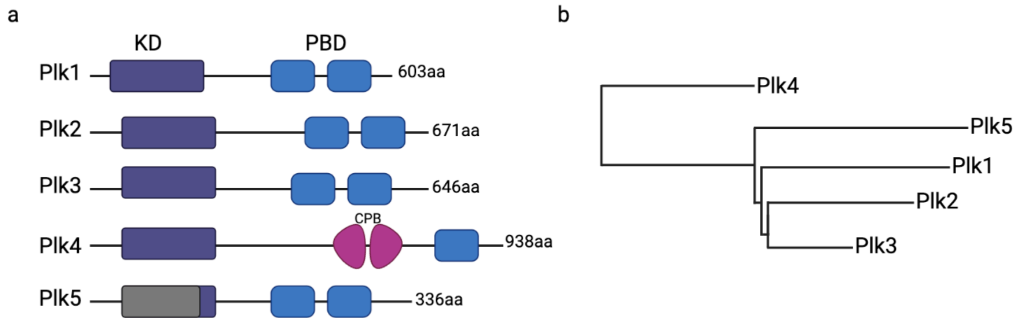

Figure 1.10. The mammalian Polo-like kinase (Plk) family. A) Comparison of the Plk's structure. Both the kinase domains (purple rectangles) and the polo box domains (PBD, blue squares) are conserved in mammalian Plks. Due to the presence of just one PBD whose sequence is not entirely homologous to the other PBDs and one cryptic polo box (CPB, pink), Plk4 is the family member with the largest structural divergence. Human Plk5 has a shortened kinase domain due to an in-frame stop codon followed by an in-frame start codon immediately after, resulting in a truncated kinase domain (purple rectangle). In other mammals, the entire Plk5 gene is encoded (grey rectangle), despite the fact that it lacks kinase activity**. B) Phylogenetic Plk's tree.** Using the VOBALT Phylogenetic Tree widget, phylogenetic analysis of Plk4 sequences shows Plk4 to be the most divergent member. *Adapted from Garvey, Debra R., et al.,2021*. Image created with BioRender online Software.

Plk4 homodimerizes in an intermolecular manner through its CPB, and it is responsible for binding to the partner CEP centrosomal proteins (CEP152 and CEP192), and other centriolar proteins, allowing centriolar targeting of Plk4 to the proximal base of each parental centriole and following downstream events(Garvey et al., 2021). Furthermore, Plk4 contains three PEST sequences that are rich in proline (P), aspartate (D), glutamate (E), serine (S), and threonine (T) residues,

which are important in controlling kinase's stability(Sillibourne & Bornens, 2010). The initiation of centriole duplication is dependent on a number of factors, including CEP192, CEP152, and CEP135, the spindle assembly six homolog protein, SAS6, the SCL/TAL1 interrupting locus protein, STIL, and the centrosome protein CPAP. Plk4 forms a ring around the mature centriole and coalesces into a single point, potentially through two mechanisms: one in which Plk4 stability is increased through the formation of a complex with STIL and SAS6(Bose & Dalal, 2019), and the other through condensation-mediated self-organization of Plk4 around the mother centriole(S. Yamamoto & Kitagawa, 2019). Plk4 phosphorylation of STIL within the STAN motif activates STIL, resulting in centriolar recruitment of SAS6, which marks the site for pro-centriole biogenesis and cartwheel assembly initiation (a stack of ring-type symmetrical assemblies that provides the structural foundation for the procentriole), which is followed by centriole elongation(McLamarrah et al., 2020; Nigg & Holland, 2018). It has also been proposed that Plk4 phosphorylates STIL at and additional site, S428, to promote STIL-CPAP binding, therefore connecting the cartwheel to centriole wall microtubules(Arquint et al., 2018). PLK4, STIL, and SAS6 are all regulated by proteasomal degradation(Arquint et al., 2018), and overexpression or depletion of either of these proteins can result in the formation of multiple centrioles or a blockage in their formation(Garvey et al., 2021). Therefore, to maintain the correct centriole number, the activity and levels of these core components in human cells must be tightly regulated.

Due to its inherent instability, Plk4's kinase activity determines its own stability through autophosphorylation and subsequent ubiquitin mediated proteolysis, which is carried out by the Skp/Cullin/F box E3 ubiquitin ligase (SCF-Slim/βTrCP-E3) complex. The SCF-Slimb/βTrCP-E3 ubiquitin ligase complex regulates Plk4 levels in human cells, via a conserved phosphodegron motive based on the PEST sequences(Cunha-Ferreira et al., 2013). When the kinase activity reaches a

certain level, Plk4 autophosphorylates within a 24-amino acid phosphodegron (Ser293 and thr297) containing a βTrCP-binding site, promoting E3 ligase recruitment and further degradation of Plk4. This autoregulatory pattern ensures Plk4 stability and correct centriole duplication. Because the degradation of Plk4 via autophosphorylation after homodimerization is limited by its activity, it is described as a suicide kinase(Cunha-Ferreira et al., 2013). Besides ubiquitin-mediated proteasomal degradation, several mechanisms have been proposed to control Plk4 kinase activity. In contrast to direct binding to the promoter, p53 inhibits Plk4 transcription via an indirect mechanism. Histone deacetylase (HDAC) repressors are a group of involved factors, since p53-dependent Plk4 downregulation was significantly restored in a dose-dependent manner after treatment with a HDAC inhibitor(J. Li et al., 2005).

Plk4 transcription and repression can also be inhibited by drug-induced DNA damage (e.g., doxorubicin). Plk4 transcript levels were reduced by approximately four-fold in HCT116 $p53^{+/+}$ colon cancer cells after doxorubicin-induced DNA damage. This effect was largely abolished in HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$, indicating that Plk4 was regulated by p53 and p21(Fischer et al., 2014). It has been demonstrated that the DNA damage response activates p53 and p21, resulting in the formation of the DP, RB-like, E2F4 and MuvB (DREAM) complex, which binds to the promoters of cell cycle genes to induce transcriptional repression(Quaas et al., 2012). As a putative transcriptional binding sites in the Plk4 promoter region, cell cycle-dependent elements (CDEs) and cell cycle gene homology regions (CHRs) were identified(Fischer et al., 2014). In fact, it has been shown that the DREAM complex components bind to the Plk4 promoter via CDEs/CHRs in G0, suppressing Plk4 expression and, Plk4 promoter activity is increased in the early stages of the cell cycle when these sites (CDE and CHRs) are mutated(Fischer et al., 2014). Furthermore, the human papillomavirus E7 oncoprotein inhibits p53-mediated Plk4 repression by interfering with DREAM complex function(Fischer et

al., 2014). These findings suggest that p53 indirectly suppresses Plk4 via the p53-p21-DREAM-CDE/CHR pathway, which has been identified as a key mechanism underlying p53-dependen cell cycle gene suppression(Fischer et al., 2014; Maniswami et al., 2018).

The nuclear factor kappa B (NFκB) family is a transcriptional factor family that regulates the expression of multiple genes, including cell cycle genes, affecting many cellular processes. Plk4 was shown to be downregulated in U2OS osteosarcoma cells when each NFκB subunit was depleted, indicating that Plk4 is an NFκB-regulated kinase. Indeed, putative NFκB binding sites in the Plk4 promoter has been identified, and all subunit have been shown to bind to the Plk4 promoter, inducing Plk4 expression(Ledoux et al., 2013). Furthermore, according to studies on promoter methylation of Plks in cancer, Plk4 is subjected to epigenetic modification, particularly under oxidative stress(Ward & Hudson, 2014). For example, during the development of hepatocellular carcinoma (HCC) in Plk4 heterozygous mice, researchers discovered increased Plk4 methylation associated with its downregulated expression(Ward et al., 2011). Moreover, Plk4 epigenetic downregulation was induced in HCC-derived cell lines after exposure to hypoxia and reactive oxygen species, and this modification may be p53 dependent(Ward & Hudson, 2014). Additionally, Plk4 hyper-methylation was found in bone marrow aspirates from patients with hematological malignancies, resulting in lower protein expression(Ward et al., 2015). Given that abnormal Plk4 methylation may contribute to tumorigenesis, hypomethylating drugs have been proposed as a potential cancer therapy in combination with conventional drugs(Ward & Hudson, 2014). Another study conducted by Fournier and colleagues discovered that acetylation of Plk4 reduces its kinase activity(Fournier et al., 2016). Lysine acetylation is a post-translational modification that affects histone and non-histone protein function. Plk4 was in fact, discovered to be a novel substrate for lysine acetyltransferases (KATs) acetylation at Lys45 and

Lys46(Fournier et al., 2016). Plk4's kinase domain is acetylated by KAT2A and KAT2B, which dampens its kinase activity by stabilizing the inactive conformation. As a result, acetylation of Plk4 prevents CA and preserves genome stability. Furthermore, endoplasmic reticulum stress has been suggested to induce activation of the transcription factor 6 (ATF6) and CCAAT/enhancer-binding protein b (C/EBPb) binding, which may inhibit transcription activity of the Plk4 gene. In either case, Plk4 transcription is cell-cycle dependent, with transcription levels undetectable in G0 and beginning to rise in G1, with maximum levels detected during mitosis.

A variety of different proteins play a role in recruiting and stabilizing centriole microtubules(Azimzadeh & Marshall, 2010; Brito et al., 2012; Gönczy, 2012). Unlike other microtubules, centriole microtubules are very stable and resistant. The number (4 in mitosis), size (400-500nm) and shape of centrioles is highly controlled. However, abnormalities in their number and size are often seen in cancer(Chan, 2011). I discuss in the next section how centrosome amplification can be a double edge sword in cancer.

1.4.2 Centrosome in Cancer

Abnormalities in centriole number, structure and function cause different diseases, including microcephaly(Marthiens et al., 2013) and cancer(Basto et al., 2008; Chan, 2011; Kayser et al., 2005; Nigg & Raff, 2009). Over a century ago, Theodor Boveri was the first to propose that centrosome amplification (>2 centrosomes per cell) generates aneuploidy and promotes tumorigenesis. His theory was based on the observation of dispermic sea urchin eggs containing multiple centrosomes, which formed multipolar spindles, leading to asymmetric distribution of the genetic material. Supporting his hypothesis, abnormalities in centrosome number and

structure have been observed in a wide range of tumors(Chan, 2011; Denu et al., 2016b; Nolte et al., 2013; Pannu et al., 2015; Y. Yamamoto et al., 2011; Zyss & Gergely, 2009) and associated with genomic instability and poor patient prognosis(S. A. Godinho & Pellman, 2014; Nigg, 2006; Nigg & Raff, 2009). By enabling the formation of multipolar spindles and chromosome missegregation, **centrosome amplification represents a mechanism leading to chromosomal instability and aneuploidy**(Ring et al., 1982). Indeed, increased expression of the master regulator of centriole duplication, PLK4(Bettencourt-Dias et al., 2005; Habedanck et al., 2005a), was observed in several large cohorts of breast cancer patients(Macmillan et al., 2001; Marina & Saavedra, 2014a).

Only recently, more than 100 years after his proposal, was Theodor Boveri hypothesis directly tested. However, whether centrosome amplification was a cause or consequence of cancer is still a debate. Experiments transiently inducing centrosome amplification were shown to be sufficient to induce tumorigenesis: (1) in normal fly models after inoculated with brain cells from flies with CA(Basto et al., 2008); (2) in human prostate epithelial cells when forced to undergo CA via SKA1 overexpression, forming tumors in nude mice(J. Li et al., 2014); (3) in mice that, spontaneously developed tumors while forced to undergo CA via Plk4 overexpression and a single truncated allele of the adenomatous polyposis coli (APC) tumor suppressor, accelerating tumorigenesis in mice that lacked the tumor suppressor p53(Levine et al., 2017); and (4) in CA mice model by overexpressing Plk4, inducing renal cysts, which are prone to renal carcinoma(Dionne et al., 2018). Moreover, in the multistep tumorigenesis Barrett's esophagus model, CA has been shown to increase from pre-malignant stage to dysplasia, and then to be present until metastasis(Lopes et al., 2018), supporting a role for CA in human cancer.

Clinically, CA levels are associated with tumor staging in different cancers, being positively correlated with tumor progression, increased invasiveness as well as histological grade(Chan, 2011; Denu et al., 2016b; J. Z. Zhao et al., 2021). CA has

also been frequently observed in hematological tumors, such as the B cell non-Hodgkin's lymphomas (follicular lymphoma, diffuse large B cell lymphoma and Burkitt's lymphoma), in which is also positively correlated with progression(Krämer et al., 2003).
Circulating tumor cells (CTCs) are a subpopulation of cancer cells that enter the circulation, and are thought to have metastatic potential(Schuster et al., 2021), and CA has been shown to be present in 75% of breast cancer CTCs(A. Singh et al., 2020). In an *in vitro* 3D culture model, extra centrosomes have been shown to promote invasive phenotypes and alteration of migration(S. a Godinho et al., 2014). This invasive behavior is triggered by the centrosomal microtubule over-nucleation, leading to an increase in Rac1 activity, a small GTPase that leads to a disruption of cell-cell adhesion, altering cytoskeletal structure, and thus promoting invasion and metastasis(S. a Godinho et al., 2014; Mack et al., 2011; Waterman-Storer et al., 1999). In accordance, CA has been shown to promote the secretion of multiple invasion-promoting factors, such as IL8, ANGPTL4 and GDF-15(Arnandis et al., 2018), which in turn interacts with Her2 receptor, activating the RAC1-Arp2/3 signaling pathway(S. E. Wang et al., 2006). The activation of this signaling pathway enhances microtubule assembly and actin polymerization, as mentioned, leading to cytoskeletal rearrangement. Furthermore, CA can promote the expression of the ninein-like protein (NLP), increasing the microtubule nucleation and stability, which stiffens cells and disrupts E-cadherin junctions, reducing cell adhesion and promoting migration and invasion(Ganier et al., 2017).

Several mechanisms, most relying on cell cycle deregulation, have been postulated to induce centrosome amplification: failure of cell division (cytokinesis failure), mitotic slippage, deregulation of the centrosome duplication machinery(S. A. Godinho & Pellman, 2014), *de novo* centriole assembly and cell-cell fusion (**Figure 1.11**). Besides Plk4 overexpression, the overexpression of other

centrosomal proteins, such as STIL, SAS6, CEP63 and CEP152 can also lead to CA, while their inhibition leads to decrease in centrosome number(Arquint & Nigg, 2016; J. Z. Zhao et al., 2021). However, besides the increase in PLK4 in certain human cancers, little has been shown *in vivo* regarding the causes of centrosome deregulation.

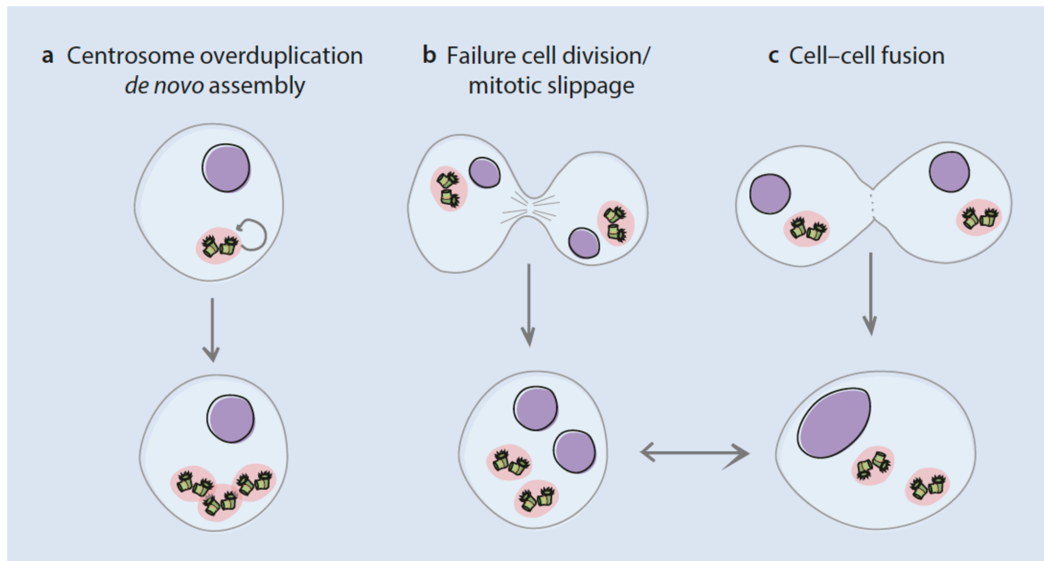

Figure 1.11. Causes of centrosome amplification. (A) Centrosome amplification might be originated by deregulated centrosome duplication machinery, which duplicate centrosomes through several rounds within a single S phase, or through *de novo* centriole assembly. (**B)** Failure in cell division or mitotic slippage as a result of aborted mitosis, might also induce centrosome amplification. (**C)** Depending on the cell cycle stage, the overcome of cell-cell fusion will originate different centrosome/genome ratios. Both cell fusion and failure in cell division/mitotic slippage will originate multinucleated cell, which then will form a single polyploid nuclei after subsequent mitosis. *Adapted from Nigg, E.A., 2002.*

1.4.3 How do cancer cells cope with supernumerary centrosomes?

Supernumerary centrosomes can be detrimental for cell proliferation for at least two reasons. First, the presence of both fewer and extra centrosomes activates the p53 signaling pathway in vertebrate cells, leading to either a G1 cell cycle arrest and a decrease in cell proliferation or directly induce apoptosis(Green & Kroemer, 2009) through PIDDosome, tumor suppressor p53-binding protein 1 (53BP1) and Ubiquitin-specific protease 28 (USP28)(M. Lin et al., 2022; Meitinger et al., 2016; Shyang Fong et al., 2016). Second, supernumerary centrosomes can lead to multipolar mitosis that can be catastrophic as cells inherit fewer chromosomes than what they need for viability.

However, cancer cells often can survive in the presence of multiple centrosomes. This is because they often do not have a functional p53 pathway. Moreover, they often also develop strategies to be able to divide, almost in a normal fashion with multiple centrosomes. To cope with extra centrosome and overcome cell death, the malignant cell gains adaptation mechanisms. One of such mechanisms, and the one which is better described is the ability to cluster centrosomes, where extra centrosomes remain close together through mitosis and still forms a pseudo-bipolar spindle, facilitating chromosome segregation(Basto et al., 2008; Brinkley, 2001; Ganem et al., 2009; Ring et al., 1982) (**Figure 1.12A)**. It should however, be said that this process may still generate some aneuploidy. This is because the process of clustering promotes merotelic kinetochore-microtubule attachments(Ganem et al., 2009) (**Figure 1.12B**). Merotely is a type of error in which single kinetochores attach to microtubules emanating from both spindle poles. This event is particularly dangerous, as it is poorly sensed by the spindle assembly checkpoint, and if not corrected, it may give rise to lagging chromosome during anaphase, leading to chromosome missegregation(Cimini, 2003, 2008; Cimini et al., 2001; Ganem et al., 2009; Salmon et al., 2005). Moreover, besides centrosome clustering, other mechanisms have been shown to allow cells to cope

with extra centrosomes, including inactivating centrosomes (**Figure 1.12A)**, centrosome loss and asymmetric segregation of centrosome during division(S. A. Godinho et al., 2009; Rhys & Godinho, 2017).

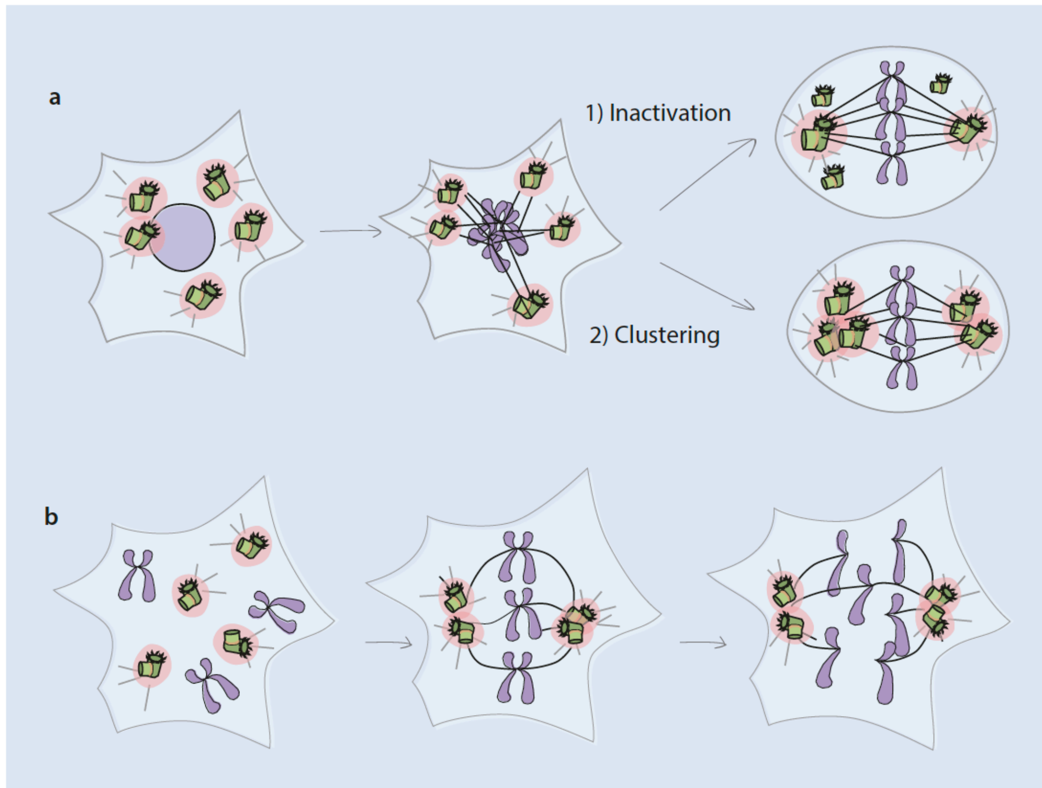

Figure 1.12. Mechanism to suppress multipolar mitosis in cells with extra centrosomes. Centrosome amplification leads to aneuploidy. **(A)** To allow bipolar mitoses in cells with extra centrosome, several distinct mechanisms are used: (a) Centrosome inactivation: by silencing MTOC activity of additional centrosomes, a bipolar mitosis can be successful achieved. Some extra centrosomes might lose PCM (yellow cloud around centrosomes) and be incapable of function as MTOC. (b) Centrosome clustering: cells can combine their extra centrosomes into two groups in order to form a bipolar spindle. **(B)** Merotelic attachment caused by centrosome amplification. Extra centrosomes can give rise to merotelic attachments - one kinetochore attaches to microtubules that emanate from opposite spindle poles, due to altered spindle geometry, and lagging chromosomes and consequently, aneuploidy daughter cells. *Adapted from Godinho, S. A. et al, 2009*; and *Rhys, A. D. et al, 2017.*

Deregulate ciliary signaling may also be an important event in cancer. Cilia are lost/compromised in multiple cancer types(Basten & Giles, 2013; Cao & Zhong, 2016; Seeger-Nukpezah et al., 2013), including breast cancer(K. Yuan et al., 2010), prostate cancer(Hassounah et al., 2013), melanoma(J. Kim et al., 2011) and pancreatic cancer(Seeley et al., 2009). As discussed above, primary cilium has an important function in regulating multiple signaling pathways. The Sonic Hedgehog (Shh) signaling is a cilia-dependent pathway, and has important functions in guiding embryonic development by regulating cell differentiation and proliferation(Bangs & Anderson, 2017; Goetz & Anderson, 2010; Mukhopadhyay et al., 2013; Rohatgi et al., 2007). Abnormal activation of Shh is observed in several types of cancer(Sarkar et al., 2010; Seeger-Nukpezah et al., 2013; S. Y. Wong et al., 2009). Moreover, the Wnt signaling is also critical to animal development and homeostasis, and regulation of Wnt signaling has also been linked to tumorigenesis, however, this is still controversial(Cao & Zhong, 2016; Wallingford & Mitchell, 2011). Despite some associations between defective cilia and cancer, a direct link of cilia in tumorigenesis is still unclear. Future studies will hopefully provide more evidences regarding this matter.

The knowledge on how centrosome abnormalities contribute to tumorigenesis is still likely not complete. Not only centrosome amplification, but also centriole loss and decrease in PCM recruitment might lead to alterations that could contribute to tumorigenesis(Nigg & Raff, 2009; Salisbury et al., 1999). Focusing more on consequences of centrosome amplification in tumors, it is known that extra centrosomes can affect cells by promoting chromosome missegregation(Ganem et al., 2009) and also by impairing asymmetric cell division in *Drosophila* neuroblasts(Basto et al., 2008), inducing the expansion of the neuronal stem cells, leading to tumors(Basto et al., 2008; Caussinus & Gonzalez, 2005; Yoo & Kwon,

2015). Moreover, centrosome amplification can also affect cilia signaling in interphase cells(Basten & Giles, 2013; Mahjoub & Stearns, 2012; Toftgård, 2009). Additionally, extra centrosomes can affect cell polarity and signaling(Basten & Giles, 2013; Cao & Zhong, 2016; Nigg, 2006; Yoo & Kwon, 2015), which can change the architecture of tumor tissue, favoring the tendency of tumors to metastasize. Furthermore, during interphase, extra centrosome, which are clustered, recruits extra PCM, leading to increased microtubule nucleation(S. A. Godinho & Pellman, 2014), which can also alter the regulation of Rho GTPases, and thus, affect the migration and invasive properties of cells (**Figure 1.13**).

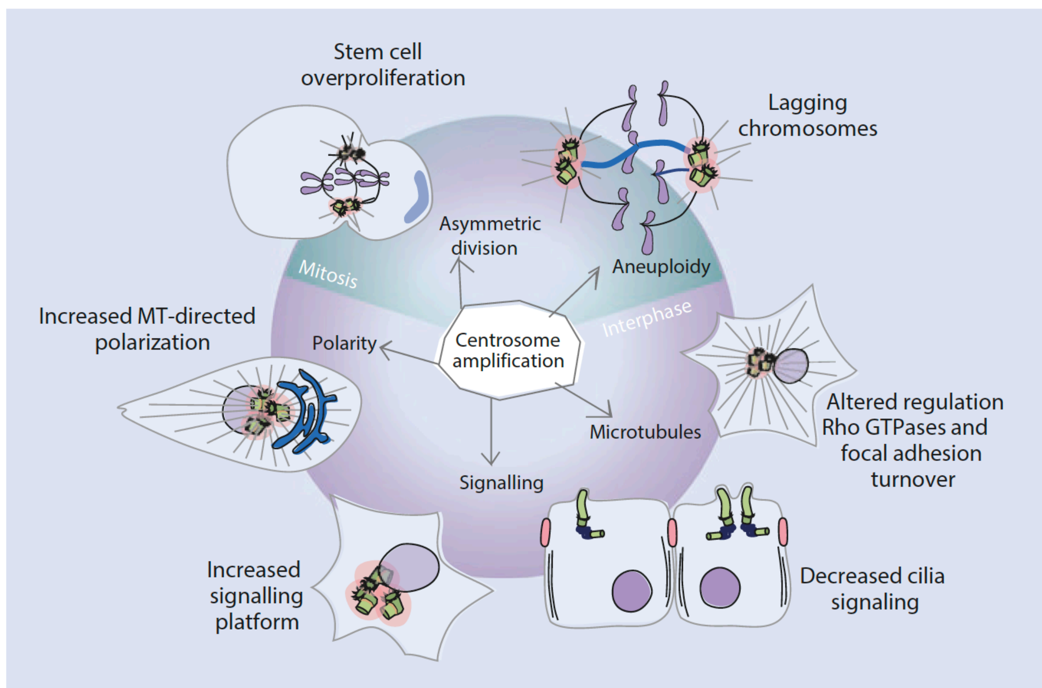

Figure 1.13. Consequences of centriole amplification in cancer. A schematic representation of how centrosome amplification could contribute to tumorigenesis. Extra centrosomes can affect cells by promoting chromosome missegregation, leading to aneuploidy, and also by impairing asymmetric cell division in Drosophila neuroblasts(Basto et al., 2008). The role of extra centrosomes is not limited to mitosis. Centrosome amplification can affect cilia signaling in interphase cells. Moreover, increased

microtubule nucleation in cells with extra centrosomes can alter the regulation of Rho GTPases and therefore affect the migration and invasive properties of cells. Furthermore, extra centrosomes could affect cell polarity and signaling. *Adapted from Godinho and Pellman*,*2014*.

1.4.4 Extra centrosomes as a therapeutic target in cancer

As discussed above, cells with extra centrosome can use unique survival requirements, including centrosome clustering. The use of drugs which **de-cluster extra centrosomes**, leading to multipolar mitoses, massive aneuploidy, causing cell cycle arrest and cell death, might stand an attractive target for a cancer treatment that does not affect normal cells (non-transformed). In fact, several drugs have been proposed to de-cluster extra centrosomes. Such drugs include Griseofulvin(Raab et al., 2012; Rebacz et al., 2007), which is a nontoxic antifungal, and a 2'-substituted derivative of griseofulvin, that has been seen to de-cluster centrosomes and selectively kill tumor cells (in concentrations that are non-toxic for normal cells). These can also include Phenantherene-derived poly-ADP ribose polymerase (PARP) inhibitors, which have also been observed to de-cluster centrosomes, inducing multipolar spindles, mitotic catastrophe and death(Castiel et al., 2011, 2013; Tong et al., 2007). Non-transformed cells treated with the same PARP inhibitor (PJ-34) showed no spindle morphology changes and cell viability was maintained(Pannu et al., 2014).

The discovery of critical players in centrosome clustering, such as the minus end directed kinesin KIFC1/HSET, looks promising for the cancer field. KIFC1/HSET, **a clustering inhibitor**, have been shown to have a non-essential function in most normal cells, inducing only multipolar divisions in a panel of cell lines with supernumerary centrosomes, impairing significantly the viability of these cells(Watts et al., 2013; Wu et al., 2013; Xiao & Yang, 2016; B. Yang et al., 2014). Because centrosome amplification can accelerate tumorigenesis, induce chromosome instability and promote cell invasion, effective treatments that

eradicate these cells harboring abnormal centrosomes within the tumor could bring promisingly impact in cancer treatment. Drugs that also **prevent centrosome duplication**, like PLK4 inhibitors(Mason et al., 2014) are also in clinical trials, looking promising for therapeutics. In fact, several Plk4 inhibitors has been described, such as **CFI-400945, CFI-400437, Centrinone, Centrinone-B, YLT-11, and YLZ-F5**(Garvey et al., 2021; X. Zhang et al., 2021; Y. Zhao & Wang, 2019b). Although, the idea of exploring drugs to target Plk4 for cancer treatment is still far from the clinic, several anticancer drugs are in preclinical and clinical trials(Garvey et al., 2021; X. Zhang et al., 2021; Y. Zhao & Wang, 2019b).

CFI-400945 was the first potent inhibitor orally available, showing a selective inhibition of Plk4 rather than other Plk family members(Mason et al., 2014). It binds to the ATP-binding pocket of Plk4 kinase domain (KD), inhibiting Plk4 autophosphorylation at Ser-305, a critical site for Plk4 activation(Mason et al., 2014). However, due to the self-regulation pattern of Plk4, CFI-400945 showed a bimodal effect on centriole number when breast (MDA-MB-468 and MCDA-MB-231) and osteosarcoma (U2OS) cells were treated with this compound. At higher doses, CFI-400945 suppressed centrosome duplication with a complete blockage of Plk4 activity, while at lower doses it drives an increase in centriole number, due to partial inhibition of Plk4, causing an increase in Plk4 levels due to its insufficient degradation, but still sufficient for substrate degradation(Mason et al., 2014; Y. Zhao & Wang, 2019b). Moreover, CFI-400945 has shown remarkable antitumor effect in several solid tumors, including pancreatic, lung, liver and breast cancer(X. Zhang et al., 2021), showing also a reduced tumor growth and prolonged survival in human pancreatic cancer xenograft models(Lohse et al., 2017). Other studies showed that CFI-400945 can also block cell proliferation and induce polyploidy, apoptosis and mitotic aberrations in lung cells, and inhibit tumor growth in murine lung cancer xenograft model(Kawakami et al., 2018). However, because CFI-400945 have also inhibits Aurora kinase B, it is still debatable whether the

cytokinesis failure induced by this inhibitor is a result of its off-target effect(Y. Zhao & Wang, 2019b). Nevertheless, CFI-400945 has entered phase I and II of five clinical trials for several solid tumors due to its dose-dependent inhibitory effects in a diverse panel of human cancer cell lines and patient-derived xenograft tumors(Y. Zhao & Wang, 2019b).

Some Plk4 inhibitors has been combined with other drugs. Kawakami and colleagues have shown that combination of CDK2 antagonists and CFI-400945 results in synergistic effect against lung cancer, causing multipolar mitosis(Kawakami et al., 2018). While CDK2 antagonism inhibited centrosome clustering, CFI-400945 at low doses generated centrosome amplification. Moreover, combination of Plk4 depletion and temozolomide (TMZ) sensitivity in glioblastoma resulted in increased antitumor effect while decreasing TMZ IC50(Z. Zhang et al., 2019). Furthermore, CFI-400945 and TMZ combination resulted in reduced tumor volume and improved the survival in tumor-bearing mice(Z. Zhang et al., 2019). Despite all these reports, the combination of therapies with Plk4 inhibitor still need further studies. Combining Plk4 inhibitors with immune checkpoints inhibitors to prevent immune responses or with drugs targeting microtubule dynamic to promote chromosome instability might also be a good strategy(Kawakami et al., 2018; Y. Zhao & Wang, 2019b). Additionally, with the recent discovery of STAT3 involvement in centrosome clustering, researchers discovered that breast cancer cells with CA caused by Plk4 overexpression were more sensitive to STAT3 inhibition. This suggests that combination of STAT3 inhibitors can be a good strategy for cancer therapy(E. J. Morris et al., 2017).

CFI-400437, is an indolinone-derived ATP-competitive kinase inhibitor, similar to CFI-400945, showing also a low inhibitory activity against other Plk4 family members(Laufer et al., 2013). In breast cancer, CFI-400437 has been shown to significantly inhibit Plk4 activity and tumor growth, both in a cell culture and in xenograft mouse tumor model(Laufer et al., 2013; Suri et al., 2019; X. Zhang et al.,

2021). However, CFI-400945 and CFI-400437 does not only selectively inhibit Plk4, but also AURKB, TRKA, TRKB and Tie2/Tek(Dominguez-Brauer et al., 2015). Because of this, other Plk4 inhibitors were developed.

Centrinone and **Centrinone-B** were both developed as highly selective Plk4 inhibitors, as they prevent centriole assembly and consequently centriole duplication, by also binding to the ATP-binding pocket of Plk4 KD.(Y. Zhao & Wang, 2019b). Centrinone treatment caused p53-mediated G1 cell cycle arrest in Hela cells, whether Centrinone-B treatment in human melanoma cell lines induced a significant decrease in cell viability and increase in apoptosis(Denu et al., 2018). However, in some cancer cell lines, Centrinone treatment resulted in cell proliferation independent of centrosome loss, but in a p53-dependent mechanism, indicating an intrinsic "set point" for centrosome number. Another disadvantage of Centrinone/Centrinone B is that it is extremely difficult for both compounds to cross the blood-brain barrier, suggesting it may not be able to treat brain tumors(Suri et al., 2019). As a result, authors proposed that centrosome depletion was not sufficient for cancer treatment, and should also be combined with other targeted drugs(Y. L. Wong et al., 2015).

YLT-11 is the most recently designed selective Plk4 inhibitor, as it fits effectively into the ATP pocket of Plk4, showing a considerable inhibition of breast cancer cell proliferation, leading to centriole replication and mitotic defects, which increased tumor cell sensitivity to chemotherapy(X. Zhang et al., 2021). YLT-11 significantly reduced tumor growth in human breast cancer xenograft models (MCF-7, MDA-MB-468, and MDA-MB -231), suggesting that YLT-11 is a promising drug for breast cancer patients(Lei et al., 2018).

Another Plk4 small-molecule inhibitor is **YLZ-F5**, which is a derivative of (E)-4-3-arylvinyl-1H-indazol-6-yl)pyrimidin-2-amine. A recent *in vitro* pre-clinical study showed that YLZ-5 treatment inhibited ovarian cancer cell proliferation and colony formation ability, as well as induced apoptosis by activating caspase 3 and 9(Zhu

et al., 2020). Furthermore, besides inhibiting Plk4 phosphorylation, YLZ-5 caused aberrant centriole duplication and promoted the accumulation of ovarian cancer cells with mitotic defects. Overall, these findings indicate that YLZ-5 is a Plk4 inhibitor with significant *in vitro* anti-proliferative activity. However, further studies in *in vivo* models and in other cancers must be performed in order to confirm YLZ-5 anti-proliferative effect.

Despite the effectiveness of the treatments targeting centrosome/Plk4 in cancer cells *in vivo,* it is critical to develop a therapeutic regimen that can be combined with Plk4 protein target therapy. Therefore, more studies are still needed to further assess the validity of such strategy as a cancer therapy.

Chapter 2.

Framework of the thesis

2.1 Rationale and Aims

Centrosomes consist of two microtubule-based centrioles (a mother and a daughter centriole), being the major microtubule organizing center of animal cells. The centrosome plays an important role in assembling the bipolar spindle during mitosis for correct cell division, participating also in the regulation of other cellular processes, such as cell polarity, differentiation, migration and signaling. Moreover, because centrosome anchor to the cellular membrane, they are also essential for extracellular signaling sensing, enabling ciliogenesis and also for species reproduction. Because centrosomes play important functions, several diseases such as reproductive and respiratory disorders, neurological disorders and neurodegenerations (microcephaly, Alstrom syndrome, Bardet-Biedl syndrome, Huntington's disease, and several others), aging and cancer, have been associated to it(Bettencourt-Dias et al., 2011; Nigg & Raff, 2009; Schatten & Sun, 2018).

In cancer, centrosome abnormalities (in their number and structure) have been widely observed in multiple tumors, including breast, colon, liver, bone marrow, cervical, and prostate cancer. Centrosome number deregulation has been extensively studied, and shown to contribute to tumorigenesis in multiple ways(Adams et al., 2021; Arnandis et al., 2018; Basto et al., 2008; Denu et al., 2016b; S. A. Godinho, 2015; S. A. Godinho et al., 2009; S. a Godinho et al., 2014; Marteil et al., 2018; Salisbury et al., 1999). Moreover, the main regulator of centrosome biogenesis, the polo-like kinase 4 (Plk4) has also been suggested to be involved in tumorigenesis. In fact, Plk4 has been found to be aberrantly expressed in several tumors, and correlated with tumor aggressiveness, metastasis and the response to chemotherapy, contributing to bad patient prognosis. Plk4 has also been linked to (EMT), one of the molecular process responsible for cancer progression and metastasis.

Because Plk4 deregulation causes loss of centrosome numerical integrity, promoting genomic instability, it is considered a good target for anti-cancer drug development. However, whether centrosome number deregulation and/or its main regulator Plk4 affect the acquisition/maintenance of a stem-like phenotype, such as *anoikis* resistance and EMT, remains unknown.

2.2 General Aim

The main purpose of this thesis was to study the direct or indirect effect of centrosome amplification on the induction of stem-like properties, such as *anoikis* resistance and EMT. Therefore, during this work, I did an overview of the literature regarding the history of cell cycle and cell cycle related mechanisms (cell cycle phases, checkpoints, DNA repair and Spindle assembly); Then, I discuss one of the biological processes by which cancer cells gain migratory and invasive capacity, the Epithelial to Mesenchymal Transition (EMT), acquiring stem-like properties, cell plasticity and resistance to apoptosis (*anoikis*) and consequently to survive therapies; Then, I introduce the centrosome, its biogenesis and cellular functions, and furthermore its role in cancer and how cells cope with extra centrosomes, and discuss the current therapeutics in cancer used to target centrosome amplification and its main regulator Plk4 (**Chapter 1**). Next, the following specific aims were addressed:

2.3 Specific Aims

1. To establish a stable p53 Knock-Out (KO) epithelial cell line model, which would allow to experimentally get an increased number of viable and cycling cells upon the induction of centrosome amplification via Plk4 overexpression **(Chapter 3)**.

2. To study the effect of Plk4-induced centrosome amplification on the induction of stem-like properties, namely the *anoikis*-resistance capacity and EMT, of epithelial non-tumorigenic cells **(Chapter 4).**

3. To discuss the relevance of our findings in the context of centrosome amplification, Plk4 and their role in contributing to tumor progression **(Chapter 5)**.

Chapter 3.

Establishment and functional validation of a p53 Knock-out mammary epithelial cell line model with Plk4-induced centrosome amplification

3.1 Abstract

Cellular stress, such as centrosome amplification, induces p53 nuclear accumulation and activation of downstream effectors to prevent cell proliferation, inducing cell cycle arrest or apoptosis. However, in pre-malignant and malignant cells, p53 is usually lost and cells with centrosome amplification can survive and accumulate within the tumor population. This chapter describes the establishment of a stable p53 knockout (KO) cell line in order to have viable cycling cells upon the induction of centrosome amplification via Plk4 overexpression. We took advantage of the previously described MCF10A-Plk4 cell line, where Plk4 is overexpressed by Doxycycline treatment, and performed p53 knockout through CRISPR/Cas9 technology. MCF10A-Plk4^{p53KO} Clone 9 was one of the p53KO clones presenting higher level of Plk4, with increased percentage of cells with centrosome amplification (75% in interphase and 93% in mitosis) and was selected to further investigate the potential role of Plk4 in the *anoikis* resistance capacity of non-tumorigenic cells. The efficiency of the KO was assessed by evaluating the expression of p53 and its downstream target p21 protein. Moreover, we characterized MCF10A-Plk4^{p53KO} Clone 9 cell cycle profile, cell viability and tumorigenic capacity in mice and in the chick chorioallantoic membrane model (CAM) model. Although the establishment of this cell line was performed for the purpose of this thesis, it can also be employed as an important tool for further studies related to centrosome amplification and Plk4 in a p53KO background.

3.2 Introduction

Cancer is one of the leading causes of premature death worldwide(World Health, 2020). Tumors usually develop within a tissue or organ when regulatory genes of the cell cycle and genome integrity lose their function, due to mutations and/or due to epigenetic and environmental factors(Collins et al., 1997; Fonseca & Bettencourt-Dias, 2019; Kastan & Bartek, 2004; Laiho & Latonen, 2003; Wei Dai, 2014).

Hundreds of genes control intricately the cell cycle and genome integrity, ensuring a balance between cell proliferation or its suppression. Thus, the cell cycle control is important to promote a regulated tissue expansion and differentiation. To avoid the transmission of genomic errors to daughter cells, there are check-point pathways that arrest cell cycle progression for DNA repair or, in case of unrepairable damage, to stimulate programed cell death – or apoptosis(Kastan & Bartek, 2004; Laiho & Latonen, 2003; Medema & MacŮrek, 2012; B. B. S. Zhou & Elledge, 2000). The tumor suppressor gene p53, also known as the "guardian of the genome", plays an important role in maintaining genomic stability. It is the most commonly mutated gene in human cancers, representing a mutation present in as many as 50% of all cancers(Greenblatt et al., 1994; Hollstein et al., 1991; Rivlin et al., 2011).

The main function of p53 is to maintain genome integrity, by acting as a checkpoint effector in the G1 and G2 phases of the cell cycle. Being a multifunctional protein, p53 acts upon different cellular stresses or abnormalities, such as DNA damage, inappropriate proliferation and oxidative stress, preventing cell cycle progression by activating its downstream factors, such as p21 (encoded by the WAF gene), Bax, MDM2 (murine double minus 2) and GADD45 (Growth Arrest and DNA Damage)(El-Deiry, 1998; Green & Kroemer, 2009; Olivos & Mayo, 2016; Riley et al., 2008; Yee & Vousden, 2005). Cell cycle arrest and senescence are mainly mediated by p53 downstream effector p21, which inhibits the hetero-dimers formed

by cyclins/CDKs, arresting cell-cycle progression(Bunz et al., 1998; Karimian et al., 2016). Moreover, while cell cycle is arrested, p53 promotes DNA repair, activating the genes GADD45 and P53R(Liu & Chen, 2006; Millau et al., 2009)se)(Liu & Chen, 2006; Millau et al., 2009). In case the DNA damage is irreparable due to sustained or severe stress signals, p53 usually induces the transcription of genes involved in apoptosis, such as PUMA (p53 upregulated modulator of apoptosis), Bax, Fas, PIG3 (P53-Inducible Gene 3), and Killer/DR5(Harris & Levine, 2005; Liu & Chen, 2006; Millau et al., 2009; Transl et al., 2016).

P53 expression and function is controlled by its negative regulators such as MDM2 and phosphatase and Tensin homolog (PTEN). The MDM2, a transcriptional target of p53, is the main player in p53 stabilization, creating a negative loop, allowing the exportation of p53 from the nucleus to the cytoplasm, where p53 is degraded by the proteosome(Haupt et al., 1997; Kubbutat et al., 1997; Vousden, 2000). In stress conditions, MDM2 is auto-poly-ubiquitinated, resulting into its degradation and a concomitant increase in p53 levels and activity(Stommel & Wahl, 2004). This regulatory feedback loop mechanism gives rise to a subtle balance between the amount of p53 and MDM2, since p53 in turn regulates MDM2 transcription levels(Kubbutat et al., 1997; Stommel & Wahl, 2004).

It is believed that perturbations in the p53 signaling pathway are required for the development of most cancers(Hainaut & Hollstein, 1999; Hollstein et al., 1991; Koifman et al., 2018; Muller & Vousden, 2014; Murphy et al., 2000; Ohtsuka et al., 2018; Transl et al., 2016; Weiss et al., 2010). Inhibition of the p53 pathway allows cells to progress in the cell cycle despite problems such as DNA damage, thus promoting tumorigenesis(Bunz et al., 1998; Hollstein et al., 1991; Lim et al., 2009; Milner et al., 1991; Rivlin et al., 2011; Weiss et al., 2010). Although p53 mutations are widespread in cancer(Kandoth et al., 2013), these are mostly due to the loss of p53 wild type tumor suppressor function and/or to the gain of a novel function, thus acting like an oncogene. In fact, the tumor suppressor role of p53 was demonstrated when mice lacking functional p53 ($p53^{-/-}$) developed various types of

tumors early in life(Donehower et al., 1992; Jacks et al., 1994; Purdie et al., 1994), while heterozygous p53 mice ($p53^{+/-}$) developed cancer at later stages, with loss or mutation of the remaining wild type p53 allele(Harvey, McArthur, et al., 1993; Harvey, Sands, et al., 1993).

Cellular stresses, as the one triggered by gain of extra centrosomes, induce the activation of the p53 pathway thus preventing cell proliferation, by cell cycle arrest or apoptosis(Contadini et al., 2019; Fava et al., 2017; Holland et al., 2012). Indeed, the presence of more than 2 centrosomes in mitosis (i.e., centrosome amplification, CA) is observed in many types of human cancers, being significantly associated with p53 inactivation and chromosome instability(Carroll et al., 1999; Chan, 2011; S. A. Godinho, 2015; Mussman et al., 2000; Nigg, 2006; Salisbury et al., 1999). Importantly, it has been shown that loss or mutation of p53 in mouse embryonic fibroblasts (MEF) leads to CA, resulting in multiple spindle poles, with chromosome mis-segregation(Fukasawa et al., 1996; Tarapore & Fukasawa, 2002). Interestingly, p53 has been shown to also localize to centrosomes during the mitotic spindle checkpoint(Ciciarello et al., 2001; V. B. Morris et al., 2000; Tarapore, Horn, et al., 2001; Tarapore, Tokuyama, et al., 2001), although its functional significance remains controversial. In fact, p53 suppresses centrosome re-duplication through multiple pathways, one of which being mediated by its downstream target p21(Tarapore & Fukasawa, 2002). In support, introduction of MDM2, promoting p53 degradation, or a gain-of-function mutant that suppresses wild-type p53 function, also results in centrosome amplification(Carroll et al., 1999; Haupt et al., 1997; Kubbutat et al., 1997; Murphy et al., 2000).

In accordance, cancer cells can survive in the presence of extra centrosomes, due to inactivation of the p53 pathway; actually, loss of p53 is required for tumorigenesis induced by extra centrosomes(Fukasawa et al., 1996; S. A. Godinho et al., 2009; Levine et al., 2017; Lopes et al., 2018). In human prostate cancer cells, centrosome number has been shown to be significantly increased in cell lines with non-functional p53 in comparison to wild-type p53 prostate cancer

cells(Ouyang et al., 2001). Interestingly, transfection of wild-type p53 gene into inactive p53 cells resulted in G2/M phase cell cycle arrest and a decrease in centrosome amplification on these cells(Ouyang et al., 2001). Moreover, in Barrett's esophagus cancer cell model, it has been shown that centrosome amplification arises early in pre-malignant conditions in p53 functional wild-type context, where p53 likely prevents the spreading of cells with centrosome amplification. Throughout malignant transformation, centrosome amplification expands and continues, both in adenocarcinoma and metastasis, being correlated and dependent on loss of the wild-type p53 or hotspot mutations(Lopes et al., 2018). All these data support the idea that p53 plays an important role in maintaining the normal function of centrosomes and prevents proliferation upon centrosome number deregulation, as well as induces apoptosis of these deregulated cells.

To elucidate the mechanisms of breast cancer initiation and progression, many *in vitro*, *in vivo* and *ex-vivo* models are usually used. While mouse models are still the best models to study breast cancer tumorigenesis, conventional two dimensional (2D) or more sophisticated three dimensional (3D) *in vitro* approaches can serve as complements to *in vivo* models, allowing to study breast cell function, mammary gland morphogenesis and also breast cancer initiation and progression. 3D culture better mimics *in vivo* conditions, allowing a better investigation of cell behavior and function of both normal and malignant cells. The use of Matrigel, an extracellular matrix (ECM)(Kleinman & Martin, 2005) mix in 3D provides the combination of ECM components similar to the *in vivo* breast microenvironment. Moreover, the combination of Matrigel and collagen I in 3D allows the generation of functional acini and ducts *in vitro*, resembling the mammary gland architecture(Krause et al., 2008; Sung et al., 2013; Vidi et al., 2013).

The MCF10A is a human mammary epithelial cell line that was first described in 1990 by Soule and colleagues, which has been widely used *in vitro* to study normal as well as transformed breast cell functions(Puleo & Polyak, 2021; Qu et al., 2015;

Soule et al., 1990). These cells were isolated from benign proliferative breast tissue and spontaneously immortalized. They are not tumorigenic, as they do not form tumors when inoculated in immunocompromised mice. Molecularly, they do not express estrogen receptor, are depleted for p16 and p14ARF genes, which are both critical for regulating cell senescence, and show Myc gene amplification(Soule et al., 1990). Moreover, they lack mutational activation of oncogenes, such as HER2, int-2, and Ha-ras(Puleo & Polyak, 2021).

The MCF10A cell line can be grown in different ways, such as in monolayer (2D), in suspension (as mammospheres), 3D "on-top" Matrigel, 3D "cell-embedded" Matrigel or in a mixed Matrigel/collagen I gel. When cultured in 2D, MCF10A presents a cuboidal epithelial morphology, expressing markers of luminal, basal and stem/progenitor phenotypes. In 3D culture ("on-top" or embedded in Matrigel), MCF10A forms spheroid structures whereas, within the mixture of Collagen I and Matrigel, they have a better microenvironment for their growth as breast epithelial cell branches and acini. These acini enclose a hollow lumen covered by a basement membrane formed by organized and polarized cells. When cultured in 3D and in suspension, MCF10A expresses both luminal and basal markers; however, these cells present low mammosphere-forming ability. Interestingly, the acinar structure formed in 3D culture was both positive for basal markers and the milk proteins ß-casein and α-lactalbumin(Qu et al., 2015). Because this cell line possesses all the properties mentioned above, MCF10A represents a good model to study cell-cell interactions, as well as the effects of the mammary microenvironment, on their cellular functions.

The MCF10A-Plk4 cell line was engineered to enable the inducible overexpression of Polo-like kinase 4 (Plk4)(S. A. Godinho et al., 2014), a regulator of centriole biogenesis required for centriole duplication(Bettencourt-Dias et al., 2005; Habedanck et al., 2005a; Kleylein-Sohn et al., 2007), whose overexpression causes CA, originating cells with more than 4 centrioles in mitosis(Coelho et al., 2015). The establishment of cell models already allowed the comprehension of the

role of centrosome amplification in cancer, where CA has been shown to trigger invasive capacity, at similar levels as to that induced by overexpression of the breast cancer oncogene ERBB2(S. A. Godinho et al., 2014). This invasive capacity was triggered by increased centrosome microtubule nucleation, leading to Rac1 activity and disruption of cell-cell adhesion. Moreover, it has been shown that CA also induces non-cell autonomous invasion via secretion of pro-invasive factors, partially regulated by increased reactive oxygen species (ROS)(Arnandis et al., 2018).

Based on the findings mentioned above, and in order to ensure cell survival upon centriole number manipulation and to mimic what happens in cancer where the tumor suppressor p53 is often mutated, the main aim of this chapter was to establish and characterize a successful knockout (KO) of the tumor suppressor gene p53 through CRISPR/Cas9 technology in a non-tumorigenic cell line (MCF10A-Plk4), where centrosome amplification can be induced by doxycycline treatment.

The knock-out of p53 gene in the MCF10A-Plk4^{p53KO} cell line was validated through p53 and its downstream target p21 protein expression upon doxorubicin and doxycycline treatment. Then, MCF10A-Plk4 and MCF10A-Plk4^{p53KO} were characterized regarding their centriole number, cell cycle profile, cell viability and ROS levels, in Plk4-induced and non-induced overexpression condition. Furthermore, the tumorigenic capacity of both cell lines was accessed in mice and in the chick choriollantoic membrane model (CAM).

3.3 Materials and methods

3.3.1 Cell culture and growth conditions

Cell lines were maintained at 37˚C with humidified 5% CO_2 atmosphere.

Human mammary epithelial MCF10A-Plk4 and MCF10A-Plk4$^{1-608}$ cells (kind gift from Susana Godinho, from Barts Cancer Institute, Queen Mary University of London) and MCF10A-Plk4-p53 knock-out cells were grown in DMEM/F12 media supplemented with 5% Horse Serum, 20ng/ml epidermal growth factor (EGF), 10µg/ml insulin, 100ng/ml cholera toxin, 0.5µg/ml hydrocortisone, 100U/ml penicillin and streptomycin.

BT20 cancer cells were grown in DMEM media supplemented with 10% fetal bovine serum (FBS), and 100U/ml penicillin and streptomycin.

3.3.2 P53-knock-out and stable cell lines

The p53 CRISPR/Cas9 Knockout plasmid (sc-416469) and p53 Homologous Direct Recombinant-HDR plasmid (sc-416469-HDR) were purchased from Santa Cruz Biotechnology. The p53 CRISPR/Cas9 Knockout plasmid consists of a pool of 3 plasmids, each encoding the Cas9 nuclease and different target-specific 20 nucleotides guide RNA (gRNA), in order to ensure a maximum knockout efficiency. The HDR-plasmid also consists of a pool of 3 plasmids, each containing a homology-directed DNA repair template corresponding to the cut sites generated by the p53 CRISPR/Cas9 plasmid. During the repair, the HDR template incorporates the puromycin resistance gene at the site of the cut, to enable selection of stable knockout cells. A negative control plasmid (sc-418922) was also used. The negative control plasmid contains a single scrambled gRNA sequence that will not bind to the genomic target DNA and the Cas9/gRNA complex will not bind to or create a double-strand brake (DSB).

3.3.2.1 Sample and plasmid preparation

MCF10A-Plk4, at a concentration of $1.2x10^6$ cells/ml, were transfected with CRISPR/Cas9 P53-KO and p53 HDR plasmids to a final concentration of 6mg/ml using the Neon® Transfection system, with the following parameters: 1400V Pulse voltage, 20ms pulse width and 2 pulses. Cells were kept in media with no antibiotic at 37˚C with humidified 5% CO_2 atmosphere incubator, and media was changed every 48h, until recovery. After recovery, cells were sorted by positivity for GFP to select the successful transfected cells using a BD FACS Aria IIu.

3.3.2.2 Antibiotic selection

In order to select p53-KO stable cell lines, different puromycin concentrations (0.2 to 1µg/ml) were titrated to determine the lowest concentration that kills 100% of non-transfected cells in 3-5 days. During 7 days, 0.7µg/ml of puromycin was added to the MCF10A-Plk4-p53KO medium, and replaced every 2-3 days by a freshly prepared selective medium. Cells that were successfully knock-out and incorporated the puromycin resistance gene survived, whether the ones who failed to incorporate the puromycin gene died.

3.3.2.3 Single cell sorting

Puromycin resistant cells were sorted into single cell clones by using a BD FACS Aria IIu in order to have single-stable p53KO clones.

3.3.3 p53 protein functional assay

To validate if p53 gene was successfully knock-out, cells were subjected to a p53 functional assay(H. S. Kim et al., 2009; Lüpertz et al., 2010). Cells were exposed to three different conditions: a treatment with 1 and 3µM of Doxorubicin (inducing mild and severe DNA damage, respectively) for 4hours and kept for 24h in drug free medium; and a third condition where 1µg/ml of doxycycline (Dox) was added to cells for 24h (inducing extra centrosomes). In parallel, a control condition (no

treatment) was also performed. After 24h, cells were harvested for western-blot. Total protein levels of p53 and p21, a downstream target of p53, were assessed by Western Blot.

3.3.4 Western Blot

Protein extracts from cultured cells were prepared by homogenizing the samples in Sample Laemli buffer 1X in PBS, boiling at 95°C for 5 minutes to denaturate samples and spinning at 14000rpm to clear the lysate.
30µg of proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% acrylamide gel (BIO-RAD), transferred to nitrocellulose membrane by semi-dry blotting using the Trans-Blot Semi-Dry Transfer Cell (Bio-Rad) at 100V for 1h. Blocking with Tris-buffered saline (TBS) (25 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2.7 mM) containing 5% dry milk and 0.1 % Tween-20 (TBS-T) was performed for 1 hour at room temperature with shaking. Membranes were incubated with primary antibodies diluted in TBS-T (with 1% milk) overnight at 4ºC with shaking. After primary antibody incubation, membranes were washed three times in TBS-T, for 10 minutes each, and incubated with an appropriate peroxidase-conjugated secondary antibody diluted in TBS-T for 1 hour at room temperature with shaking. Membranes were then washed in TBS and incubated with 1mL of Enhanced ChemiLuminescence (ECL) (Amersham) reagent for 5 minutes to allow protein visualization on an x-ray Amersham Hyperfilm ECL (GE Healthcare).

Table 3.1: Western Blot primary antibodies

Primary antibody	Specie	Dilution	Seller	Reference	Molecular Size
Anti-p53(Ab-6) (Pantropic) (DO-1)	Mouse	1/1000	Merck	OP43-100UG	53kDa
p21 Waf1/Cip1 (12D1)	Rabbit	1/1000	Cell Signaling	2947S	21kDa
HSP70	Mouse	1/8000	Santa Cruz	B-6	70kDa
GAPDH	Rabbit	1/1000	Cell Signaling	14C10	37kDa

3.3.5 *In vitro* Doxycycline Induction

Doxycycline (Dox) is preferable to tetracycline as an inducer in the Tet-dependent regulatory systems, as induction due to Dox has high potency, superior tissue penetration and widespread availability(Marx et al., 2014). For every experiment, cells were treated with 1µg/ml of Dox to induce PLK4 overexpression for 24h. After 24h of treatment, cells were washed with 1X phosphate buffer solution (PBS) twice and trypsinized and counted, following with the assay. In parallel, a control condition (no Dox) was also performed.

3.3.6 Immunofluorescence

Adherent cell lines were grown on glass coverslips (VWR, #631-0150) and fixed using cold methanol for 10min at -20˚C. After fixation, cells were washed with 1X PBS and incubated for 30min at room temperature with 1X PBS containing 10% FBS. Afterwards, cells were incubated for 1h30min at room temperature with primary antibodies (table 2) diluted in 1X PBS + 10%FBS. After the incubation with the primary antibodies, cells were washed with 1X PBS three times, 10 min each. Cells were then incubated for 1h at room temperature with secondary antibodies (Alexa 488 and Alexa 549), diluted at 1:500 in 1X PBS with 10%FBS. After the secondary antibodies, cells were washed four times with 1X PBS, in which DAPI (1:500) was added on the second wash to stain DNA. The coverslips were then

mounted on slides using DAKO Faramount Aqueous Mounting Medium (Agilent, #S302580-2). The slides were kept for 24h to allow the mounting media to solidify before use.

Table 3.2: Immunostaining primary antibodies

Primary antibody	Specie	Dilution	Seller	Reference
Centrin-1(20H5)	Mouse	1/1000	Merck	04-1624
CP110	Rabbit	1/250	Homemade	Jiang et al, 2012(Jiang et al., 2012)

Centrin-1 primary antibody was conjugated with the secondary antibody Alexa Fluor 594 (Alfagene, #A11032), and CP110 primary antibody was conjugated with secondary antibody Alexa Fluor 488 (Alfagene, #A11034).

3.3.7 Image acquisition and centrosome quantification

Cells were observed and images were acquired using a commercial Nikon High Content Screening microscope, based on Nikon Ti equipped with a Andor Zyla 4.2 sCMOS 4.2Mpx camera, using the 100x 1.45 NA objective, DAPI, FITC and TRITC fluorescence filter sets and controlled with the Niko Elements software.
Centrioles were always stained with two centriolar markers, a distal (CP110) and a proximal (Centrin-1) marker, to search for co-labelling and avoid false positives. Only structures positive for the two markers were considered as centrioles. At least 100 cells for each cell line per condition were analyzed, and centriole numbers were manually counted on the Fiji/Image J Software. We consider as centriole amplification, when cells presented more than four (>4) centrioles.

3.3.8 Cell Cycle status

For cell cycle analysis, cells were harvested and centrifuged at low speed 1500rpm for 5min. The pellet cells were then washed in PBS, followed by a 5min spin at

1500rpm. Supernatant was removed and pellet was gently resuspended in 300µL of PBS, and adding slowly 700µl of 100% -20°C Ethanol. To ensure proper mixing and a good fixation of the cells, tubes were slowly inverted a few times. For DNA staining, a 5000rpm for 5 min spin was pursued in order to remove all the ethanol. Cell pellet was washed three times with PBS, and resuspended in 1mL of PBS with 0.5µg/ml RNAse A and with 2.5µg/ml of Propidium Iodide. Samples were incubated for 30min at 37°C, protected from light. Prior to FACS, cells were passed thought a 25-gauge needle to avoid doublets and ensure single cells. Labelled cells were analyzed on a FACS Canto II (BD Biosciences).

3.3.9 Cell Viability

Cell viability was assessed using the PrestoBlue™ Cell Viability Reagent (Invitrogen, #A13262). One day prior to doxycycline induction, $2x10^4$ cells were seeded into a 96 multiwell plate. 24h after PLK4 overexpression, cells were washed twice with PBS and 50µL of 1:20 PrestoBlue Reagent was added (diluted in media). The plate was incubated for 30min at 37°C and fluorescence was read at excitation 569nm, Emission 590nm and 75% sensibility, using the BioTek's Synergy™ MX microplate reader.

3.3.10 Doxorubicin treatment

Cells were plated in 96 well plates and treated/not treated with 1µg/ml of Doxycycline for 24h, prior of Doxorubicin treatment. Doxorubicin (Abcam, #ab120629) was dissolved in DMSO. Therefore, DMSO was used as a vehicle control for Doxorubicin treatment. We determined the concentration of Doxorubicin based on the IC50 of MCF10A-PLK4 cells grown in monolayer. 30nM of Doxorubicin was added to cells and cell viability was assessed 48h after Doxorubicin treatment. Results were normalized to MCF10A-Plk4 control condition.

3.3.11 Reactive oxygen species (ROS) assay

MCF10A-Plk4 and MCF10A-Plk4^{P53KO} Clone 9 were plated in black 96 well plates with clear bottom. 24h after absence and treatment with 1µg/ml Doxycycline for Plk4 overexpression, cells were washed with PBS and cellular ROS were assessed with the DCFDA-Cellular ROS Assay Kit (Abcam, ab113851). According to manufacturer's instructions, DCFDA was added to a final concentration of 10µM for 30min at 37°C. After washing twice, 200µL of PBS was added to each well and fluorescence was read on λex = 485 nm and λem = 535 nm. 200µM of Tert-Butyl hydroperoxide solution (TBHP) for 10min was used as a positive control for ROS induction. DCFDA values were normalized to the cell mass of respective conditions, determined by the Sulforhodamine B (SRB) assay.

3.3.12 *In vivo* Tumorigenesis

$1x10^6$ cells in 100µl cell suspension were xenografted into the subcutaneous region of 4-5 week-old female N:NIH(s)II:nu/nu nude mice, using a 25-gauge needle. Mice were maintained and housed at i3S Animal House, in a pathogen-free environment, under controlled conditions of light and humidity. Animal Guidelines for the Care and Use of Laboratory Animals, directive 2010/63/UE. Mice (four to seven per group) were weighted, and tumor width and length were measured with calipers every week. Tumor volume was estimated by using the equation, $V=0.5 x a x b^2$, where V is the volume, *a* is the length of the major axis of the tumor, and *b* is the length of the minor axis.

Mice were fed with 1mg/ml of Dox (Merck, D3447) in water supplemented with 5% sucrose (ThermoFisher, 15503022) during the experimental procedure of tumor growth to keep the overexpression of PLK4 in the injected cell lines. Water was changed once per week for the duration of the treatment. Mice were euthanized two months after the first inoculation.

3.3.13 *Ex-vivo* Tumorigenesis

To evaluate the effect of Pllk4 on tumorigenic response in the chick Embryo chorioallantoic membrane (CAM) model, fertilized chick (*Gallus gallus*) eggs were obtained from commercial sources and incubated horizontally at 37°C in a humidified atmosphere and referred to the embryonic development day (EDD). According to the European Directive 2010/63/EU, ethical approval is not required for experiments using embryonic chickens. Correspondingly, the Portuguese law on animal welfare does not restrict the use of chicken eggs for experimental purposes. In detail, at EDD3, a square window was opened in the shell after the removal of 1.5–2 mL of albumen to allow detachment of the developing CAM. The window was sealed with a transparent adhesive tape and the eggs returned to the incubator. On EDD9, $1x10^6$ cells were resuspended in Matrigel, to mimic the normal microenvironment, serving as a surface for attachment and allowing cells to grow and proliferate. These were then inoculated, pairwise, into a 5mm silicone ring under sterile conditions into a total of 24 eggs. At the endpoint (EDD16, 7days post inoculation), the ring was removed and CAMs were excised from the embryos and fixed with 10% neutral-buffered formalin, paraffin-embedded for slide sections and stained with Hematoxylin Eosin for histological examination or processed for immunohistochemistry and photographed *ex-ovo* using a stereoscope at 20× magnification (Olympus, Tokyo, Japan, SZX16 coupled with a DP71 camera). The area of CAM tumors was determined using the Olympus cell Sens Standard 1.14 program.

Four test conditions were tested: MCF10A-Plk4 and MCF10A-Plk4^{P53KO} Clone 9 with and without PLK4 overexpression. Posteriorly, $2x10^6$ MCF10A-Plk4^{P53KO} Clone 9, with and without PLK4 overexpression, resuspended in Matrigel, were also inoculated, pairwise, into a total of 24 eggs, to evaluate tumor formation.

3.4 Results and Discussion

3.4.1 P53 Knock-Out clones

The CRISPR/Cas9 is a versatile and popular tool that has been widely used, both *in vitro* and *in vivo* for efficient gene modification and/or disruption(Bauer et al., 2015; Sander & Joung, 2014). For the disruption of the p53 gene, we used a commercially available pool of 3 plasmids designed for maximum knockout efficiency. The p53 CRISPR/Cas9 knockout plasmid is designed to cause a double-strand break (DSB) in a 5' constitutive exon within the TP53 gene, therefore, disrupting the gene.

After transfection recovery, cells were treated with puromycin in order to select the successfully edited cells. These cells contain a puromycin resistance gene in their genomic DNA that was incorporated by the homology direct repair (HDR) plasmid during the DSB caused by the p53KO-Cas9 plasmid.

In order to avoid p53KO populational heterogeneity, different puromycin resistant p53KO single-clones were obtained by single cell sorting. From 18 MCF10A-Plk4^{p53KO} clones, we selected, randomly, some clones to validate the knock-out efficiency by western blot for p53 protein expression, as a first approach.

The Flo-1 (Distal esophageal adenocarcinoma) cell line was used as a positive control for p53 expression, while the HeLa cell line was used as a negative control. As observed in figure 3.1, several clones were successfully knock-out for the p53 protein (clones 9, 12, 15, 16), when compared to the parental cell line MCF10A-Plk4, showing complete absence of expression of p53. In clone 4, however, p53 was not successfully knock out, being its expression even increased when compared to MCF10A-PLK4 control cells.

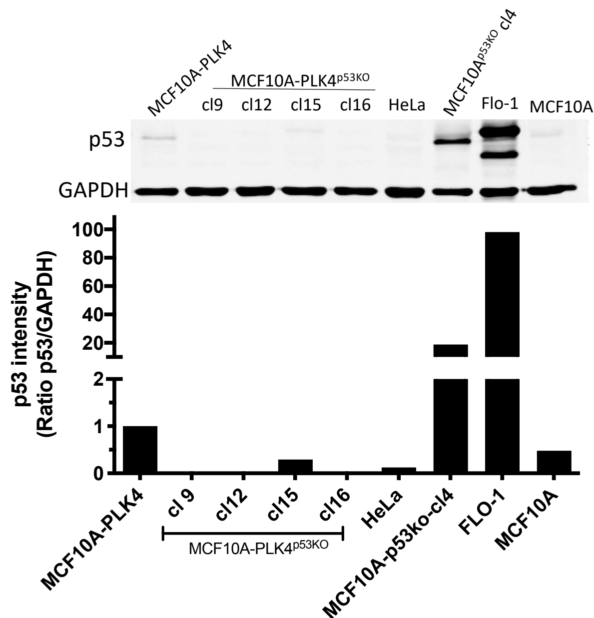

Figure 3.1. Efficient p53 knock out in MCF10A-Plk4 and p53 knock-out clones. Western b protein quantification of p53 expression on the MCF10A-PLK4, MCF10A-PLK4^{p53KO} clones, Flo-1 a HeLa cell lines. GAPDH was used as a loading control, Flo-1 and HeLa cell lines were used positive and negative controls for p53 expression, respectively. All conditions were normalized to t control condition (MCF10A-PLK4 control).

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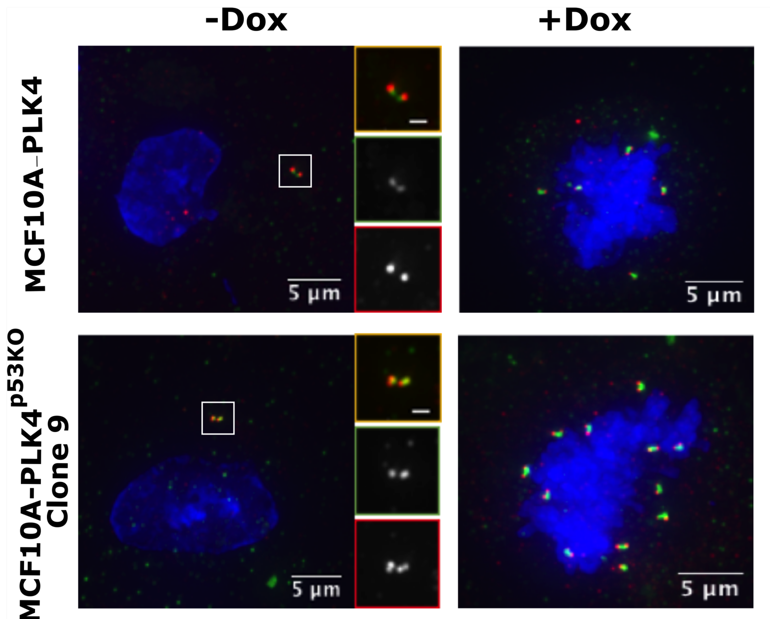
3.4.2 Centriole number

Next, we decided to characterize the centriole number of MCF10A-Plk4 and MCF10A-Plk4^{p53KO} stable cell lines. Cells were treated with 1µg/ml of Dox to induce Plk4 overexpression for 24h, and centriole number was manually scored by immunofluorescence, using two centriolar markers (CP110 and Centrin-1) (Figure 3.2A). As can be appreciated in figure 3.2B, MCF10A-Plk4 presented 46% of cells with 2 centrioles, 52% of cells with 3 to 4 centrioles, and a small percentage of cells having more than 4 centrioles (2%). When Plk4 was overexpressed by Dox treatment, MCF10A-PLK4 showed a decrease in the percentage of cells with 2 centrioles (34%) and 3 to 4 centrioles (41%) with increasing percentage of cells

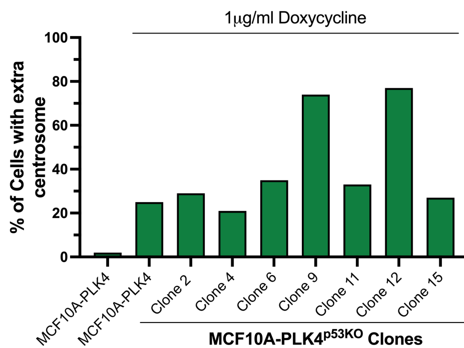
with >4 centrioles (25%) in interphasic cells. Interestingly, MCF10A-Plk4^{p53KO} stable cell lines presented different profiles concerning centrosome number when CA was induced by PLK4 overexpression. The p53KO clones 2, 4, 6, 11 and 15 showed similar levels of CA as the parental cell line MCF10A-Plk4 (28 ±5%). Interestingly, clones 9 and 12 presented a higher percentage of cells with CA upon Dox treatment. It is important to point out that, when the parental cell line MCF10A-PLK4 was originated, no clones were selected at any point and all the selected cells were pooled to make the cell line population. This might explain why we have clones with different levels of CA.

To conduct further experiments, we decided to select a MCF10A-Plk4^{p53KO} clone possessing a high percentage of CA in order to evaluate the direct or indirect role of CA on the induction of autocrine cellular properties, as well as the paracrine regulation of other cells from the tumor microenvironment. With this purpose, MCF10A-Plk4^{p53KO} clone 9 was chosen for further experiments, as it was one of the clones that presented a high percentage of cells with centriole amplification, with 74% of cells with more than 4 centrioles in interphase. In mitosis, which have a fixed number of 4 centrioles, MCF10A-Plk4 shows an increase from 12% to 76% of cells with CA when PLK4 is overexpressed, whether MCF10A-Plk4^{p53KO} clone 9 presents an increase from 26% to 93% of cells with CA (figure 3.2C).

A



B



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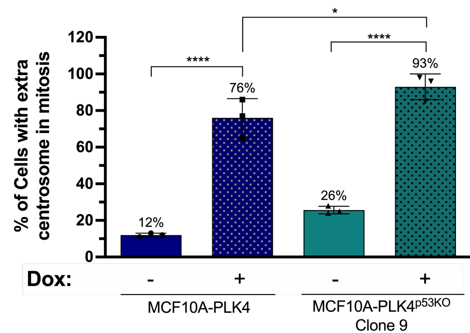

Figure 3.2. Plk4 overexpression in MCF10A-Plk4-^{p53KO} clones induces centriole amplification. A) Representative figures displaying cells with normal centriole number (-Dox) and cells with centriole amplification through Plk4 overexpression (+Dox). Centrioles are stained with two centriole markers Centrin-1 (green) and CP110 (red). Scale bars: 5µm (main images), 1µm (insets). **B)** Centriole number quantification in MCF10A-Plk4 and MCF10A-Plk4^{p53KO} interphasic and **C)** mitotic cells using two centriole markers. PLK4 was overexpressed using 1µg/ml of Dox for 24h. Data shown is average ± SD for three independent experiments, $p<0.05$, Oneway ANOVA test.

3.4.3 P53 protein knock-out validation

With the intention to validate if p53 gene was successfully disrupted using the CRISPR/Cas9 technique, cells were subjected to a p53 functional assay. Cells were treated with 1 or 3µM of Doxorubicin in order to induce DNA damage (mild and severe DNA damage, respectively), and centriole amplification using 1µg/ml of Dox. Doxorubicin induces DNA damage, in a dose-dependent manner, and this triggers p53 phosphorylation (at serine 392), induction of p21, sustained cell cycle arrest in the G2 phase of the cell cycle, and increase of other apoptotic factors (such as Bax, MDM2, among others)(Bunz et al., 1998; H. S. Kim et al., 2009; Lüpertz et al., 2010).

In order to validate de knock-out efficiency, p53 protein expression was analyzed by western blot. As observed in figure 3.3, the knock-out in the MCF10A-Plk4^{p53KO} Clone 9 was efficiently achieved, as the p53 protein intensity was undetectable, when compared to the MCF10A-Plk4, which is p53 positive. One the other hand, when compared to the control condition (no treatment), MCF10A-Plk4 showed increased p53 intensity, when treated with 1 and 3µM of Doxorubicin, but no significant difference when treated with 1 µg/ml of Dox. This suggests that CA induced by Plk4 overexpression in the MCF10A-Plk4 does not trigger a significant p53 activation. Moreover, we also analyzed the expression of p21, a downstream target of p53. P21 is a well-known inhibitor of cell cycle promoting the arrest of cells in the G1/S and G2/M cell cycle transitions, inhibiting CDK4,6/cyclin-D and CDK2/cyclin-E, respectively(Bunz et al., 1998; Karimian et al., 2016). P21 expression was not detected in the MCF10A-Plk4^{p53KO} Clone 9, but was increased in MCF10A-Plk4 when treated with 1µM and 3µM of Doxorubicin. With this, we concluded that in MCF10A-Plk4^{p53KO} Clone 9, p53 was successfully knock-out by CRISPR/Cas9, which was validated by p53 and p21 expression when different stimuli were given to cells.

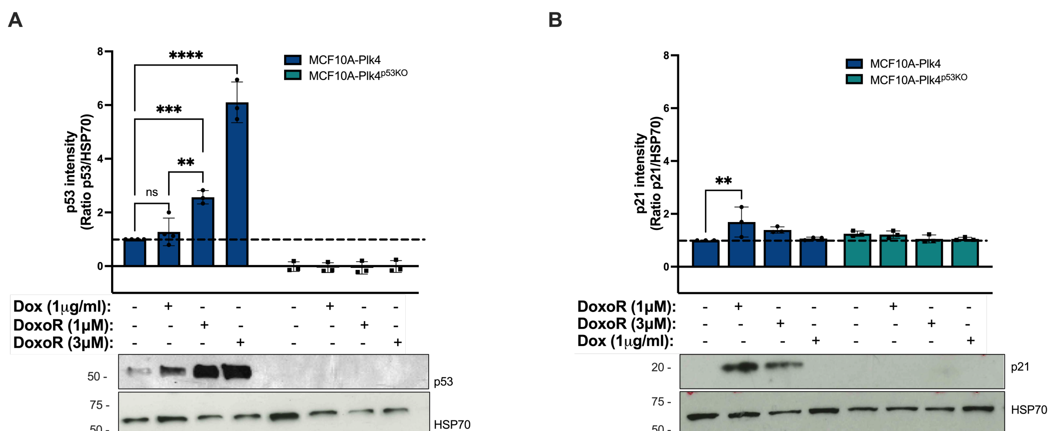

Figure 3.3. MCF10A-PLK4^{p53KO} Clone 9 did not show p53 and p21 upregulation upon doxorubicin and doxycycline treatment. Western blot protein quantification of p53 and its downstream target p21 protein on the MCF10A-PLK4 and MCF10A-PLK4^{p53KO} Clone 9 after the exposure of cells to 1 and 3μM of Doxorubicin for 4 hours and 24h in drug free medium and 1 μg/ml of Doxycycline for 24h. HSP70 was used as a loading control. All conditions were normalized to the control condition (MCF10A-PLK4 control). Data shown is average ± SD for three independent experiments, $p<0.05$, 2way ANOVA test

3.4.4 Cell cycle status and cell viability

In order to determine if Plk4 overexpression has an effect on cell viability, we took advantage of the PrestoBlue Cell Viability Reagent. This reagent is reduced by metabolically active cells, providing a quantitative measurement of viable and proliferating cells(Life Technologies Corporation, 2010). Interestingly, the removal of p53 did not alter cell viability of MCF10A-Plk4^{p53KO} Clone 9 when compared to the parental cell line MCF10A-Plk4 (Figure 3.4A). By treating cells with 1μg/ml of Dox for 24h to induce Plk4 overexpression, we observed that in the MCF10A-Plk4 cell line, Plk4 overexpression, leading to centrosome amplification, significantly altered cell viability when compared to the control condition. Moreover, MCF10A-Plk4^{p53KO} Clone 9 presented a higher viability when compared to its respective non-

treated condition. Furthermore, when Plk4 was overexpressed in both cell lines, the cell viability of MCF10A-Plk4^{p53KO} Clone 9 was significantly higher than MCF10A-Plk4 (Figure 3.4A). When analyzing the cell cycle profile on both cell lines (Figure 3.5), we did not observed changes in cell cycle progression in the MCF10A-Plk4 when centriole amplification was induced, when compared to the control condition. However, in MCF10A-Plk4^{p53KO} Clone 9 +Dox there was a decrease in the percentage of cells in G1, when compared to its respective control condition, but no significant change in the other cell cycle stages. When compared to MCF10A-Plk4 (+Dox), MCF10A-Plk4^{p53KO} Clone 9 (+Dox) also presented significant decrease of G1 cell cycle phase, but with no alteration in the other cell cycle stages. This might suggest that, in the absence of p53, Plk4 overexpression might be inducing an increase in cell proliferation, showed by increased percentage of viable cells (figure 3.4), or that cells are dying less. An apoptosis assay might explain better the results regarding cell death upon Plk4 overexpression in the p53KO background.

Our results demonstrated that when Plk4 is overexpressed, MCF10A-Plk4 cells presents an increased cell viability, with no changes in cell cycle profile. With the removal of p53, cells presented a 2.5-fold increase in cell viability, when compared to control conditions. It is important to point out that we only analyzed cell viability and cell cycle profile of these cell lines 24h after inducing CA. Thus, different outcomes might be obtained in the long term after CA induction. Nevertheless, it is clear from our results that, in the absence of p53, CA potentiates cell viability/proliferation in our cell line model.

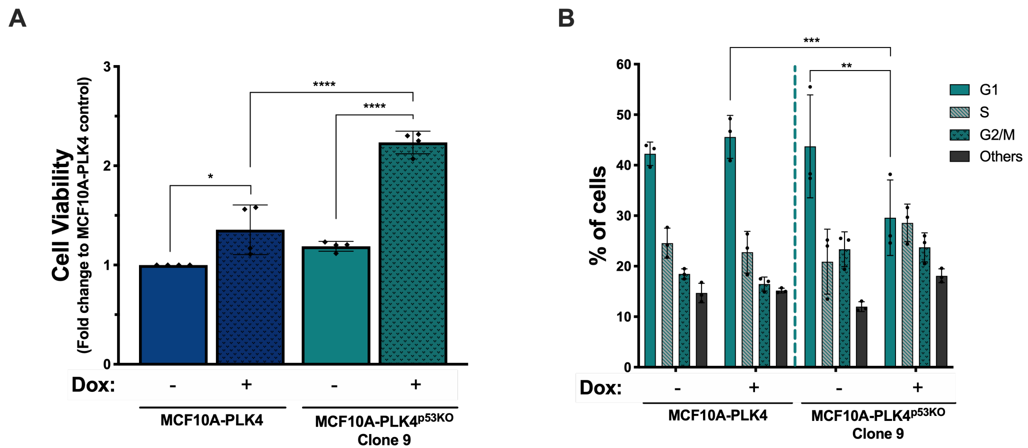

Figure 3.4. Centrosome amplification in p53KO background potentiated cell viability of MCF10A-Plk4 and MCF10A-Plk4^{P53KO} clone 9. (A) Cell viability (comparing to MCF10A-Plk4 control) and **(B)** Cell cycle stages of MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clones with Plk4 overexpression using 1µg/ml of Doxycycline for 24h. Data shown is average ± SD for three (for cell cycle an) and four (for cell viability) independent experiments, $p<0.05$, One-way ANOVA test.

3.4.5 Drug resistance capacity

The predictive value of p53 mutations in terms of cancer progression and treatment outcomes is still a contradictory subject. Studies on breast cancer patients have shown that p53 mutations were associated with resistance to the therapeutic drug Doxorubicin, but sensitive to 5-fluorouracil or γ-radiation(Aas et al., 1996; Formenti et al., 1997). Still, other groups have found no significant correlation between p53 mutations and patient's response to treatment(Rozan et al., 1998). Thus, we decided to validate in our model the therapeutic resistance to doxorubicin in the presence and absence of p53. For this purpose, cells were treated with 30nM of doxorubicin for 48h, based on the IC_{50} of MCF10A-Plk4 (Figure 3.5A) and cell viability was assessed using the PrestoBlue reagent. As appreciated in figure 3.5B, doxorubicin treatment in both MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9 resulted in a significant decrease in cell viability when compared to no treatment

conditions. Interestingly, MCF10A-Plk4^{p53KO} clone 9 is significantly more resistant to doxorubicin than MCF10A-Plk4. This means that, in this cell line model, p53KO ensures a protective mechanism against doxorubicin treatment.

PLK4 overexpression, as well as CA, has been suggested to have implications in breast cancer therapy, contributing to its failure by promoting resistance to tamoxifen and trastuzumab(Marina & Saavedra, 2014a). Therefore, we decided to explore whether cells with CA were also resistant or sensitive to doxorubicin treatment. Thus, cells where pre-treated with doxycycline for 24h to induce PLK4 overexpression, and therefore CA, and then treated with doxorubicin for 48h, and next evaluated cell viability on both cell lines. As observed in figure 3.5B, CA did not confer doxorubicin resistance to either MCF10A-Plk4 nor MCF10A-Plk4^{p53KO} Clone 9, showing a significant decrease in cell viability when compared to no-treatment condition. Moreover, in line with previous results, MCF10A-Plk4 showed to be more sensitive to doxorubicin, as it presented significantly lower percentage of viable cells than MCF10A-Plk4^{p53KO} Clone 9. This suggests that the loss of p53, but not CA, confers resistance to doxorubicin, but still not sufficient to promote a fully resistance, at similar levels as observed in the no drug treatment condition.

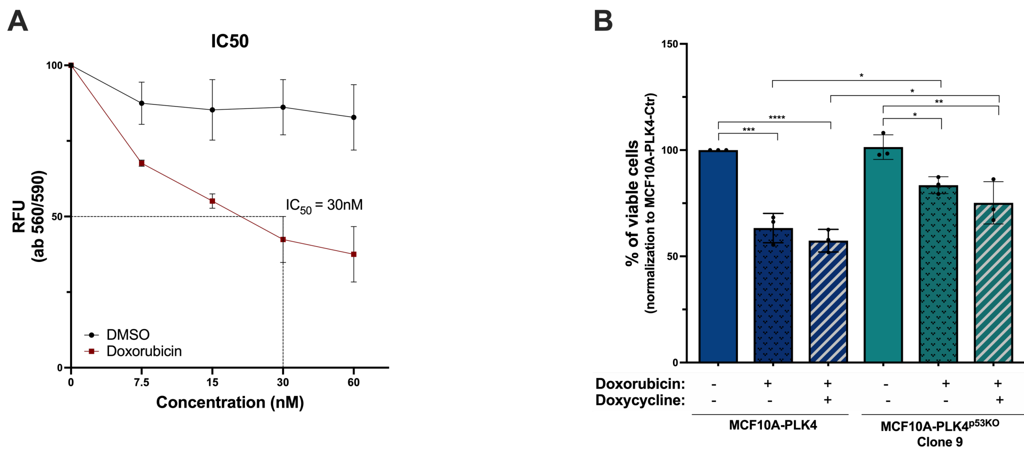

Figure 3.5. Centrosome amplification in WT and P53 knock-out does not confer doxorubicin resistance. A) Half maximal inhibitory concentration (IC_{50}) of Doxorubicin in the MCF10A10A-PLK4 in comparison to DMSO (control). All values are expressed as average ± SD for three independent experiments. **B)** Cell viability of cells treated with 30nM of Doxorubicin for 48h and 1μg/ml of Doxycycline for 24h prior of Doxorubicin treatment for 48h. Data shown is average ± SD for three (for cell cycle an) and four (for cell viability) independent experiments, $p<0.05$, One-way ANOVA test.

3.4.6 Reactive Oxygen Species (ROS)

The role of extra centrosomes in increasing cellular ROS has already been described(Arnandis et al., 2018). In summary, centrosome amplification induced by Plk4 overexpression (2μg/ml Dox for 48h) induces a 1.5-fold increase in ROS, with an increase of genes that control intracellular ROS. This increasing ROS alters cell secretion, inducing paracrine invasive phenotype, mediated by centrosome amplification.

Thus, we decided to characterize ROS levels in our cell line models with and without centrosome amplification in the wild-type and p53KO background. We used the DCFDA fluorescent dye and TBHP as a positive control for ROS induction (Figure 3.6). DCFDA diffuses into cells, where it is deacetylated to DCF, which in turn fluoresces upon reaction with the presence of a variety of ROS, such as hydroxyl, peroxyl and other ROS(Eruslanov & Kusmartsev, 2010). Intracellular ROS were measured in the MCF10A-Plk4 and MCF10A-Plk4^{p53KO} Clone 9 cells and significant differences were observed in cellular oxidative stress in MCF10A-Plk4^{p53KO} Clone 9 cells when compared to MCF10A-Plk4 cell line, suggesting therefore that, in our cell line model, the absence of p53 triggers an increase in intracellular ROS. P53 has important roles in regulating pro and antioxidant genes, depending on ROS levels(Cordani et al., 2020). In case of high ROS levels, p53 activates pro-oxidant genes, while with lower ROS levels it switches on antioxidant genes, suggesting that p53 modulates ROS according to stress severity(Cordani et al., 2020; Sablina et al., 2005). In fact, a study was conducted to determine the

effects of p53 on ROS levels in a set of human normal and carcinoma cell lines (which exhibit functional p53) shows that 48h after inhibition of p53, those cells presented increased ROS levels. In contrast, there was no increase in ROS in p53-negative cell lines MDAH041 and H1299, but conditional expression of WT p53 in MCFAH041-derived TR9-7 cells resulted in a 50% decrease in ROS(Sablina et al., 2005). Thus, p53 protects cells from ROS before damaged DNA is inherited to daughter cells, whereas p53 deficiency leads to elevated intracellular ROS(Sablina et al., 2005).

Moreover, the induction of Plk4 overexpression in the MCF10A-Plk4 and MCF10A-Plk4^{p53KO} Clone 9 cell line did not induce a significant cellular ROS increase. Our data differ from others(Arnandis et al., 2018), probably due to the difference on the duration of treatment between ours and others studies. Previous study from *Arnandis et al.*(Arnandis et al., 2018) where CA was induced for 48h, a 1.5-fold increase in ROS levels was observed. Because we only induced Plk4 overexpression for 24, it might not be sufficient to increase ROS levels within the cell.

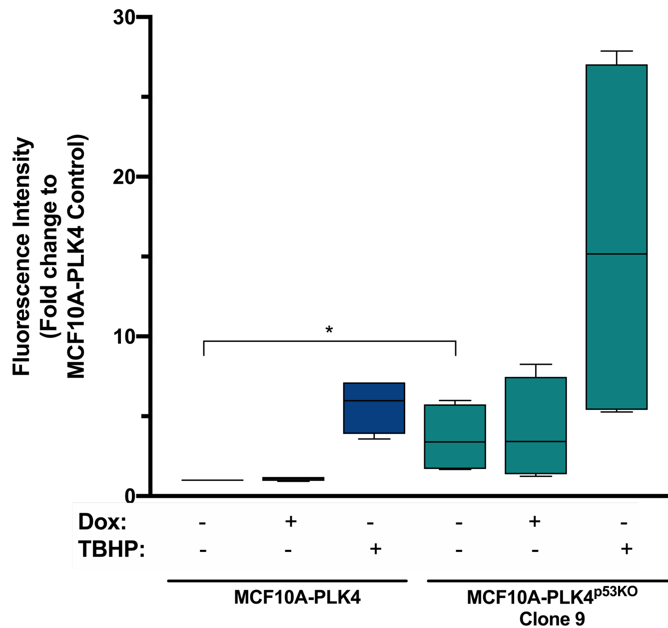

Figure 3.6. Knockout of p53, but not centrosome amplification, increases intracellular ROS. MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9 with PLK4 overexpression using 1µg/ml of Doxycycline for 24h did not show statistical difference in comparison to non-treated condition. However, the absence of p53 induced an increase in cellular ROS in the MCF10A-Plk4^{p53KO} clone 9. TBHP was used as positive control for ROS induction. DCFDA data was normalized to cell mass, measured by SRB assay. Data shown is average ± SD for three independent experiments, $p<0.05$, One-way ANOVA test (Kruskal-Wallis test).

3.4.7 Tumorigenicity

3.4.7.1 Mice

To determine the tumorigenicity capacity of cells, 1 million MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9 cells suspended in Matrigel with and without doxycycline treatment were injected into 4 to 7 nude mice (3 months of age). Matrigel was used to mimic the cellular microenvironment, serving as a surface for cell attachment, cell growth and proliferation(Kleinman & Martin, 2005). Mice were

being continuously fed with 1mg/ml of doxycycline in water supplemented with 5% sucrose during the experimental procedure of tumor growth to keep the overexpression of Plk4 in the injected cell lines. The MCF10A-Ca1a, a malignant cell line with metastatic capabilities generated from the MCF10A(Santner et al., 2001), was used as a positive control for tumor growth. Mice inoculated with MCF10A-Ca1a showed tumor growth 7 days after inoculation and were sacrificed on day 21 due to massive tumor size and according to Ethic Committee standards. However, we did not observe tumor growth on mice inoculated with MCF10A-Plk4 and MCF10A-Plk4^{p53KO} Clone 9 with and without Plk4 overexpression after 60 days of cell inoculation (Figure 3.7.1). Our results show that transplanting the MCF10A cell line model, in wild-type or knock-out p53 context, is not sufficient to drive tumor formation in mice, even if harboring centrosome amplification.

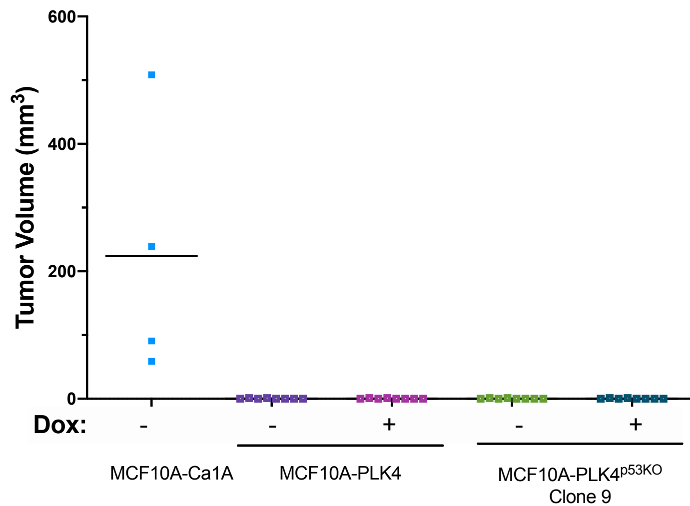

Figure 3.7.1. No tumor formation was observed in MCF10A-Plk4 and p53KO cells with/without centrosome amplification when inoculated in mice. 1 million cells with Matrigel were subcutaneously injected into 4 to 7 nude mice in both shoulder area. MCF10A-Ca1a was used as a positive control for tumor growth. No tumor growth was observed on mice inoculated with MCF10A-Plk4 and MCF10A-Plk4^{p53KO} Clone 9 with and without Plk4 overexpression after 60 days. Each point represents the number of mice used in the experiment.

3.4.7.2 Chick Choriollantoic Membrane model (CAM assay)

Traditionally, the tumorigenic capacity of cells is determined by xenografting into the subcutaneous region of immunocompromised mice. However, it encompasses several issues, such as financial costs, ethical limitations and time consuming. The chick chorriollantoic membrane model (CAM) has been shown to account several advantages regarding the *in vivo* gold standard mice model. It is a low-cost technique, reproducible and reliable model used to investigate several functional features of tumor biology, such as tumorigenesis, invasive and metastatic capacity of cells, susceptibility to anticancer drugs and radiation therapy(Pinto et al., 2021; Ribatti, 2017). For this reason, and to better explore the tumorigenic capacity of MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9, we decided to use CAM as a second model for tumorigenesis.

Four different test conditions, MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9 cells, with and without doxycycline treatment, were inoculated, pairwise, into a total of 24 eggs. CAMs were inoculated at embryonic development day (EDD) 9 with 1 million cells resuspended in Matrigel. The experiment ended at EDD16. Matrigel was also used in CAM to mimic the cellular microenvironment, serving as a surface for cell attachment for cell growth and proliferation. At the endpoint, CAMs were fixed with paraformaldehyde, excised from the embryo and photographed *ex-ovo*. Pictures were used to determine tumor area. No significant differences were found between control and treatments in the MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9 (Figure 3.7.2A). Because no tumor formation was observed by inoculating with 1 million cells, we also tested their tumorigenic capacity by inoculating 2 million MCF10A-Plk4^{p53KO} clone 9 (with and without centrosome amplification) cells resuspended in Matrigel into a total of 10 eggs (Figure. 3.7.2B). Despite being p53 negative, which potentiate tumorigenesis (Koifman et al., 2018; Lopes et al., 2018; Ohtsuka et al., 2018; Serçin et al., 2016), we did not observe tumor formation in CAM, confirming the non-tumorigenic potential of this cell line.

Previous studies have shown that CA (induced by overexpressing the *Drosophila melanogaster* homolog of Plk4, *Sak*) impairs asymmetric cell division in *Drosophila*(Basto et al., 2008). The transplantation of these neuroblasts from *Sak* overexpressed flies into WT flies originated tumors. Moreover, in mammals, transient overexpression of Plk4, followed by aneuploidy, has been shown to accelerate tumorigenesis in p53-deficient epidermis and also to promote spontaneous tumor formation in p53-deficient mice(Coelho et al., 2015; Levine et al., 2017; Serçin et al., 2016). Despite these *in vivo* studies showing the role of CA in tumorigenesis, our results show that transplanting the MCF10A cell line model, in wild-type or knock-out p53 context with CA, is not sufficient to drive tumor formation in mice or CAM. Many aspects can be discussed regarding this matter: first, despite the fact that MCF10A provides a unique tool for the investigation of molecular changes during human breast cancer progression, the use of a non-tumorigenic cell line model might be already a limitation for this cell line to induce tumor formation in complex *in vivo* models. Second, despite the high frequency of CA, it might be too early after CA for cells to acquire tumorigenic features, in order to induce tumor formation in mice/CAM. We cannot exclude the possibility that over time upon CA, cells accumulate mitotic errors and mutations that can favor their survival and tumorigenic capacity. We have observed that, in the absence of p53, MCF10A-Plk4^{p53KO} Clone 9 is able to maintain, until at least 9 days after the first induction of Plk4 overexpression, high levels of cells with CA (discussed on Chapter 3. Figure 1B). This means that despite supernumerary centrosomes can be cells detrimental for cells, in the absence of p53 they can overcome this and continue to proliferate. The same is not observed in the MCF10A-Plk4 cell line, as one day after Dox removal, MCF10A-Plk4 presented a sustained decrease of the percentage of cells with CA, throughout time, equalizing to control levels. Therefore, our cell line models, despite harboring CA and p53 absence, do not possess the capacity to form tumors.

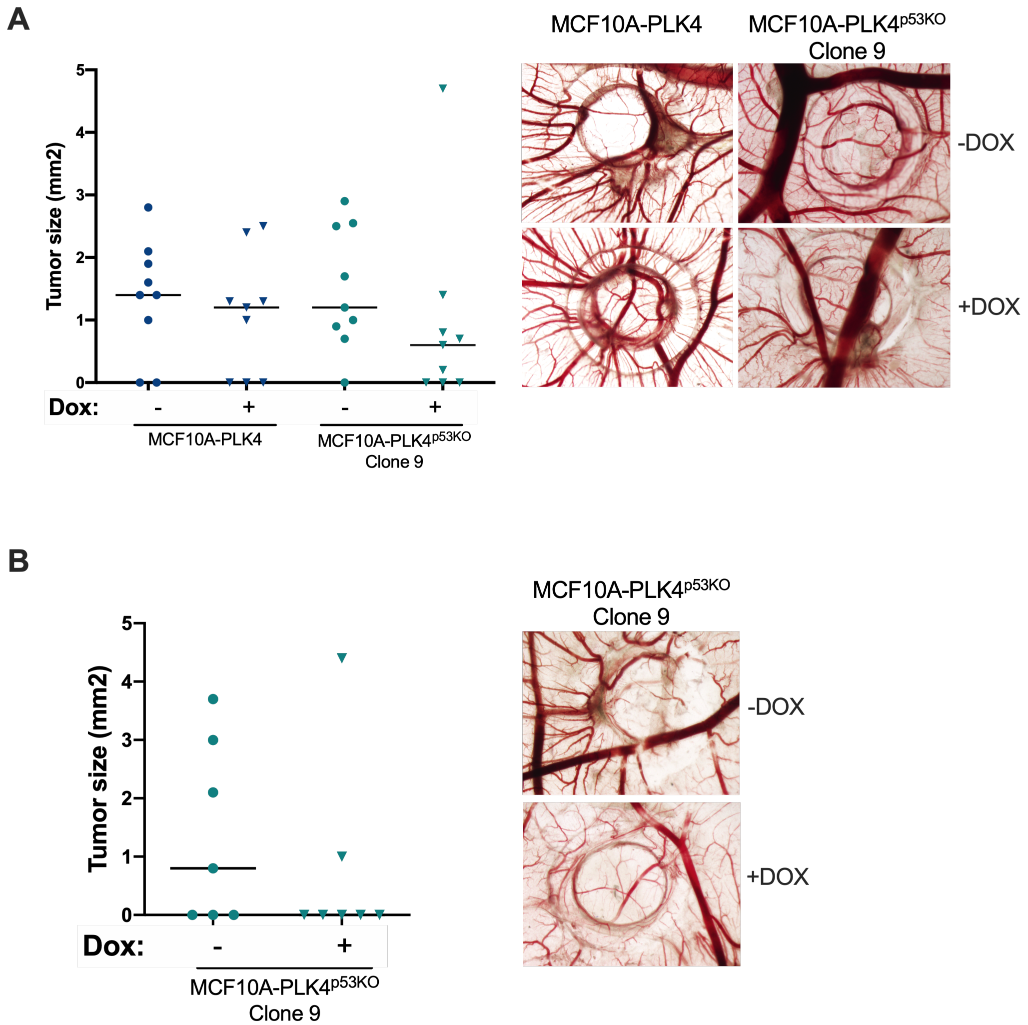

Figure 3.7.2. No tumor formation was observed in CAM when inoculated with MCF10A-Plk4 and p53KO cells with/without centrosome amplification. (A) Tumorigenic response (left) and representative images of CAM xenograted (right) of four different test conditions, $1x10^6$ MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9 cells, with and without doxycycline treatment. Cells were inoculated, pairwise, into a total of 24 eggs. **B)** Tumorigenic response (left) and representative images of CAM xenograted (right) of two different test conditions of $2x10^6$ MCF10A-Plk4^{p53KO} clone 9 cells, with and without doxycycline treatment, inoculated, pairwise, into a total of 10 eggs.

3.5 Conclusion

We have successfully disrupted the p53 gene in the non-tumorigenic MCF10A-Plk4 breast epithelial cell line model by using the CRISPR/Cas9. Using this technique, different MCF10A-Plk4 p53 Knock-out clones were originated, showing different centriole amplification levels. MCF10A-Plk4^{p53KO} clone 9 was chosen for further characterization, as it presented higher levels of CA than the parental cell line, MCF10A-Plk4. The knockout was validated by showing no p53, nor p21 protein expression, upon challenging the cells with different stimuli, such as doxorubicin (causing mild and severe DNA damage in a dose dependent matter). Moreover, we characterized MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9 cell lines for centriole number in mitosis, cell viability/proliferation, cell cycle profile, drug resistance to doxorubicin, ROS, and their tumorigenic capacity in mice and in the CAM model upon centrosome amplification. We found that MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9 cells differs on centriole number, where MCF10A-PLK4 presents 12% of mitotic cells with CA, whereas MCF10A-Plk4^{p53KO} clone 9 shows 26% of cells with CA. When Plk4 is overexpressed, MCF10A-Plk4 presented an increase of 76% of cells with CA, and MCF10A-Plk4^{p53KO} clone 9 shows 93% of cells with CA.

MCF10A-Plk4^{p53KO} clone 9 did not present changes in cell viability/proliferation when compared to MCF10A-Plk4. However, PLK4 overexpression induced an increase in cell viability in both cell lines, being more striking in MCF10A-Plk4^{p53KO} clone 9. Therefore, although the removal of p53 did not alter cell viability/proliferation, MCF10A-Plk4^{p53KO} clone 9 became more permissive for the increase in cell viability upon CA than MCF10A-Plk4. In regards to cell cycle length, I also did not observe cell cycle changes between MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9, showing again that the removal of p53 does not impair the cell cycle on these cells. No cell cycle change was observed in WT p53 MCF10A-Plk4 cells when CA is induced, however, when CA is induced in the p53

KO background cells presented a significant decrease in G1 cell cycle phase when compared to non-induced condition and to MCF10A-Plk4 with CA. This could imply that, in the p53KO background, the induction of CA for 24h via Plk4 overexpression can induce minor cell cycle changes, but not sufficient trigger major cell cycle arrest, as cells were cycling normally.

To investigate whether p53 loss correlates with the response to drugs, we found that, in our cell line model, the p53KO clone showed increased sensitivity to a 48h doxorubicin treatment. Importantly, MCF10A-Plk4, possessing WT p53, presented higher sensitivity than MCF10A-Plk4^{p53KO} clone 9. Along this, loss of p53 in human cancers has been shown to alter the response to therapeutic agents(Bunz et al., 1999). In regards to drug resistance, MCF10A-Plk4^{p53KO} clone 9 showed to be significantly more resistance to doxorubicin treatment than MCF10A-Plk4, however, it is still more sensitive to doxorubicin than no treatment condition. Moreover, Plk4 overexpression, inducing CA amplification, did not induce doxorubicin resistance in both cell lines. Still, MCF10A-Plk4^{p53KO} clone 9 showed significantly more viable cells then MCF10A-Plk4, when Plk4 was overexpressed prior of doxorubicin treatment. This suggests that CA does not induce a protective role against drugs, being the absence of p53 the main protective mechanism of drug treatment. This conclusion was based on the observation that with or without CA, cells were still sensitive to doxorubicin. However, cells with WT p53 were more sensitive to doxorubicin than cells without p53.

We assessed the tumorigenicity capacity of both MCF10A-Plk4 and MCF10A-Plk4^{p53KO} clone 9 cells and observed no tumor formation in mice or CAM after inoculating $1x10^6$ or $2x10^6$ cells. Despite a study showing that p53-deficient cells injected into athymic mice demonstrated considerable increase in tumor growth rate when compared to the control A549 cells(Sablina et al., 2005), it is important to point out that these cells are already cancerous, harboring other mutations that favor tumor formation. In accordance with our results, a study from Weiss and colleagues has shown that MCF10A p53-null clones possess heterogeneous

capabilities in anchorage-independent growth, invasion, EGF-independent growth and differential sensitivity the DNA damaging drug doxorubicin(Weiss et al., 2010). Moreover, they showed that regardless of p53 status of the different clones, they did not form tumors in athymic nude mice 30 days after inoculation, when compared to MDA-MB-231 cells used as positive control. This suggests that, similar to our model, loss of p53 can induce changes *in vitro* and impart features of transformation, but it is not sufficient for *in vivo* tumorigenesis. Additional changes besides the loss of p53 is necessary for the tumorigenic capability of these cells.

The characterization of these cell line models will be useful to further understand the breast cancer phenotypic changes that accompany p53 loss and centrosome amplification, as well as help to provide future insights in cancer initiation and progression.

3.6 Autor contributions and acknowledgements

3.6.1 Author Contribution

All the experiments presented in this chapter were performed and analyzed by Irina Fonseca. *In vivo* tumorigenesis assays were performed by Irina Fonseca and Ana Sofia Ribeiro (mice tumorigenesis assay), and CAM tumorigenesis assay was performed by Irina Fonseca and Marta Teixeira Pinto. The experiments were designed by Irina Fonseca, with the supervision of Gaëlle Marteil, Barbara Sousa, Joana Paredes and Mónica Bettencourt-Dias.

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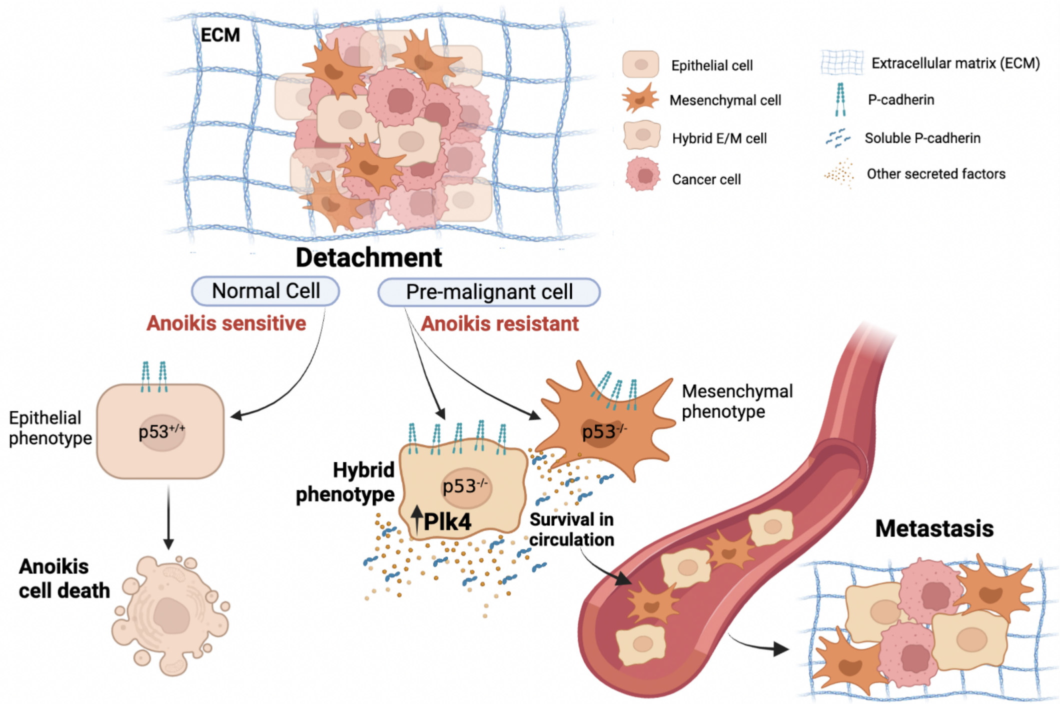
Chapter 4.

Polo-like kinase 4 (Plk4) potentiates *anoikis*-resistance of p53KO mammary epithelial cells by inducing a hybrid EMT phenotype

This section is adapted from: **Fonseca, I.**, Horta, C., Ribeiro, A.S. *et al.* Polo-like kinase 4 (Plk4) potentiates *anoikis*-resistance of p53KO mammary epithelial cells by inducing a hybrid EMT phenotype. *Cell Death Dis* **14**, 133 (2023). https://doi.org/10.1038/s41419-023-05618-1

4.1 Graphical Abstract



4.2 Abstract

Polo-like kinase 4 (Plk4), the major regulator of centriole biogenesis, has emerged as a putative therapeutic target in cancer due to its abnormal expression in human carcinomas, leading to centrosome number deregulation, mitotic defects and chromosomal instability. Moreover, Plk4 deregulation promotes tumor growth and metastasis in mouse models and is significantly associated with poor patient prognosis.

Here, we further investigate the role of Plk4 in carcinogenesis and show that its overexpression significantly potentiates resistance to cell death by *anoikis* of non-tumorigenic p53 knock-out (p53KO) mammary epithelial cells. Importantly, this effect is independent of Plk4's role in centrosome biogenesis, suggesting that this kinase has additional cellular functions. Interestingly, the Plk4-induced *anoikis* resistance is associated with the induction of a stable hybrid epithelial-mesenchymal phenotype and is partially dependent on P-cadherin upregulation. Furthermore, we found that the conditioned media of Plk4-induced p53KO mammary epithelial cells also induces *anoikis* resistance of breast cancer cells in a paracrine way, being also partially dependent on soluble P-cadherin secretion.

Our work shows, for the first time, that high expression levels of Plk4 induce *anoikis* resistance of both mammary epithelial cells with p53KO background, as well as of breast cancer cells exposed to their secretome, which is partially mediated through P-cadherin upregulation. These results reinforce the idea that Plk4, independently of its role in centrosome biogenesis, functions as an oncogene, by impacting the tumor microenvironment to promote malignancy.

4.3 Introduction

Centrosomes are the primary microtubule-organizing center (MTOC) of dividing animal cells. Consisting of two centrioles surrounded by a pericentriolar matrix (PCM), they participate in essential cellular processes, such as cell division, signaling, migration, and polarity(Bettencourt-Dias & Glover, 2007; Brito et al., 2012). Centriole biogenesis is tightly regulated and occurs once per cell cycle, in S-phase, during which centrioles duplicate to ensure the assembly of a bipolar spindle in mitosis, an essential structure for proper chromosome segregation(Brito et al., 2012; Kleylein-Sohn et al., 2007; Nigg & Holland, 2018; Nigg & Stearns, 2011).

Polo-like kinase 4 (Plk4), is a regulator of centriole biogenesis that is required for centriole duplication(Bettencourt-Dias et al., 2005; Habedanck et al., 2005b; Holland et al., 2010; Kleylein-Sohn et al., 2007) via the phosphorylation and interaction with different centriolar proteins. In consequence, when Plk4 is overexpressed, it leads to centrosome amplification (CA, i.e., the presence of supernumerary centrosomes) resulting in cells with more than 4 centrioles(Coelho et al., 2015), promoting genomic instability and tumorigenesis(Anderhub et al., 2012; Ganem et al., 2009; S. A. Godinho & Pellman, 2014; Lopes et al., 2018; Shirley Jusino, 2017; A. Singh et al., 2020). In fact, Plk4 overexpression, as well as CA and centrosome structure abnormalities, have been already found in many types of tumors(Chan, 2011; Denu et al., 2016a; Kayser et al., 2005; Lopes et al., 2018; Marteil et al., 2018; A. Singh et al., 2020) and shown to be significantly associated with poor clinical outcomes for cancer patients(Chan, 2011; de Almeida et al., 2019; Levine et al., 2017; Z. Li et al., 2016).

Despite strong evidence of Plk4's role in cancer and its therapeutic potential, it is still unclear how it contributes to tumorigenesis. Plk4 has been demonstrated to enhance cell migration through RhoA activation and to be associated with the expression of matrix metalloproteinases (MMPs) and other pro-motility

genes(Rosario et al., 2015). In addition, Plk4 has been shown to promote cancer metastasis through the regulation of the actin cytoskeleton by the Arp2/3 complex(Kazazian et al., 2017) in breast cancer cells, whereas its downregulation suppresses tumorigenesis and metastasis in neuroblastoma(Tian et al., 2018). Plk4 has also been shown to have a prognostic value, since its overexpression is associated with worse disease-free survival (DFS) and overall survival (OS), demonstrating also a negative impact on the response to chemotherapy(Z. Li et al., 2016; Marina & Saavedra, 2014b; Pannu et al., 2012). Levine *et al.* showed that transient Plk4 overexpression causes CA and aneuploidy *in vivo* in a mouse model of intestinal neoplasia and accelerates the occurrence of spontaneous tumor formation(Levine et al., 2017), and Serçin *et al.* demonstrated that transient Plk4 overexpression accelerates tumorigenesis in p53-deficient mice(Serçin et al., 2016).

The loss of the p53 tumor suppressor gene has been strongly associated with tumorigenesis and malignant progression in the majority of epithelial cancers(Hayashi et al., 2016; Ohtsuka et al., 2018; Rivlin et al., 2011). One of the molecular mechanisms potentiated by the absence of p53 is the induction of epithelial to mesenchymal transition (EMT)(C. J. Chang et al., 2011; Pinho et al., 2011). EMT is the process by which cells lose their epithelial characteristics and undergo several molecular, biochemical, and morphological changes, acquiring a more undifferentiated and mesenchymal phenotype. EMT is well known for conferring stem-like properties and cell plasticity, leading to the acquisition of a migratory and invasive phenotype by weakening cell-cell adhesion, facilitating metastatic capacity, as well as resistance to chemo and radiotherapy(Jing et al., 2011; Mani et al., 2008; Pastushenko & Blanpain, 2019). EMT also allows evasion to *anoikis*, a specific mode of apoptotic cell death that occurs due to insufficient cell-matrix interactions(Frisch & Screaton, 2001; Grossmann, 2002). Resistance to *anoikis* is a characteristic found in cells with stem-like properties, being thus, considered a critical contributor to cancer cells dissemination within the

bloodstream and, consequently, to their metastatic capacity(Rennebeck et al., 2005; Tan et al., 2013). Interestingly, high Plk4 expression has been shown to induce EMT in neuroblastoma and colorectal cancer by regulating PI3K/Akt and Wnt/ß-catenin signaling pathways, respectively(Liao et al., 2019; Tian et al., 2018), which are well known oncogenic pathways.

Although EMT has been initially assumed as a binary process, the concept of hybrid epithelial-mesenchymal phenotype has recently emerged and has been shown to be more relevant than previously thought for cells to become metastatic. By expressing both epithelial and mesenchymal markers, cells increase their plasticity and are able to better respond to external stimuli, potentiating their capacity to resist apoptosis (as *anoikis*) and enhancing their metastatic features(Kröger et al., 2019; Pastushenko & Blanpain, 2019). P-cadherin, which is a cell-cell adhesion protein, is expressed in cancer cells harboring epithelial and mesenchymal features, being a putative marker of a hybrid EMT phenotype(Ribeiro & Paredes, 2015). In fact, P-cadherin expression is early promoted by EMT-inducers, such as hypoxia, driving *anoikis* resistance capacity in breast cancer cells(Sousa et al., 2020). Moreover, its overexpression has been found in many types of tumors(Paredes et al., 2002, 2007; Vieira & Paredes, 2015) and is associated with tumorigenic and metastatic potential, stem cell activity, and collective cell invasion(Mandeville et al., 2008; Ribeiro et al., 2010; Vieira, André Filipe, Ribeiro et al., 2014). For all these reasons, P-cadherin is considered an important biomarker of poor prognosis in breast cancer (Paredes et al., 2005; Turashvili et al., 2011).

In this work, we show that Plk4 overexpression potentiates *anoikis* resistance of non-tumorigenic p53 knock-out mammary epithelial cells, by the induction of a stable hybrid epithelial-mesenchymal phenotype. Moreover, we also show that cells with high Plk4 expression secrete factors that promote *anoikis* resistance of cancer cells in a paracrine way, being P-cadherin one of the mechanistic players in that process. Furthermore, tumors with high expression of Plk4 and P-cadherin

are significantly correlated with a worse DFS and OS, as revealed by Kaplan-Meier plots. Therefore, our findings demonstrate that Plk4 overexpression influences the communication between cells and the tumor microenvironment, impacting malignancy in different ways.

4.4 Materials and methods

4.4.1 Cell culture and growth conditions

Cell lines were maintained at 37˚C with humidified 5% CO_2 atmosphere.

Human mammary epithelial MCF10A-Plk4 and MCF10A-Plk4$^{1-608}$ cells (kind gift from Susana Godinho, from Barts Cancer Institute, Queen Mary University of London) and MCF10A-Plk4-p53 knock-out cells were grown in DMEM/F12 media supplemented with 5% Horse Serum, 20ng/ml epidermal growth factor (EGF), 10µg/ml insulin, 100ng/ml cholera toxin, 0.5µg/ml hydrocortisone, 100U/ml penicillin and streptomycin.

BT20 cancer cells were grown in DMEM media supplemented with 10% fetal bovine serum (FBS), and 100U/ml penicillin and streptomycin.

RPE-Plk4 cells were grown in DMEM/F12 media supplemented with 10% Tet-free fetal bovine serum (FBS), and 100U/ml penicillin and streptomycin.

4.4.2 P53 Knock-out cell line

For the generation of p53 knock-out (p53KO) cell line, CRISPR/Cas9 plasmid (sc-416469) and p53 Homologous direct Recombinant-HDR plasmids (sc-416469-HDR) were transfected into MCF10A-Plk4 cells using the Neon® Transfection system. After transfection recovery, cells were selected with puromycin in order to select p53KO stable cell line. To have monoclonal stable p53KO clones, puromycin resistant cells were sorted into single cell clones by using a BD FACS Aria IIu.

To validate if p53 was successfully knocked-out, cells were exposed to three different conditions to determine the functionality of the p53 protein: treatment with 1 and 3µM of Doxorubicin (mild and severe DNA damage, respectively) for 4h and kept for 24h in drug free medium and, a third condition where 1µg/ml of Doxycycline was added to cells for 24h (inducing extra centrosomes). After the 24h, cells were harvested for western-blot analysis. Total protein levels of p53 and p21, a downstream target of p53, was assessed by Western Blot.

4.4.3 Plk4 overexpression - Doxycycline (Dox) treatment

Cells were grown until 80-85% confluence and for every experiment, cells were treated with 1µg/ml of Dox (Merck, Darmstadt, Germany) to induce Plk4 overexpression for 24h. After 24h of treatment, cells were washed twice with 1X phosphate buffer solution (PBS), trypsinized and counted, before starting any specific assay. In parallel, a control condition (no doxycycline) was also performed.

4.4.4 Immunofluorescence

Adherent cell lines were grown on glass coverslips (VWR, #631-0150) and fixed using cold methanol for 10 min at -20 ˚C. After fixation, cells were washed with 1X PBS and incubated for 30min at room temperature (RT) with 1X PBS containing 10% FBS. Afterwards, cells were incubated for 1h30min at RT with primary antibodies Centrin-1(20H5) (1/1000, Merck 04-1424) and CP110 (1/250, Jiang et al, 2012(Jiang et al., 2012)) diluted in 1X PBS + 10%FBS. After the incubation with the primary antibodies, cells were washed with 1X PBS three times, 10 min each. Cells were then incubated for 1h at RT with corresponding secondary antibodies Alexa Fluor 488 (Alfagene, #A11034) and Alexa Fluor 549 (Alfagene, #A11032), diluted at 1:500 in 1X PBS with 10%FBS. After the secondary antibodies' incubation, cells were washed four times with 1X PBS. DAPI (1:500) was added on the second wash to stain DNA. The coverslips were then mounted on slides

using DAKO Faramount Aqueous Mounting Medium (Agilent, #S302580-2). The slides were kept for 24h to allow the mounting media to cure before use.
Two centriolar markers were always used, in order to avoid false positive. Only structures positive for the two markers were considered as centrioles.

4.4.5 Image acquisition and centrosome quantification

Mitotic cells were observed using a Leica DMI6000 (Leica Microsystems, Germany) Microscope and images were acquired with a Hamamatsu FLASH4.0 (Hamamatsu, Japan) camera, using the HCX PL APO CS 63x/1.30 GLY 21ºC objective, and the LAS X Software. Images were taken in Z-Stacks in a range of 10-14μm, with a distance between planes of 0.2μm.
We consider as centriole amplification, when mitotic cells presented more than four (>4) centrioles. In order to obtain the percentage of cells with extra centrioles, at least 100 cells were analyzed for centriole number per condition and per experiment and only centrioles positive for the two centriolar markers (Centrin-1 and CP110) were considered. Centrosomes were quantified manually, using the Fiji/Image J Software (National Institutes of Health).

4.4.6 Mammosphere forming efficiency (MFE) assay

Cells were enzymatically harvested and manually disaggregated to form a single-cell suspension and resuspended in cold PBS. Cells were then plated at the density of 750cells/cm^2 in non-adherent culture conditions, in 6-well plates coated with 1.2% poly (2-hydro- xyethylmethacrylate)/95% ethanol (Sigma-Aldrich) and allowed to grow for 5 days, in DMEM/F12 containing B27 supplement (Invitrogen), 500 ng/mL of hydrocortisone, 40 ng/mL insulin, 20 ng/ mL EGF in a humidified incubator at 37°C and 5% (v/v) CO_2. MFE was calculated as the number of mammospheres (≥60 μm) formed divided by the number of cells plated, and presented as percentage.

4.4.7 Protein extraction and western blot analysis

Protein extracts from cultured cells were prepared by using catenin lysis buffer [1%(v/v) Triton X-100 and 1%(v/v) NP-40 (Sigma-Aldrich, USA) in deionized phosphate-buffered saline (PBS)] supplemented with 1:7 proteases inhibitors cocktail (Roche Diagnostics GmBH, Germany) for 10 min, at 4 °C. Cell lysates were vortexed and centrifuged at 14000rpm at 4°C, for 10min. Supernatants were collected and protein concentration determined using the Bradford assay (BioRad Protein Assay Kit, USA). Proteins were homogenized in Sample buffer 1X in PBS [Laemmli, 5%(v/v)2-b-mercaptoethanol and 5% (v/v) bromophenol blue], boiled at 95°C for 5 minutes for protein denaturation and spinning at 14000rpm to clear the lysate.

40 µg of proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% acrylamide gel (BIO-RAD) and transferred to nitrocellulose membrane by semi-dry blotting using the Trans-Blot Semi-Dry Transfer Cell (Bio-Rad) at 100 V for 2 h. Blocking with PBS containing 5% non-fat dry milk and 0.1 % Tween-20 was performed for 1 h at room temperature with agitation. Membranes were incubated with primary antibodies (Table 1) diluted in PBS-Tween-20 (PBS-T) with 1% non-fat dry milk overnight at 4 ºC with agitation. After primary antibody incubation membranes were washed three times in PBS-T, for 15 min each, and incubated with an appropriate peroxidase-conjugated secondary antibody diluted 1:2000 in PBS-T for 1 h at room temperature with agitation. Membranes were then washed in PBS-T and incubated with 1 mL of Enhanced ChemiLuminescence (ECL) (Amersham) reagent for 5 minutes to allow protein visualization on an x-ray Amersham Hyperfilm ECL (GE Healthcare). Fiji-Image J Software was used for quantification of the difference in protein expression comparing with GAPDH or HSP70 expression.

4.4.8 siRNA transfection

P-Cadherin gene silencing (*CDH3*) was performed in MCF10A-Plk4p53KO using a validated siRNA, specific for *CDH3* (50nM, Hs_GCDH3_6), with the following target sequence 5' AAGCCTCTTACCTGCCGTAAA 3′, from Qiagen (USA). P53 gene silencing was performed in MCF10A-Plk4 and MCF10A-Plk4$^{1\text{-}608}$ using a specific siRNA (100nM, L-003329-00-0020, Dharmacon). Transfections were carried out using Lipofectamine 2000 (Invitrogen), according to manufacturer's recommended procedures. A scrambled siRNA targeting sequence 5' AAGCCTCTTACCTGCCGTAAA 3′, with no homology to any gene, was used as a negative control (Qiagen, USA). Cells were incubated with the transfection mix for 6h. After siRNA transfection, cells were incubated for 24h in the presence or absence of Dox for 24h (1µg/ml) to induce extra centrosomes. Gene inhibition was evaluated by western blot after 31h of cell transfection for the MFE assay and after 47h for condition media collection.

4.4.9 Condition media collection

For the conditioned medium assays, $5x10^5$ cells/well of MCF10A-Plk4 and MCF10A-Plk4^{P53KO} were grown until 80% - 85% confluence in 6 well plates. 24h after presence or absence of 1µg/ml Dox treatment for Plk4 overexpression, in serum-free media, conditioned media was collected, centrifuged at 1200 rpm for 5 min and filtered through a 0.2 µm pore filter. Condition media was then added to BT20 cells for 48 h.

4.4.10 P-Cadherin ectodomain cleaved (soluble P-cadherin, sP-cad)

For P-Cadherin ectodomain cleaved detection, $5x10^5$ cells were grown until confluence in 0.2 mg/ml collagen-type I-coated 6 well plates (Merck, Darmstadt, Germany) and incubated in serum-free medium for 24 h with/without Dox treatment. For better detection of the soluble P-cadherin, serum-free conditioned media was filtered with 0.2µm filter (Cytiva, Germany) for 5min at 1200rpm and

concentrated using 30K Microcon Centrifugal Filter Devices (Merck, Darmstadt, Germany), according to manufacturer's instruction. The proteins secreted were quantified in the recovered supernatant using the Bradford assay, and Western Blot was performed.

4.4.11 qRT-PCR

qRT-PCR was performed in order to determine *PLK4* and P-Cadherin (*CDH3*) RNA expression. Cells were harvested and RNA extraction was performed using the RNeasy Kit (Qiagen, USA) according to manufacturer's instructions. Concentration was determined in a ND-1000 spectrometer (Nanodrop) and 1 µg of total RNA was converted to cDNA using the Omniscript Reverse Transcriptase RT Kit (Invitrogen, USA). Quantitative Real-Time PCR (qRT-PCR) reaction was performed with TaqMan Gene Expression Assays (Applied Biosystems, USA), using gene-specific *PLK*4 and P-Cadherin (*CDH3*) PrimeTime probes (Integrated DNA Technologies, Inc., USA), recognizing specifically the corresponding cDNA sequences, which were amplified for 40 cycles. Analysis was performed with the 7500 Fast Real Time PCR Systems Instrument and software (Applied Biosystems), following the manufacturer's recommendations. PrimeTime probe for *GAPDH* was also used as a housekeeping gene, and relative gene expression was determined by normalization. Data was analyzed by the comparative 2(-DDCT). All reactions were done in triplicate and the results presented as mean of the values from three or more independent experiments. For *PLK4* mRNA detection on the MCF10A-Plk4 and MCF10A-Plk4^{p53KO}, a probe annealing on the exons 15-16 was used, while on MCF10A-Plk4$^{1-608}$ Plk4 probe annealing on exons 1-3 was used, due to the truncation on the C-terminus (Table 2).

4.4.12 Clonogenic assay

MCF10A-Plk4^{p53KO} cells were treated and not treated (negative control) with 1µg/ml of Doxycycline for 24h in order to induce Plk4 overexpression, and then harvested

and replated equally (300 cells) in petri dish plates (7cm) and allowed to grow for 10days, where media was changed every 3days. Colonies were fixed with cold methanol and stained with 0,1% Sulforhodamine B (SRB), washed with PBS and air-dried followed by digital photography (Black-white image for better contrast visualization).

4.4.13 Cell Viability

Cell viability was assessed using the PrestoBlue™ Cell Viability Reagent (Invitrogen, #A13262). One day prior to doxycycline induction, $2x10^4$ cells were seeded into a 96 multiwell plate. 24h after Plk4 overexpression, cells were washed twice with PBS and 50µL of 1:20 PrestoBlue Reagent was added (diluted in media). The plate was incubated for 30min at 37°C and fluorescence was read at excitation 569nm, Emission 590nm and 75% sensibility, using the BioTek's SynergyTM MX microplate reader.

4.4.14 Analysis of survival curves

For survival curves, Univariate survival curves were estimated with Kaplan-Meier and compared using the log-rank test. *P* values <0.05 were considered statistically significant.

4.4.15 Data Availability

The dataset supporting de findings of this study are available within the article and its supplementary information files

4.5 Results

4.5.1 Plk4 overexpression potentiates *anoikis* resistance and increases colony formation of mammary epithelial cells with a p53 knock-out background

Due to its role in controlling centriole duplication and its deregulation in multiple tumors, participating in tumorigenesis, metastasis and in the chemotherapy response, Plk4 has been put forward as a potential therapeutic target in cancer. However, tumors are known to be very heterogeneous in their constitution. They are composed of distinct pools of cancer cells, including cancer stem cells, non-stem cancer cells and other cells from the tumor microenvironment. Because of the complexity of the tumor microenvironment, the exact molecular mechanism of how Plk4 contributes to tumorigenesis, and its effect in stem-cell features, is still poorly understood.

In the present study, we hypothesized that high levels of Plk4 could contribute to tumorigenesis by inducing stem-like properties, such as *anoikis* resistance, in non-transformed p53-mutated cells, thus potentiating cancer progression.

In order to test this hypothesis, we used the MCF10A-Plk4 cell line, a human mammary non-cancerous cell line, engineered to promote Plk4 overexpression in an inducible manner (S. A. Godinho et al., 2014). Upon Doxycycline (Dox) treatment, Plk4 is transiently overexpressed, and consequently CA is promoted. Extra centrosomes affect cells by promoting the formation of multipolar spindles and chromosome misegregation, leading to chromosomal instability and aneuploidy(Ganem et al., 2009; S. A. Godinho & Pellman, 2014). Moreover, CA can also be detrimental for cell proliferation, as they can activate the p53 signaling pathway in vertebrate cells, leading to either a G1 cell cycle arrest or apoptosis(Fava et al., 2017; Green & Kroemer, 2009; Holland et al., 2012). However, cancer cells can usually survive to the presence of multiple centrosomes, because they often do not have a functional p53 pathway(Fukasawa et al., 1996;

Ward & Hudson, 2014). To ensure cell survival upon centriole number manipulation, and to mimic what occurs in cancer, where the tumor suppressor p53 is often mutated, we successfully knocked out (KO) p53 in the MCF10A-Plk4 cells by using the CRISPR/Cas9 technology (Supplementary fig. 4.1).

Plk4 overexpression was induced for 24h using 1µg/ml of Dox in MCF10A-Plk4 and MCF10A-Plk4^{p53KO} cell lines. We analyzed *PLK4* mRNA expression by RT-qPCR and, as expected, a significant 4-fold-increase and 9-fold increase were observed after 24h of Dox treatment, in MCF10A-Plk4 and MCF10A-Plk4^{p53KO} respectively, followed by a significant decrease immediately after 24h of Dox removal (Fig. 4.1A) in both cell lines. Centriole number was quantified in mitotic cells, which normally have a fixed number of 4 centrioles, 2 at each pole of the mitotic spindle (Fig. 4.1B and Fig. 4.1C). MCF10A-Plk4^{p53KO} cells showed continuously high percentage of cells with extra centrosomes, even at 96h after Dox removal. However, MCF10A-Plk4 cells presented a sustained decreased of the percentage of cells with centrosome amplification throughout time after Dox removal, equalizing to control levels at 96h (Fig. 4.1B). The presence of p53 in MCF10A-Plk4 cells might explain why the number of cells with CA decreases after Dox removal, since it is described that, CA activates p53, leading to the elimination of cells with this phenotype(Contadini et al., 2019; Fava et al., 2017). On the other hand, MCF10A-Plk4^{p53KO} cells with extra centrosomes are maintained for longer in the population (Fig. 4.1B).

Anoikis resistance capacity was assessed by the Mammosphere Forming Efficiency (MFE) assay of MCF10A-Plk4 and MCF10A-Plk4^{p53KO} cell lines. The MFE enables the survival of cells with stem-like features, such as *anoikis* resistance, from single cells, in non-adherent and serum free conditions (Lombardo et al., 2015). Only mammospheres equal or greater than 60µm of diameter were counted, as previously described(Vieira et al., 2012). In the control condition, we observed that MCF10A-Plk4^{p53KO} cells formed more mammospheres in comparison to MCF10A-Plk4, suggesting that p53 loss promotes *anoikis*

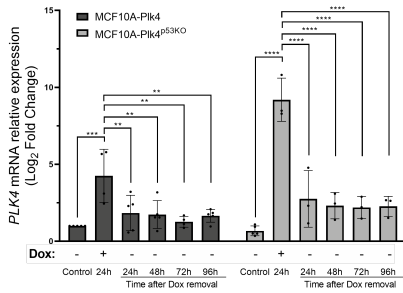
resistance (Fig. 4.1E). This result was expected since it has been demonstrated that loss of p53 confers *anoikis* resistance capacity to breast cancer cells(Akaogi et al., 2013; C. J. Chang et al., 2011; Grossmann, 2002; Ohtsuka et al., 2018). We also observed that upon Plk4 overexpression in both models, MFE was significantly potentiated, but only in the p53KO background (4-fold increase), suggesting that Plk4 significantly promotes *anoikis* resistance in the absence of p53. Nevertheless, when Dox was removed for 96h, MCF10A-Plk4^{p53KO} cell line lost its capacity to form mammospheres, showing a decreased percentage of MFE. In order to confirm Plk4's role in inducing carcinogenesis, MCF10A-Plk4^{p53KO} cell line was subjected to the clonogenic assay. Cells were treated/not treated with 1μg/ml Dox to induce Plk4 overexpression and then replated at low cell density (300 cells/plate) and allowed to grow for 10 days. We observed that Plk4 overexpressed cells formed significantly more colonies than non-overexpressed cells (Fig. 4.1F, G). This result is supported by other studies where Plk4 has been demonstrated to increase colony formation, as well as treatment of Plk4 inhibitor (CFI-400945) significantly reduces cell growth, viability and colony formation in human prostate cancer cells, in multiple embryonal tumor cell lines, and other cancers (Liao et al., 2019; C. K. Singh et al., 2022; Sredni et al., 2017; Suri et al., 2019).

Additionally, we also evaluated whether Plk4 overexpression has an effect on cell viability by taking advantage of the PrestoBlue Cell Viability Reagent. This reagent is reduced by metabolically active cells, providing a quantitative measurement of viable and proliferating cells. Interestingly, the knock-out of p53 did not alter cell viability of MCF10A-Plk4^{p53KO} when compared to the parental cell line MCF10A-Plk4 **(**Supplementary fig. 4.2**)**. By treating cells with 1μg/ml of Dox for 24h to induce Plk4 overexpression, we observed that it significantly altered cell viability when compared to the control condition in both cell lines. However, cell viability of MCF10A-Plk4^{p53KO} was significantly higher than in MCF10A-Plk4, when Plk4 was overexpression was induced by Dox. These results demonstrate that, besides

contributing to *anoikis* resistance, Plk4 overexpression (in the p53KO background) also potentiates cell viability, demonstrating its putative role in tumorigenesis.

As Plk4's effect in *anoikis* resistance might be specific to the MCF10A epithelial cell line, we therefore explored Plk4's role in *anoikis* resistance in the RPE cell line. The RPE cell line is an hTERT-immortalized retinal pigment epithelial cell line, which we engineered to enable the inducible expression of Plk4 upon doxycycline (Dox) treatment (RPE-Plk4 cell line). To confirm that Plk4 is overexpressed upon Dox treatment, cells were treated for 24h with 1µg/ml of Dox, and *PLK4 mRNA* levels and centrosome number was quantified. We observed that, upon Plk4 overexpression, the RPE-Plk4 cell line presented a significant 4-fold increase in *PLK4* mRNA levels (in comparison to not treated condition) (Supplementary fig. 4.3A). Moreover, we also observed an increase from 5% (not treated condition) to 92% of mitotic cells with >4 centrioles upon Plk4 overexpression (Supplementary fig. 4.3B), confirming that this cell line is responsive to Dox treatment inducing Plk4 overexpression. After, we silenced p53 expression in the RPE-Plk4 cell line, and performed spheres assay upon Dox-treatment to induce Plk4 overexpression**.** As it can appreciate in Supplementary fig. 3C, RPE-Plk4 cells were able to form spheres, but only in the condition where p53 expression was silenced. Moreover, when Plk4 was overexpressed upon Dox treatment, the sphere efficiency of RPE-Plk4 was potentiated, demonstrating that Plk4 overexpression, in the absence of p53, induces *anoikis*-resistance capacity of normal epithelial cells. These findings support our previous results in the MCF10A cell line, showing that Plk4 contributes to tumorigenesis by increasing the ability of p53 knock-out mammary epithelial cells to circumvent *anoikis*.

A



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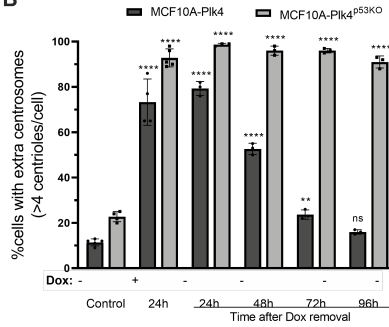

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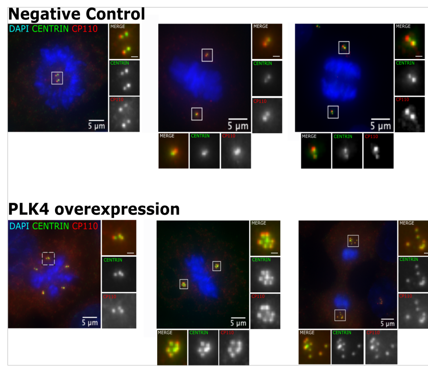

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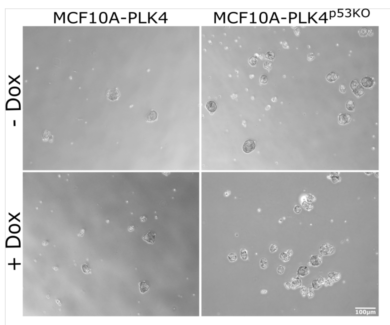

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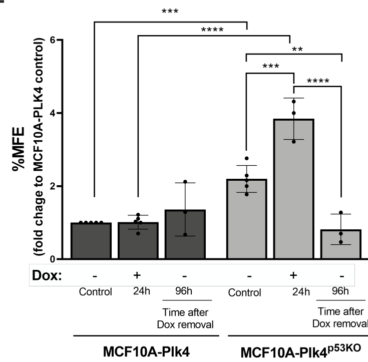

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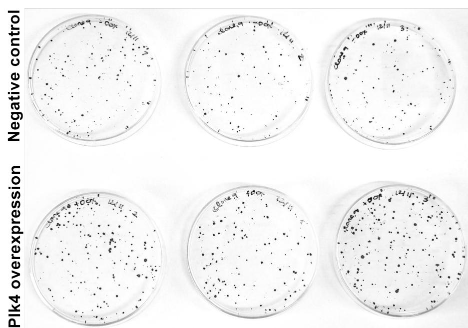

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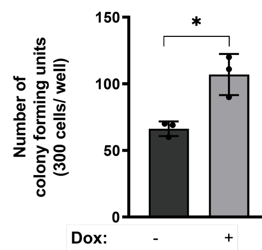


Figure 4.1. Plk4 overexpression potentiates *anoikis* resistance in MCF10A mammary epithelial cells in a p53 knock-out background.

A) *PLK*4 mRNA expression levels in MCF10A-Plk4 and MCF10A-Plk4^{p53KO} cells after induction of Plk4 overexpression. After the first stimulus (1µg/ml of Dox for 24h), Dox was removed and cells were kept in Dox-free media, by replenishing media with new fresh media, and collected at different time points (24h, 48h, 72h and 96h) for mRNA analysis. *GAPDH* mRNA expression was used as a housekeeping gene and all conditions were normalized to the control condition (MCF10A-Plk4 control). Data represents average ± SD for at least three independent experiments, $p<0.05$, One-Way ANOVA test.

B) Quantification of centriole number in MCF10A-Plk4 and MCF10A-Plk4^{p53KO} mitotic cells 24h of Dox treatment and upon Dox removal. Plk4 overexpression induced centriole amplification in 73% of MCF10A-Plk4 and 92% of MCF10A-PLK4^{p53KO} cells 24h after Dox. 96h following Dox removal, MCF10A-PLK4^{p53KO} maintained high percentage of cells with extra centrosomes, while MCF10A-Plk4 reverted to control levels. To obtain the percentage of cells with >4 centrioles, ≥100 cells were quantified per condition and per experiment. Data represents average ± SD for three independent experiments, and significance to each cell line's respective control condition. $p<0.05$, One-Way ANOVA test.

C) Representative figures displaying cells in negative control conditions (with normal centriole number) and cells with Plk4 overexpression (displaying CA) after Dox treatment. Centrioles were stained with two centriole markers Centrin-1 (green) and CP110 (red). Scale bars: 5µm (main images), 1µm (insets).

D) Phase contrast images of representative mammospheres formed by MCF10A-Plk4 and MCF10A-PLK4^{p53KO} cells with and without Dox treatment in non-adherent conditions. Magnification: 5X; Scale bar 100µm.

E) *In vitro* quantification of Mammosphere Forming Efficiency (MFE) of MCF10A-Plk4 and MCF10A-Plk4^{p53KO} cells with and without Plk4 overexpression using 1µg/ml of Dox 24h prior to Mammosphere Forming. MCF10A-Plk4 was used as a control. Moreover, after the first Dox stimulus with 1µg/ml, cells were kept in Dox-free medium for 96h to evaluate MFE. The data is reported as the fold change in the percentage of mammospheres formed/7500 seeded cells ± SD, $p<0.05$, Ordinary One-Way ANOVA.

F) Representative image and **G)** quantification of colony forming units of MCF10A-Plk4^{p53KO} cells treated and not treated with 1ug/ml Dox to induce Plk4 overexpression. Plk4 overexpression significantly induces an increase in colony formation in the MCF10A-Plk4^{p53KO} cells. Data shown is average ±SD for three independent experiments, $p<0.05$, Unpaired t-test

4.5.2 The impact of Plk4 overexpression on *anoikis* resistance is independent of its role in centrosome amplification

Since we observed that, at 96h following Dox removal, MCF10A-Plk4^{p53KO} cells maintained a high percentage of CA, but low levels of Plk4, we wondered whether *anoikis* resistance capacity of these cells was being mediated by CA or by Plk4 overexpression. To tackle this question, we used a cell line with a truncated form of Plk4 (Plk4$^{1-608}$), which retains kinase activity, but does not induce CA, when Plk4 is transiently overexpressed(Gernot et al., 2010; S. A. Godinho et al., 2014). To confirm the increase in Plk4 levels, but not in centrosome number after Dox treatment in MCF10A-Plk4$^{1-608}$ cells, we analyzed *PLK4* mRNA levels (Fig. 4.2A) and centrosome number (Fig. 4.2B) after overexpressing Plk4 by using 1μg/ml of Dox. As appreciated in Figure 4.2A, a significant 3.5-fold increase in Plk4 mRNA levels was observed in MCF10A-Plk4$^{1-608}$ cells upon 24h of Dox, while, as expected, no CA was observed (Fig. 4.2B). We next asked whether the observed *anoikis* resistance was due to increased Plk4 levels or to CA. In order to test this, MCF10A-Plk4$^{1-608}$ cells was subjected to the MFE assay. Interestingly, when Plk4 is overexpressed, MCF10A-Plk4$^{1-608}$ was able to form more mammospheres, when compared to control conditions, suggesting that Plk4 catalytic activity, promotes *anoikis* resistance, independently of its role in CA (Fig. 4.2C).

As previously mentioned, loss of p53 confers *anoikis* resistance capacity to breast cancer cells. Thus, we finally asked whether after p53 removal, MCF10A-Plk4 and MCF10A-Plk4$^{1-608}$ cells would have a higher ability to form mammospheres. By silencing p53 with a specific siRNA (Fig. 4.2C), both cell lines showed an increase in their ability to resist to *anoikis*, and this effect was significantly potentiated when Plk4 was overexpressed upon Dox treatment (Fig. 4.2C). Furthermore, we observed that in the absence of p53, MCF10A-Plk4 and MCF10A-Plk4$^{1-608}$ cell lines presented a small increase in the percentage of cells with extra centrosomes after Dox treatment (Fig. 4.2B). These results reinforce the role of p53 in Plk4-induced *anoikis* resistance, as well in the control of centrosome number.

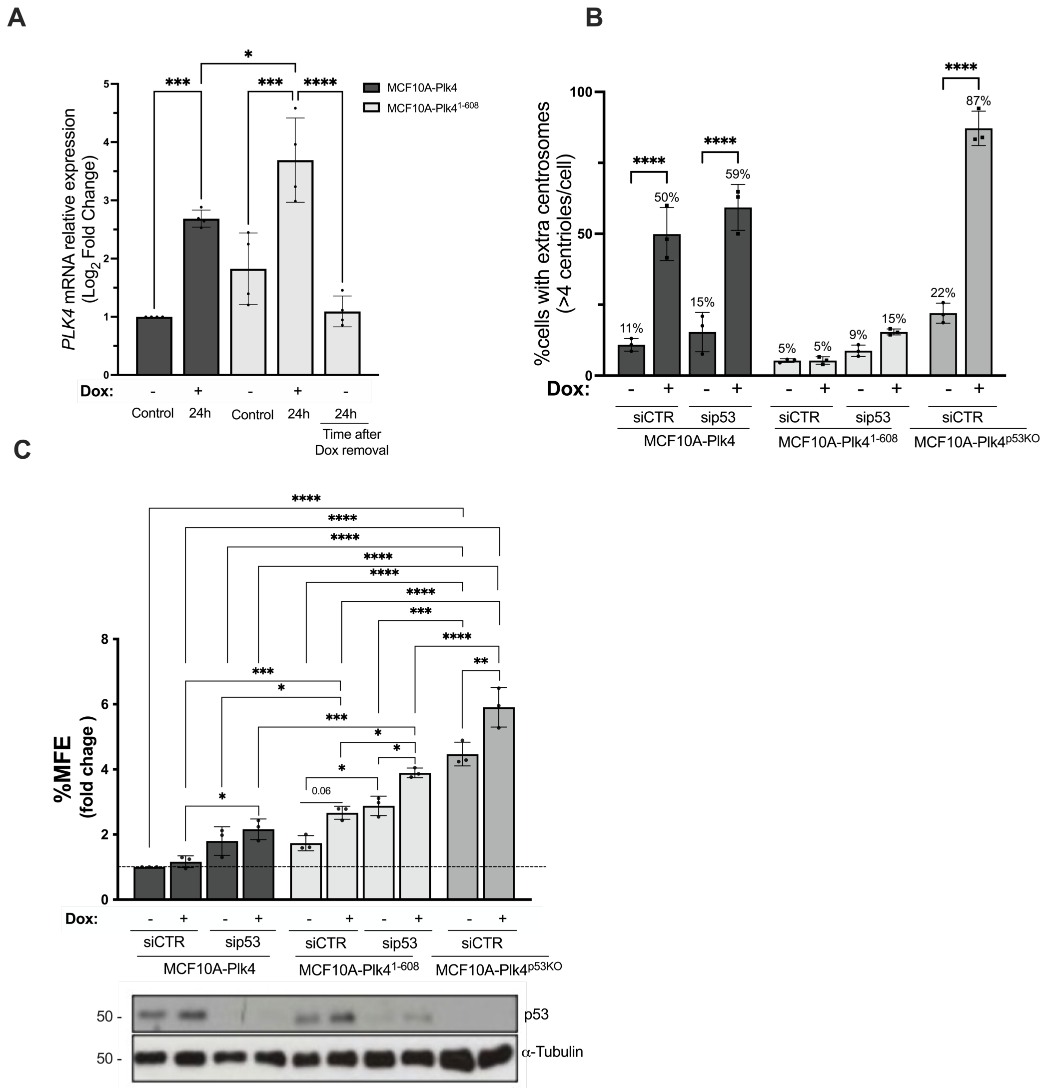


Figure 4.2. Plk4 catalytic activity is involved in *anoikis* resistance of mammary epithelial cells, independently of its role in centrosome amplification.

A) *PLK*4 mRNA expression levels in MCF10A-Plk4$^{1-608}$ cells after induction of Plk4 overexpression using 1µg/ml of Dox for 24h. After this stimulus, Dox was removed by replenishing media with new fresh media and collected 24h after Dox removal for mRNA analysis. *GAPDH* mRNA expression was used as a housekeeping gene and all conditions were normalized to the MCF10A-Plk4 control condition. Data represents average ± SD for at least three independent experiments, $p<0.05$, One-Way ANOVA test.

B) Quantification of centriole number in MCF10A-Plk4, MCF10A-Plk4^{p53KO} and MCF10A-Plk4$^{1\text{-}608}$ mitotic cells 24h after Plk4 overexpression in silenced p53 (sip53) and not silenced (siCTR) conditions. Plk4 overexpression induced centriole amplification in 50% of MCF10A-Plk4 and 87% of MCF10A-PLK4^{p53KO} cells, but not in MCF10A-Plk4$^{1\text{-}608}$ (5%). When Plk4 is overexpressed in a p53 silenced background, MCF10A-Plk4 showed an increase of 59% of cells with extra centrosome, and MCF10A-Plk4$^{1\text{-}608}$ presented a 15% increase of CA, in comparison to the respective controls. To obtain the percentage of cells with >4 centrioles, ≥100 cells were quantified per condition and per experiment. Data represents average ± SD for three independent experiments, and significance to each cell line's respective control condition. $p<0.05$, One-Way ANOVA test.

C) *In vitro* quantification of Mammosphere Forming Efficiency (MFE) assay of MCF10A-Plk4, MCF10A-Plk4^{p53KO} and MCF10A-Plk4$^{1\text{-}608}$ cells with and without Plk4 overexpression using 1µg/ml of Dox 24h prior to the assay with and without p53 silencing. MCF10A-Plk4 was used as a control. The data is reported as the fold change in the percentage of mammospheres formed/7500 seeded cells ± SD, $p<0.05$, Two-Way ANOVA.

4.5.3 Plk4 overexpression induces the acquisition of a hybrid E/M phenotype on MCF10A-Plk4 p53KO mammary epithelial cells

Loss of p53 by epithelial cells has been described to confer stem-like properties and to induce EMT, allowing them to circumvent *anoikis*, contributing to tumor progression(C. J. Chang et al., 2011; Ohtsuka et al., 2018; Olivos & Mayo, 2016; Pinho et al., 2011). Thus, we went to explore the EMT profile of MCF10A-Plk4 and MCF10A-Plk4^{p53KO} cells. Non-induced MCF10A-Plk4 cells showed a clear epithelial phenotype, with high levels of E-cadherin and low expression of mesenchymal markers, such as Vimentin, Snail/Slug and Zeb2 (Fig. 4.3). On the other hand, MCF10A-Plk4^{p53KO} cell line exhibited a mesenchymal phenotype, with low E-cadherin expression, and high levels of Vimentin and Snail/Slug. However, when Plk4 was induced in the p53KO context, there was a reversion of the mesenchymal phenotype to a hybrid EMT phenotype, with an increase in the expression of E-cadherin, as well as of the EMT transcription factors Snail/Slug and Zeb2, and a concomitant decrease in Vimentin levels (Fig. 4.3). Altogether, these results indicate that Plk4 overexpression, in a p53KO context, induces the

stabilization of a hybrid EMT phenotype in non-tumorigenic mammary epithelial cells.

In neuroblastoma, Plk4 promotes EMT through PI3K/Akt signaling pathway, by downregulating E-cadherin and upregulating EMT-related factors, such as Snail/Slug(Tian et al., 2018). On the other hand, Plk4 also promotes EMT via Wnt/β-catenin in colorectal cancer(Liao et al., 2019). Interestingly, in esophageal squamous cell carcinoma, Plk1 overexpression, another member of the polo-like kinase family, has been demonstrated to trigger *anoikis* resistance through regulation of β-catenin expression, by directly binding to the NF-kB subunit RelA, which inhibits the ubiquitination and degradation of β-catenin(D. C. Lin et al., 2011). Importantly, NF-kB signaling pathway, which is activated upon cellular stress, can regulate the expression of genes required for centrosome duplication, being Plk4 a direct NF-kB target gene(Ledoux et al., 2013). Studies, have also documented a crosstalk between NF-kB and p53(Perkins, 2012). It is important to point out that p53 has been shown to downregulate Plk4 and Plk1 expression(McKenzie et al., 2010; Nakamura et al., 2013). These data suggests that regulation of these kinases provides another rout of potential crosstalk within cells potentiating cancer progression. However, the exact mechanism through which Plk4 might be mediating a hybrid EMT, protecting cells against *anoikis* is still poorly explored and needs further study.

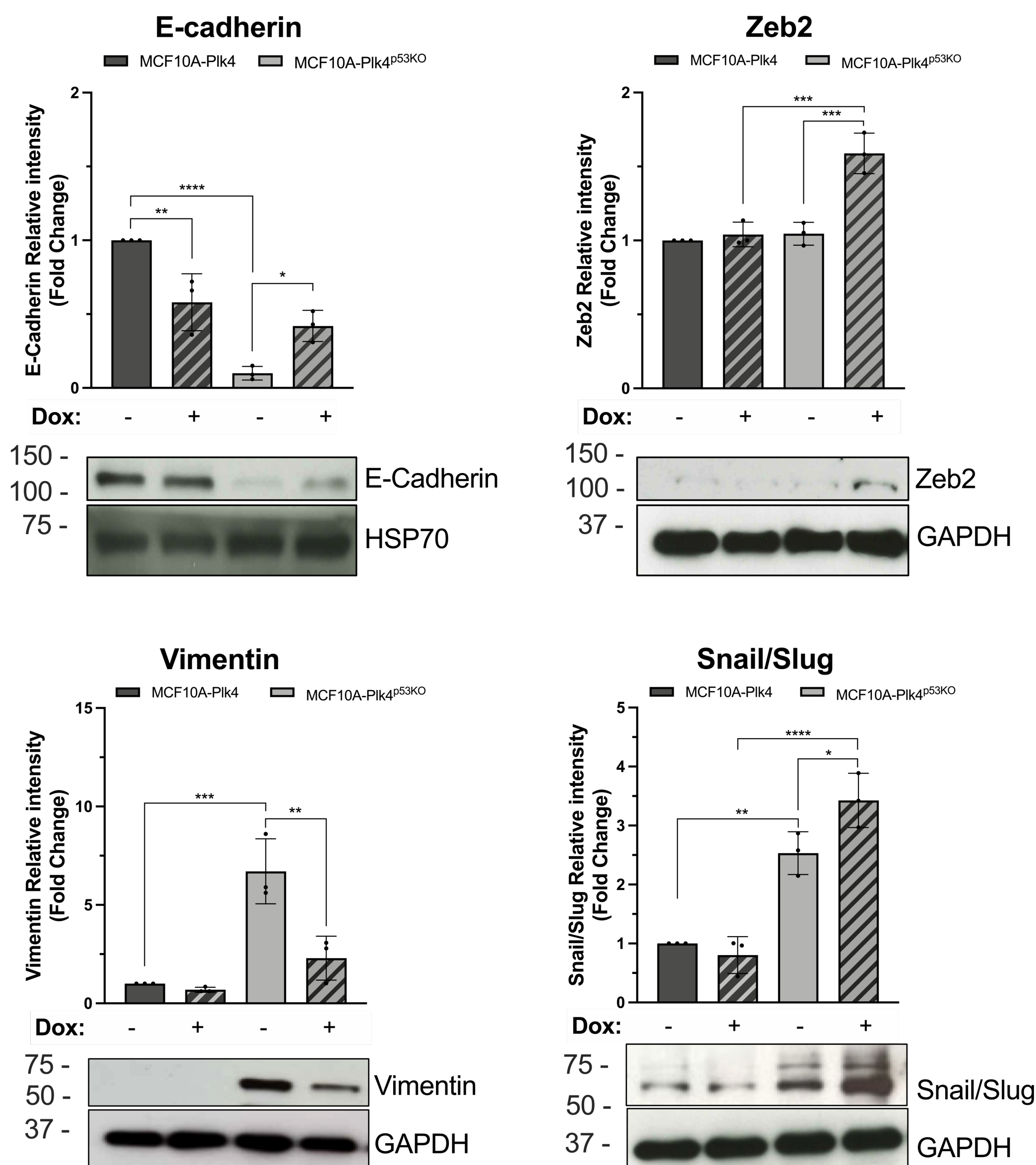


Figure 4.3. MCF10A-Plk4 p53KO mammary epithelial cells with centrosome amplification show a hybrid epithelial-mesenchymal phenotype

Western Blot analysis of MCF10A-Plk4 and MCF10A-Plk4^{p53KO} cells with and without Plk4 overexpression using 1µg/ml of Dox for 24h. Representative western blot images and quantification of their relative integrated intensity of Epithelial (E-cadherin) and Mesenchymal markers (Vimentin, Zeb2, Snail/Slug). MCF10A-Plk4 cells presents an epithelial profile, with high levels of E-cadherin

and low levels of Zeb2, Vimentin and Snail/Slug. On the other hand, the p53KO cells showed a mesenchymal profile, with low expression of E-cadherin and increased expression of Vimentin and Snail/Slug. When Plk4 is overexpressed, p53KO cells acquire a Hybrid EMT phenotype, with high Zeb2, Snail/Slug and E-cadherin expression. Hsp70 (for E-cadherin) and GAPDH were used as loading controls. Integrated intensity was measured by ImageJ Software (National Institutes of Health). All conditions were normalized to the control condition (MCF10A-Plk4, non-induced). Data shown is the fold change in pixels integrated intensity ± SD for three independent experiments, $p<0.05$, Ordinary One-Way ANOVA.

4.5.4 P-cadherin expression is increased in Plk4 induced-MCF10A-PLK4^{p53KO} cells and is required for *anoikis* resistance in an autocrine and paracrine way

Recently, the concept of hybrid EMT has emerged and has been shown to be extremely important for cancer cells to become invasive and metastatic in a collective manner(Aggarwal et al., 2021; Jolly et al., 2015). We have previously demonstrated that overexpression of the cell-cell adhesion molecule P-cadherin promotes collective cell invasion, stem cell properties and tumorigenesis(Ribeiro & Paredes, 2015; Sousa et al., 2020; Vieira et al., 2012; Vieira, André Filipe, Ribeiro et al., 2014). Moreover, P-cadherin expression is early promoted by EMT-inducers, such as hypoxia, driving *anoikis* resistance in breast cancer cells(Sousa et al., 2020) and being a putative biomarker and stability factor of a hybrid EMT phenotype(Ribeiro & Paredes, 2015; Turashvili et al., 2011).

Therefore, we analyzed P-cadherin mRNA and protein expression in both MCF10A-Plk4 and MCF10A-Plk4^{p53KO} cells. Interestingly, we observed that P-cadherin is mainly expressed by MCF10A-Plk4^{p53KO} cells (Fig. 4.4A, 4B), and its expression is significantly increased, both at mRNA and protein levels, when Plk4 is overexpressed. Nevertheless, no alterations in P-cadherin expression were observed in MCF10A-Plk4 cells, where p53 is normally expressed.

Based on these observations, we next evaluated whether P-cadherin expression was required for the Plk4-induced *anoikis* resistance in p53KO cells. Thus,

CDH3/P-cadherin was silenced in MCF10A-Plk4^{p53KO} cells using specific siRNA for the *CDH3* gene. A scrambled siRNA, with no homology to any gene, was used as a negative control (siCTR). Following siRNA transfection, cells were treated or not treated with 1µg/ml of Dox for 24h (siCTR+Dox and si*CDH3*+Dox) and MFE assay was performed. As expected, and previously shown, by inducing Plk4 overexpression with Dox, cells formed significantly more mammospheres in comparison to non-treated cells (Fig. 4.4C). Interestingly, the silencing of P-cadherin expression in the MCF10A-Plk4^{p53KO} cells significantly decreased their capacity to form mammospheres, independently of Plk4 overexpression. However, the percentage of MFE observed upon si*CDH3* and induction of Plk4 in MCF10A-Plk4^{p53KO} cells did not reach the MFE percentage observed without induction, whether *CDH3* was silenced or not. This suggests that Plk4 overexpression is able to induce *anoikis* resistance in p53KO MCF10A cells, and this effect is partially mediated by P-cadherin expression.

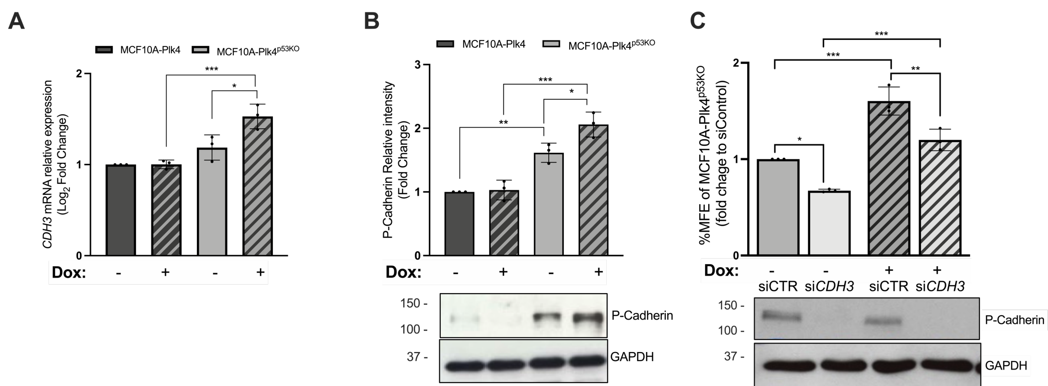


Figure 4.4. P-cadherin expression is increased in Plk4 induced-MCF10A-Plk4^{p53KO} cells and is partially required for resistance to *anoikis*.

P-Cadherin expression is increased in p53KO cells treated with doxycycline, but not observed in MCF10A-Plk4, both at the **A)** (*CDH3*) mRNA and **B)** at the protein level. P-cadherin gene (*CDH3*) expression was assessed by qRT-PCR and protein levels by Western Blot of MCF10A-Plk4 and MCF10A-Plk4^{p53KO} cell lines treated and not treated with doxycycline. All conditions were normalized

to the MCF10A-Plk4 control condition. Data shown is average ± SD for three independent experiments, $p<0.05$, Ordinary One-Way ANOVA.

C) Silencing of P-cadherin in Plk4-induced MCF10A-Plk4^{p53KO} significantly decreased their capacity to form mammospheres. *CDH3* gene silencing was performed in MCF10A-Plk4^{p53KO} cells using a validated siRNA, specific for *CDH3*. A scrambled siRNA with no homology to any gene, was used as a negative control (siCTR). Following siRNA transfection, cells were treated or not treated with 1µg/ml of Dox for 24h. MFE was performed after Dox treatment. All conditions were normalized to siControl. Western Blot bellow represents the validation of P-Cadherin silencing. Data shown is average ± SD for three independent experiments, $p<0.05$, Ordinary One-Way ANOVA.

A non-cell autonomous role for CA has been previously reported(Adams et al., 2021; Arnandis et al., 2018), where cells with extra centrosomes caused by Plk4 overexpression induce paracrine invasion of other cells by secreting extracellular vesicles with pro-invasive factors. Since there is differential protein secretion in cells with centrosome amplification via Plk4 overexpression, we decided to investigate whether high Plk4 expression would also play a role in potentiating paracrine *anoikis* resistance of cancer cells in the tumor microenvironment. Thus, BT20 breast cancer cells were treated with the conditioned media of MCF10A-Plk4^{p53KO} cells for 48h and performed the MFE assay. We observed a significant increase on the capacity of these cancer cells to form mammospheres when treated with the conditioned media of Plk4-induced MCF10A^{p53KO} (Fig. 4.5A) when compared to control condition. Because the increased mammosphere capacity in BT20 might be due to BT20's exposure of a CM derived from a different cell line, rather than due to Plk4 overexpression, we also treated MCF10A-Plk4^{p53KO} cells (CM receiver) with the CM of Plk4 overexpressed MCF10A-Plk4^{p53KO} cells (CM donor) for 48h and measured their MFE (supplementary fig. 4.4). We observed that MCF10A-Plk4^{p53KO} "CM receiver" cells formed significantly more mammospheres than control (not treated condition) and serum-free condition (CM was produced in serum-free media). Interestingly, the CM from MCF10A-Plk4^{p53KO} "donor" cells without Plk4 overexpression did not induce an increase in mammosphere

formation in MCF10A-Plk4^{p53KO} "receiver" cells, suggesting that this effect is mainly mediated by Plk4 overexpression.

We have previously shown that P-cadherin overexpression in breast cancer cells promotes an increase in cell migration and invasion due to the secretion of pro-invasive factors, such as MMP1 and MMP2, which then leads to P-cadherin ectodomain cleavage (soluble Pcad, sP-cad) that also has pro-invasive activity by itself(Ribeiro et al., 2010). Given that the conditioned media of MCF10A-Plk4^{p53KO} cells increased *anoikis* resistance of cancer cells, we investigated if the soluble/cleaved P-cadherin form (sP-cad) was also being secreted by these cells. We found that, sP-cad expression was significantly increased in MCF10A-Plk4^{p53KO} cells (not induced condition), which was not observed in MCF10A-Plk4 (Fig. 4.5B). Importantly, sP-cad expression was significantly potentiated when Plk4 was induced in the MCF10A-Plk4^{p53KO}, suggesting that Plk4 plays a direct role in sP-cad expression in the p53KO context. Finally, we went to evaluate if sP-cad was also required for the induction of paracrine *anoikis* resistance of breast cancer cells, by treating BT20 breast cancer cells with the conditioned media of MCF10A-Plk4^{p53KO} upon *CDH3* silencing. We observed a significant 0.5-fold decrease in the ability of BT20 cells to form mammospheres when treated with conditioned media of MCF10A-Plk4^{p53KO}, with *CDH3* silencing, independently of Plk4 overexpression (Fig. 4.5C). Moreover, we also observed that cancer cells treated with the conditioned media of cells with both Plk4-overexpressed and silenced P-cadherin presented a decreased capacity to form mammospheres, but they still have higher MFE than control cells. This emphasizes our previous result, where high levels of Plk4 induces *anoikis* resistance partially depending on P-cadherin, also in a paracrine way.

Overall, our results highlight the role of Plk4 and P-cadherin, in influencing the communication between different cells to promote malignancy.

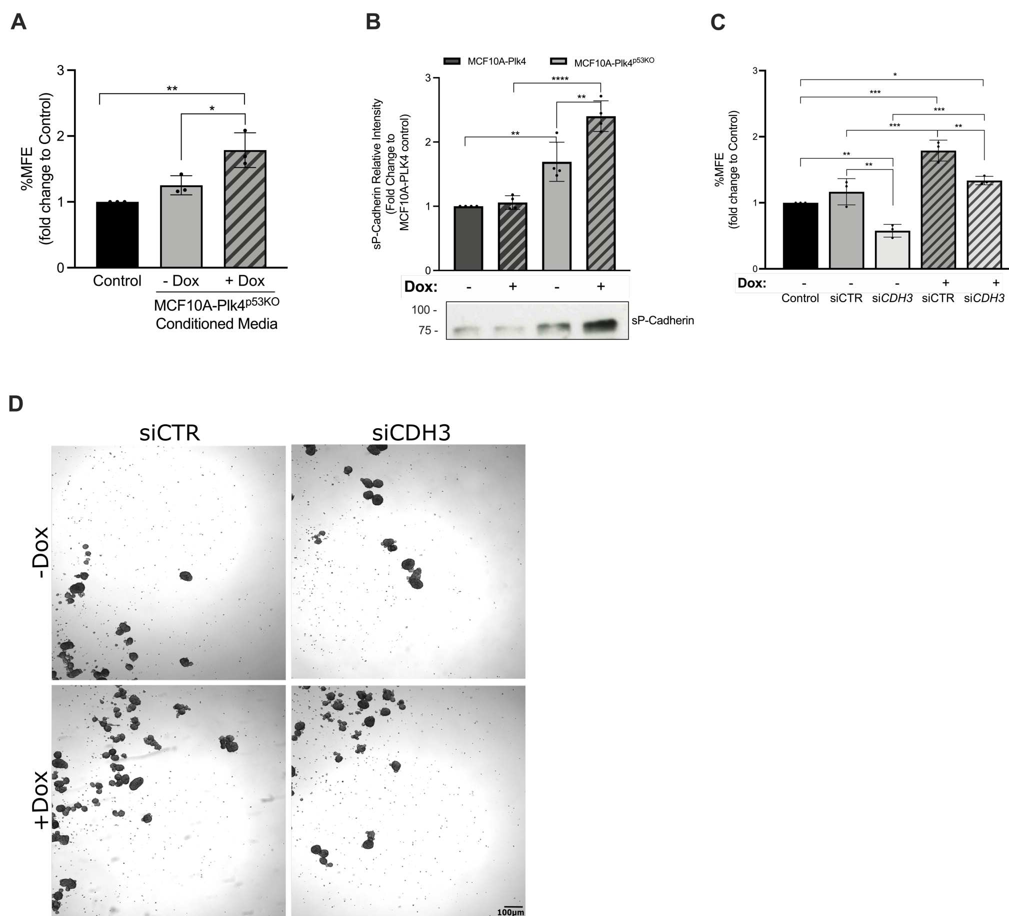


Figure 4.5. Soluble P-Cadherin (sP-cad) is partially required for *anoikis* resistance of breast cancer cells mediated through the conditioned media of p53KO cells with centrosome amplification.

A) MFE assay of BT20 breast cancer cells under the following conditions: (control) nontreated; (Dox-) treated with conditioned media from MCF10A-Plk4^{p53KO} without Plk4 OE; (Dox+) treated with conditioned media from MCF10A-Plk4^{p53KO} with Plk4 OE. There is a significantly increase MFE when BT20 cells were treated with the conditioned media of MCF10A-Plk4^{p53KO} with induced Plk4 overexpression. All conditions were normalized to the control condition (BT20 control). Data shown is average ± SD for three independent experiments, $p<0.05$, Ordinary One-Way ANOVA.

B) Western blot analysis of conditioned media from MCF10A-Plk4 and p53KO cell line showing different expression levels of sP-cad. P-cadherin ectodomain cleaved (sP-cad) expression is increased in p53KO cells treated with Dox. MCF10A-Plk4 cells have lower P-cadherin basal levels,

which is not affected by Plk4 overexpression. Data shown is average ± SD for four independent experiments, $p<0.05$, Ordinary One-Way ANOVA.

C) MFE assay of BT20 breast cancer cells incubated with the following conditioned media: (Control) no treatment; (siCTR; Dox-) MCF10A-PLK4^{p53KO}-siCTR without Plk4 OE; (siCDH3; Dox+) MCF10A-Plk4^{p53KO} depleted of P-cadherin without Plk4 OE; (siCTR; Dox+) MCF10A-Plk4^{p53KO}-siCTR with Plk4 OE; (siCDH3; Dox+) MCF10A-Plk4^{p53KO} depleted of P-cadherin with Plk4 OE. P-cadherin-silencing significantly affects mammosphere forming capacity. Data shown is average ± SD for three independent experiments, normalized to BT20 control, $p<0.05$, Ordinary One-Way ANOVA.

D) Phase contrast images of representative mammospheres formed by BT20 breast cancer cells treated with MCF10A-Plk4^{p53KO}'s conditioned media of siCTR and siCDH3 cells with and without Dox treatment in non-adherent conditions. Magnification: 2X; Scale bar 100μm.

4.6 Discussion

Dysregulation of Plk4 has been found in several types of cancers and shown to cause loss of centrosome numerical integrity, promoting genomic instability. Furthermore, increased Plk4 expression has been linked to cancer metastasis and to chemotherapy resistance. However, the control of centriole duplication may not be the only relevant function of Plk4 in carcinogenesis.

Herein, we investigated if Plk4 could play a role in carcinogenesis, specifically by inducing stem-like features, such as *anoikis* resistance. We demonstrated that loss of the tumor suppressor p53 is necessary to maintain Plk4 induced-CA throughout time. Moreover, we also showed that Plk4 overexpression promotes *anoikis* resistance in non-tumorigenic mammary epithelial cells, in a p53 knockout background. As centrosome amplification is a direct cause of Plk4 overexpression, CA could also be contributing to *anoikis* resistance. By taking advantage of a cell line with truncated form of Plk4 (Plk4$^{1\text{-}608}$) that retains kinase activity but does not induce centrosome amplification when Plk4 is overexpressed, we observed that these cells are able to resist *anoikis* when Plk4 is overexpressed. These results demonstrates that the *anoikis* resistance observed is directly mediated by Plk4

kinase activity, independently of its role in promoting CA. Moreover, we also showed that p53-silenced $Plk4^{1-608}$ cells are more resistant to *anoikis* than p53 WT $Plk4^{1-608}$ cells, which is then potentiated when Plk4 is overexpressed. The same was observed by transiently inducing Plk4 in the MCF10A-$Plk4^{p53KO}$, where cells became *anoikis* resistant, confirming that Plk4 potentiates *anoikis* resistance in the p53 knock-out background. Moreover, we also demonstrated that in the MCF10A-$Plk4^{p53KO}$ cell line, Plk4 overexpression significantly increases cell viability and colony formation, reinforcing its putative role in tumorigenesis.

Loss of p53 has been described to confer stem-like properties, to potentiate *anoikis* resistance of cells, and to induce an epithelial to mesenchymal transition (EMT)(C. J. Chang et al., 2011; Ohtsuka et al., 2018). In this work, we showed that high Plk4 levels in a p53KO context leads to a hybrid EMT phenotype, with increased expression of P-cadherin. In fact, *anoikis* resistance and hybrid EMT phenotype are crucial features for cancer progression and metastatic colonization(Paoli et al., 2013; Pastushenko & Blanpain, 2019; Saitoh, 2018a). In accordance to other studies, in which P-cadherin has been shown to be promoted by EMT-inducers, such as hypoxia, driving *anoikis*-resistance capacity in breast cancer cells(Ribeiro & Paredes, 2015; Sousa et al., 2020) , we demonstrate that P-cadherin expression is partially required for *anoikis* resistance observed in the MCF10A-$Plk4^{p53KO}$ cells, as well as in breast cancer cells exposed to their conditioned media.

Previous reports have shown that the hybrid EMT state is critical to stemness, independently of phenotypic plasticity(Kröger et al., 2019). Both epithelial and mesenchymal traits need to be co-expressed within an individual cancer cell for efficient tumorigenicity, as mesenchymal properties are important for the intravasation from the primer tumor and survival in blood circulation, whereas epithelial traits are essential for the metastatic colonization of distant organs(Kröger et al., 2019; Their, 2002). P-cadherin has been demonstrated as a promising biomarker of the hybrid EMT state, based on the rationale that P-cad expression disturbs epithelial cell-cell adhesion and promotes the acquisition of a

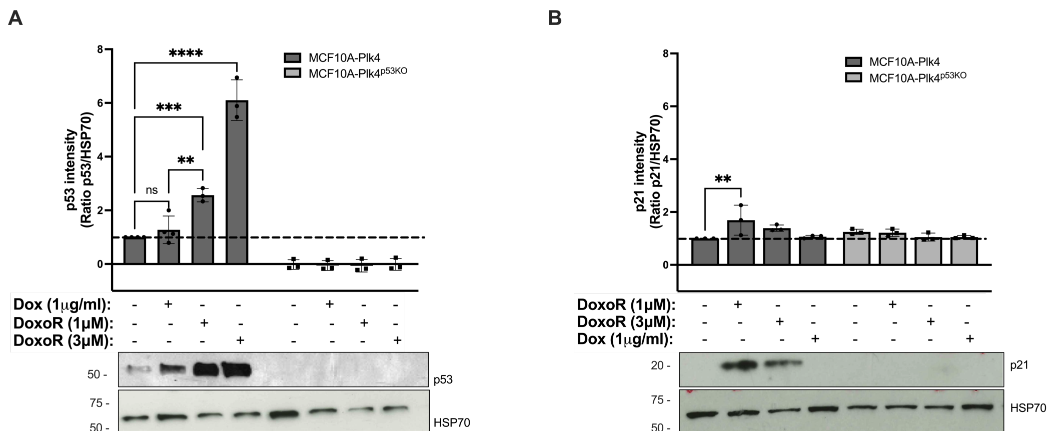
more undifferentiated cell phenotype, giving these cells a phenotypic state between epithelial and mesenchymal morphology(Ribeiro & Paredes, 2015; Vieira et al., 2012). Moreover, cells overexpressing P-cadherin show increase therapy resistance, stem cell properties, a more aggressive and invasive behavior(Ribeiro & Paredes, 2015). These properties point to a hybrid EMT state, with increased plasticity and metastatic capacity. In fact, P-cadherin has been described as a poor prognostic biomarker in basal-like breast tumors, and to correlate with histological grade, being essentially present in high-grade tumors(Paredes et al., 2002, 2005; Turashvili et al., 2011). Interestingly, overexpression of Plk4 has also been shown to have a prognostic value associated with worse DFS and OS of breast cancer patients(X. Zhang et al., 2021; Y. Zhao & Wang, 2019a). Moreover, unpublished data from our lab shows that P-cadherin expression is significantly associated to the expression of the EMT transcription factor Zeb2. Zeb2 has also been pointed out as possible hybrid-EMT marker in breast cancer, as its mRNA is highly expressed in cells with hybrid E/M and Mesenchymal phenotypes (Mooney et al., 2017). In accordance, we also showed that in the absence of p53, Plk4 overexpression induces an increase in Zeb2 expression, reinforcing the role of Plk4 in inducing a hybrid EMT phenotype.

By taking advantage of the publicly available data from Kaplan Meier plotter (https://kmplot.com/analysis/), where RNA seq data is available for n=2976 breast cancer patients, we observed that patients with tumors co-expressing high Plk4 and P-cadherin expression show a worse prognosis (Supplementary fig. 4.5). Besides breast cancer, Plk4 has been found to be upregulated in most solid tumors, such as gastric, pancreatic, lung, melanoma, cervical, osteosarcoma and brain (neuroblastoma, glioblastoma, medulloblastoma) tumors, and associated with shortened patient survival in these tumors(Chan, 2011; X. Zhang et al., 2021; Y. Zhao & Wang, 2019a).

In conclusion, our observations have important implications for understanding the potential link between Plk4 levels, cancer and tumor microenvironment, as well

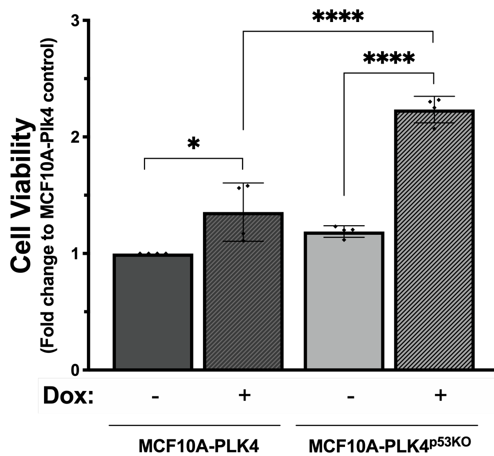
as clinical outcomes. Our data shows that high Plk4 expression is associated with malignant features such as *anoikis* resistance and EMT in cancerous and non-cancerous cells in the tumor microenvironment, endorsing its role in tumor initiation and/or progression. As emerging data has been supporting the idea that Plk4 plays an important role in tumorigenesis, more comprehensive research on the exact signaling pathways and on the present and next-generation Plk4 inhibitors, following successful clinical studies, may provide a new dimension for innovative cancer therapies.

4.7 Supplementary information

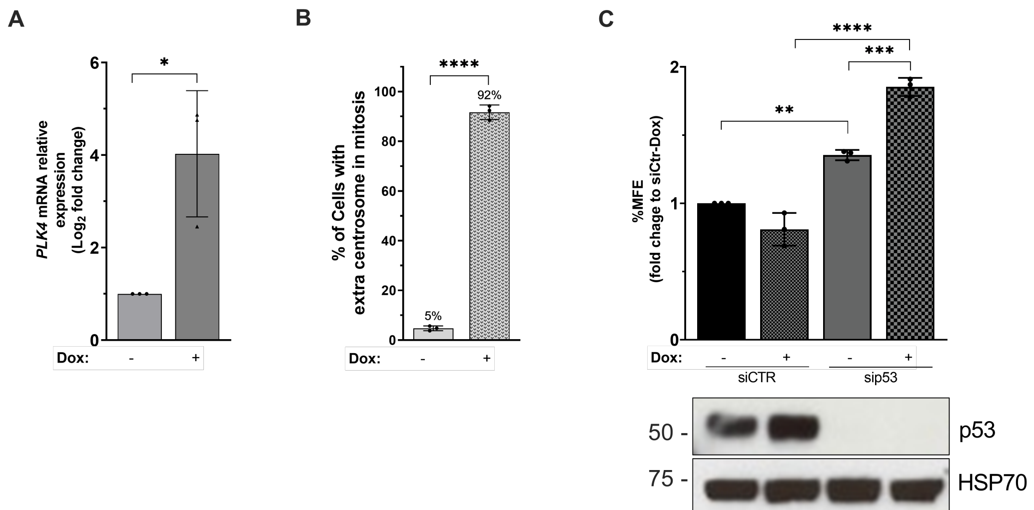


Supplementary Figure 4.1. p53 knock-out in the MCF10A-PLK4 cell line.

Western blot protein quantification of **(A)** p53 and its downstream target **(B)** p21 protein on MCF10A-Plk4 and MCF10A-PLK4^{p53KO} cells after exposure to 1 and 3 μM of Doxorubicin (DoxoR) in order to induce DNA damage (mild and severe DNA damage, respectively), for 4 hours, and 24h in drug free medium and 1 μg/ml of Doxycycline (Dox) for 24h. Integrated intensity was measured by ImageJ Software (National Institutes of Health). HSP70 was used as loading control, and all conditions were normalized to the control condition (MCF10A-Plk4 control). Data shown is average ± SD for three independent experiments, $p<0.05$, two-way ANOVA test.

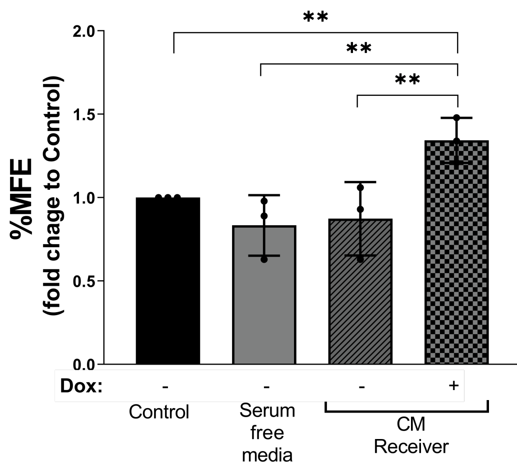


Supplementary Figure 4.2. Plk4 overexpression significantly potentiates cell viability of MCF10A-Plk4 and MCF10A-Plk4^{P53KO} cells. Plk4 overexpression induced by Dox treatment (1µg/ml for 24h) induced a significand 0.5fold increase in the MCF10A-Plk4 and a 2.5fold increase in the MCF10A-Plk4^{P53KO} cell viability. This increase is more evident in the p53 knock-out background, demonstrating that the absence of p53 potentiates Plk4's effect in cell viability. Data shown is average ± SD for four independent experiments, p<0.05, One-way ANOVA test.

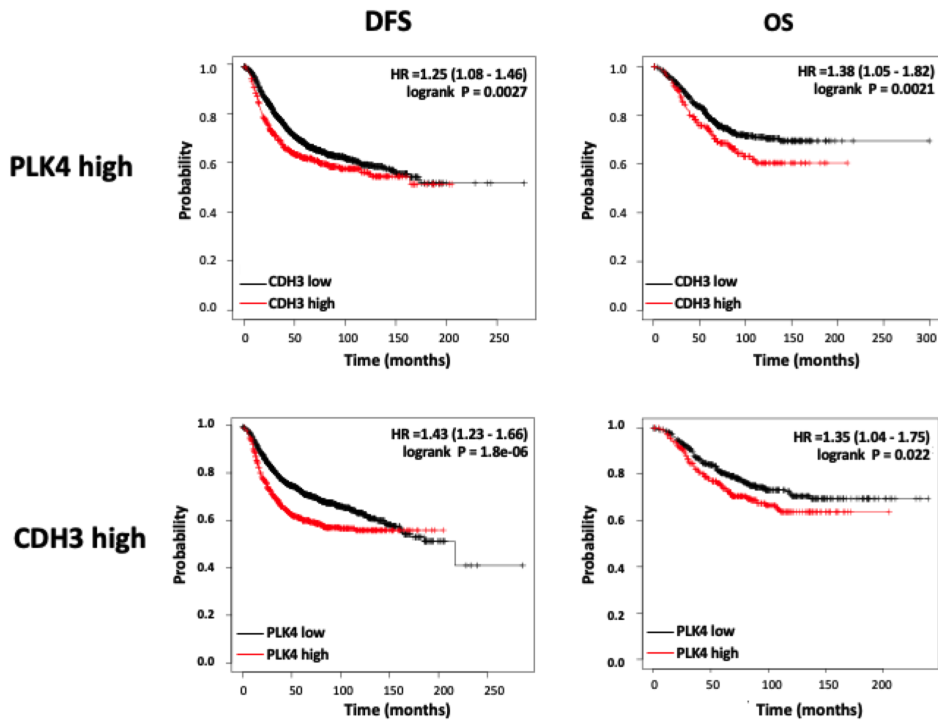


Supplementary Figure 4.3. Plk4 overexpression potentiates anoikis resistance of RPE-Plk4 cells in the absence of p53. (A) *PLK4* mRNA expression levels in the RPE-Plk4 cell line after

induction of Plk4 overexpression with Doxycycline treatment (1µg/ml of Dox for 24h). *GAPDH* mRNA expression was used as a housekeeping gene and condition was normalized to control (-Dox) condition. Data represents average ±SD for three independent experiments, $p<0.05$, Unpaired t test. (**B)** Quantification of centriole number in the RPE-Plk4 mitotic cells 24h of Dox treatment. Plk4 overexpression induced centriole amplification in 90% of cells 24h after Dox. To obtain the percentage of >4 centrioles, ≥100 cells were quantified per condition and per experiment. Data represents average ±SD for three independent experiments. $p<0.05$, Unpaired t test. **C)** *In vitro* quantification of Mammosphere Forming Efficiency (MFE) of RPE-Plk4 cells with and without Plk4 overexpression in the presence (siControl) and absence of p53 (sip53). The data is reported as the fold change in the percentage of mammospheres formed/7500 seeded cells ±SD, $p<0.05$, Two-Way ANOVA.



Supplementary Figure 4.4. Conditioned medium (CM) from Plk4 overexpressed cells significantly increases *anoikis* resistance of MCF10A-Plk4^{p53KO} treated cells. To promote Plk4 overexpression, MCF10A-Plk4^{p53KO} "CM donor" were treated with 1µg/ml of Dox for 24h, and MCF10A-Plk4^{p53KO} "CM receiver" cells were treated for 48 with CM from donor cells, following MFE assay. CM+Dox induced a significant increase in the MFE of MCF10A-Plk4^{p53KO} "CM receiver" cells when compared to control conditions (not treated and serum free media). Moreover CM-Dox did not induce an increase in MFE, suggesting that this effect is Plk4 mediated. Data represents three independent experiments and reported as the fold change in the percentage of mammospheres formed/7500 seeded cells ±SD, $p<0.05$, Two-WAY ANOVA.



Supplementary Figure 4.5: *Co-expression of high Plk4 and high P-cadherin expression in breast tumors are significantly associated with worse prognosis.*

Patients with breast tumors co-expressing high Plk4 and P-cadherin expression showed a significant correlation with worse DFS and OS, as revealed in Kaplan-Meier plots. Univariate survival curves were estimated with Kaplan-Meier and compared using the log-rank test. *P* values <0.05 were considered statistically significant.

Table 4.1: Western Blot primary antibodies.

Primary antibody	Specie	Dilution	Seller	Reference
P-Cadherin (clone 56)	Mouse	1/500	BD Transduction	610228
E-Cadherin	Rabbit	1/1000	Cell Signaling	24E10
Vimentin (21H3)	Rabbit	1/1000	Cell Signaling	5741T
Snail/Slug	Rabbit	1/400	Abcam	Ab85931
Zeb2	Rabbit	0.1ug/ml	Invitrogen	PA5-82094
GAPDH (0411)	Mouse	1/1000	Santa Cruz	Sc47724
HSP70	Mouse	1/8000	Santa Cruz	B-6

Table 4.2: List of probes used for qRT-PCR.

Gene	Assay ID	Exon	RefSeq Number
PLK4	Hs.PT.58.2404526	15-16	NM_001190799
PLK4	Hs.PT.58.19849206	1-3	NM_001190799
CDH3	Hs.PT.58.2656432	7-8	NM_001793
GAPDH	Hs.PT.39a.22214836	2-3	NM_002046

4.8 Acknowledgements

We thank Susana Godinho for the MCF10A-Plk4 and MCF10A-Plk4$^{1-608}$ cell lines. We also thank all the members of the Cancer Metastasis group and Cell Cycle Regulation (CCR) group (specially Sascha Werner, Mariana Faria and Paulo Duarte) for helping in the discussion of the work and for critical reading of the manuscript.

4.9 Conflict of interest

The authors declare that they have no conflict of interest.

4.10 Author contributions

I.F. carried out experiments, analyzed data and wrote the manuscript (original draft, review and editing). G.M. supervised the establishment and validation of the stable p53 knock-out cell line experiments. C.H. performed the epithelial and mesenchymal markers western blot and Plk4/P-cadherin qRT-PCR experiments, analyzed data and helped in discussion of the work. A.S.R. performed the analysis of Plk4 and CDH3 association in breast cancer cohort in the Kaplan Meier plotter. B.S. helped in the MFE and qRT-PCR analysis and discussion of the work. M.B.D. and J.P. supervised experiments, obtained funding and revised the manuscript. All authors read, revised and approved the final manuscript.

4.11 Ethics Statement

This study did not require ethical approval.

4.12 Funding

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Chapter 5.

General Discussion

In the majority of animal cells, centrosomes, which are non-membranous organelles, act as microtubule organizing centers (MTOCs). They play important cellular functions, regulating cell shape, motility, division and signaling(Bettencourt-Dias & Glover, 2007; Brito et al., 2012). The biogenesis of centrioles is tightly regulated and occurs once per cell cycle, during S-phase, where they duplicate to ensure the assembly of a bipolar spindle in mitosis, an essential structure for appropriate chromosome segregation(Brito et al., 2012; Nigg & Holland, 2018; Nigg & Stearns, 2011). Polo-like kinase 4 (Plk4) is a regulator of centriole biogenesis required for centriole duplication(Bettencourt-Dias et al., 2005; Habedanck et al., 2005a; Kleylein-Sohn et al., 2007) via the phosphorylation and interaction with different centriolar proteins. In consequence, when Plk4 is overexpressed, it causes centrosome amplification (CA, i.e., increase in centrosome number) originating cells with more than 4 centrioles(Coelho et al., 2015). In fact, centrosome amplification, as well as high Plk4 expression, is frequently observed in cancer(Chan, 2011; Denu et al., 2016b; Ganier et al., 2017; Kayser et al., 2005; Marteil et al., 2018; A. Singh et al., 2020), and already shown to be significantly associated with poor clinical outcomes in cancer patients(Chan, 2011; de Almeida et al., 2019; Denu et al., 2016b; Levine et al., 2017; Marteil et al., 2018; Ogden et al., 2017).

Deregulation of centriole duplication has been suggested to play a crucial role in tumor progression. Extra centrosomes have been shown to affect cells by promoting the formation of multipolar spindles and chromosome misegregation, leading to aneuploidy(Ganem et al., 2009; S. A. Godinho, 2015; S. A. Godinho & Pellman, 2014). Furthermore, extra centrosomes also have been shown to impair asymmetric cell division in *Drosophila* neuroblasts(Basto et al., 2008) and to affect cilia signaling(Bettencourt-Dias et al., 2011; Mahjoub & Stearns, 2012; Toftgård, 2009; K. Yuan et al., 2010). In addition, CA has been shown to promote invasive characteristics to non-tumorigenic cells, due to enhanced microtubule nucleation, as well as to invasive capacity of surrounding cells, by the induced secretion of

pro-invasive factors(Adams et al., 2021; Arnandis et al., 2018; S. A. Godinho et al., 2014). This invasive behavior is triggered by the centrosome microtubule over-nucleation, which lead to an increase in Rac1 activity and disruption of cell-cell adhesion, thus promoting invasion and metastasis(S. A. Godinho et al., 2014; Mack et al., 2011; Waterman-Storer et al., 1999).

On the other hand, Plk4 has also drawn the attention as a potential cancer biomarker. In normal and proliferating tissues, Plk4 is expressed in low-abundance, being required for centriole biogenesis(Habedanck et al., 2005a; Maniswami et al., 2018). Plk4 overexpression results in centriole amplification, causing further genomic instability and tumorigenesis(Holland et al., 2010). In fact, aberrant Plk4 expression has been linked to several human cancers(Z. Li et al., 2016; Shinmura et al., 2014; X. Zhang et al., 2021; Y. Zhao & Wang, 2019b; Q. Zhou et al., 2020). Li *et al.,* for instances, discovered that *Plk4* mRNA is highly expressed in breast cancer, and correlates with lymph node and distant metastasis, acting as a potential prognostic factor in this disease(Garvey et al., 2021; Z. Li et al., 2016). The same was observed in non-small lung cancer, where increased Plk4 expression was associated with metastasis, higher tumor size and poor disease-free and overall survival in patients(Q. Zhou et al., 2020). Thus, strong evidences support that Plk4 might have a critical role in carcinogenesis. However, mechanistically, the oncogenic properties of overexpressed Plk4 can be attributed to the consequent centriole amplification associated with its upregulation, while CA can also be just a consequence of Plk4 overexpression.

Besides its central role in regulating centriole duplication, and because it interacts with different other centrosomal proteins, Plk4 has been shown to affect Arp2/3-mediated actin branching and cell migration, while Plk4 knockdown leads to defects in cell spreading and migration(Kazazian et al., 2017). Moreover, Pelletier and colleagues showed that Plk4 is essential for the recruitment of centrosomal proteins CEP85 and STILL to the leading edge of migrating cells, where they are involved in the phosphorylation of Arp2 and actin filament assembly(Kazazian et

al., 2017) . In accordance, Plk4 has also been shown to play a role in EMT(Liao et al., 2019; Tian et al., 2018). EMT is important in cancer progression, since it occurs when tumor cells lose their epithelial characteristics while gaining mesenchymal characteristics, becoming invasive and metastatic. *In vitro* studies demonstrated that Plk4 overexpression in colorectal cancer cells activate the Wnt/β-catenin pathway and alter the expression of several EMT-related proteins, including the upregulation of N-cadherin and Snail, and downregulation of Occludin. Plk4 knock-down also prevented the Wnt/β-catenin pathway from being activated and also suppressed the growth of xenograft tumors in nude mice(Liao et al., 2019). Similar findings were made in neuroblastoma (NB), where NB tissues showed noticeably enhanced Plk4 levels, and high Plk4 levels were inversely correlated with patient survival, suggesting an oncogenic role of Plk4 in NB(Tian et al., 2018). Interestingly, EMT was inhibited in NB cells when Plk4 was downregulated, showing an increased expression of the epithelial marker E-cadherin, followed by a downregulation of mesenchymal markers, such as N-Cadherin, Vimentin and Slug, as well as p-Akt. By using a specific PI3K pathway inhibitor (LY294002), the p-Akt was inhibited, followed by a modulation of the EMT markers in Plk4-overexpressing NB cells, suggesting Plk4's involvement in controlling the EMT process via PI3K/Akt pathway in NB(Tian et al., 2018). Furthermore, *in vivo* carcinogenesis and metastasis in nude mice were significantly reduced when Plk4 was downregulated in SK-N-BE(2) NB cells. Studies have also evidenced Plk4's role in promoting EMT in breast cancer. shRNA-mediated Plk4 knockdown has been shown to inhibit cancer invasion and promoted an epithelial phenotype in poorly differentiated breast cancer cells, by increasing E-cadherin expression while decreasing N-cadherin and fibronectin mesenchymal markers(Kazazian et al., 2017). Using murine xenografts of Plk4-depleted human breast cancer cells (MDA-MB-231), researchers discovered that, while there were no effects on primary tumor growth, 25% to 45% reduction in Plk4 expression was sufficient to completely inhibited invasive and metastatic progression of the breast

cancer xenografts *in vivo*(Kazazian et al., 2017). Therefore, emerging data has been pointing out the Plk4's involvement in EMT and metastasis, facilitating cancer progression.

The tumor suppressor p53 acts as a transcription factor, regulating a variety of downstream effectors, causing growth arrest and/or apoptosis. Therefore, p53 plays an essential role during mitosis, preventing CA, aneuploidy and chromosome missegragation, preserving genomic stability. Plk4 as a CA trigger and driver of tumorigenesis, is thought to collaborate with p53 inactivation in cancer development. Indeed, studies have demonstrated that, in the context of p53 dysfunction, transient Plk4 overexpression promoted the development of squamous cell carcinomas in mice(Serçin et al., 2016). Similar findings were observed in lymphoma and sarcoma, where elevated Plk4 expression accelerated tumorigenesis in the absence of p53(Coelho et al., 2015). Because Plk4 overexpression resulted in CA and in p53-dependent cell cycle arrest in mammalian cells, these findings supported the theory that cells with high Plk4 expression and p53 deficiency were more likely to form tumors. It has been demonstrated that Plk4 transcriptional repression by p53 occurs via HDAC transcriptional repressors or through the p53-p21-DREAM-CDE/CHR pathway(Fischer et al., 2016; J. Li et al., 2005). According to Nakumura *et al.*, Plk4 expression was suppressed, and the risk of Plk4-induced CA was reduced, when p53 was activated(Nakamura et al., 2013). The p53 pathway, on the other hand, was found to be partially inactivated in thymic tumors from Plk4-overexpressing mice. These findings suggest a possible feedback loop between p53 and Plk4 in tumor development(Levine et al., 2017).

Due to all these reasons mentioned before regarding Plk4's role in EMT and in promoting tumorigenesis, and as CA is a consequence of Plk4 overexpression, we wondered if Plk4 could also have a role in *anoikis* resistance and EMT, independently of its role in centriole duplication. Our first observation showed that wild-type p53 is needed for the elimination of cells with CA induced by Plk4

overexpression. Cells with functional p53 presented a decreased percentage of CA throughout time, when compared with cells with absent p53, where these presented a high percentage of CA overtime. These results confirm the existence of a reciprocal function between p53 and Plk4.

In the work presented in this thesis I observed that, only in p53 knock-down background, Plk4 overexpression promoted *anoikis* resistance in non-tumorigenic mammary epithelial cells. *Anoikis* refers to a cell death mechanism triggered when cells lose their cell-matrix attachment, avoiding cell survival, dissemination and distant organ colonization. This process is used by cancer cells due to the loss of epithelial (E) markers and gain of mesenchymal (M) markers (EMT). This feature is associated with stem cell-like capacities, as cells become more plastic and can circumvent cell death, facilitating metastasis. The relationship between EMT and *anoikis* is considered a crucial step for cancer progression. In fact, Plk4 has been associated with EMT as previously mentioned; however, its role in *anoikis* resistance is a new way of linking Plk4 with tumor progression.

Interestingly, I show that Plk4's role in inducing *anoikis* resistance is mediated by its kinase activity but independent of its role in centrosome biogenesis. This conclusion was based on the observation that, a truncated cell line with a mutation in the C-terminal of Plk4, which fails to induce CA, was still capable of inducing *anoikis* resistance. Note that the C-terminal coiled-coil region is responsible for Plk4 dimerization, which is necessary for centriole duplication, and scaffolds for Plk4 trans-autophosphorylation, priming for SCF^{Slimb} binding and ubiquitination, while the N-terminal is responsible for Plk4's kinase activity. Moreover, by removing p53 in cells with mutated C-terminal, *anoikis* resistance was potentiated when Plk4 was overexpressed, confirming that Plk4's role in inducing *anoikis* resistance is dependent on p53. This result was surprising as it provides an important mechanistic insight into Plk4's role in cancer, but also raises interesting new questions, such as the signaling pathways involved. Signaling pathways, such as Wnt/β-catenin, has been shown to be involved in promoting EMT in colorectal

cancer and also *anoikis* resistance in esophageal squamous cell carcinoma via Plk's family member, Plk1. Plk1 directly binds to NF-κB subunit RelA, inhibiting the ubiquitination and degradation of β-catenin. Interestingly, Plk4 has also been shown to interact with NF-κB, being a direct target gene. Therefore, Plk4 could also be potentiating *anoikis* resistance in non-tumorigenic mammary cells through its interaction with NF-κB, avoiding β-catenin degradation, and thus inducing stem-like features, such as *anoikis* circumvention.

In accordance with other studies, we observed a shift from epithelial markers (decrease in E-cadherin) to mesenchymal markers (increase Vimentin and EMT transcriptional factors Snail/Slug) only by removing p53 from cells, confirming p53's role in EMT(C. Chang et al., 2011; Pinho et al., 2011), confering stem-like properties and *anoikis* resistance. By overexpressing Plk4 in the p53KO background, cells reversed from a full mesenchymal phenotype to a hybrid E/M phenotype, showing a reduction on Vimentin expression, and an increase in E-cadherin and on the EMT transcriptional factors Snail/Slug and Zeb2.

Moreover, Plk4 overexpressed cells in p53KO background also presented an increased *mRNA* and protein expression of P-cadherin, a cell-cell adhesion molecule shown to have a role in *anoikis* resistance and hybrid EMT phenotype. P-cadherin has been extensively studied in the cancer context, being a putative biomarker for hybrid EMT and bad prognostic factor for breast cancer patients. Interestingly, we observed that P-cadherin expression is partially required for *anoikis* resistance induced by overexpressed Plk4 in the p53KO context, shown by cells decreased capacity to form mammospheres when P-cadherin was silenced. More importantly, it has been demonstrated that cells with CA induced by Plk4 overexpression secrete pro-invasive factors, inducing invasive capacities of other cells. In fact, we observed that cells with high Plk4 expression are also able to induce *anoikis* resistance of other cells through the secretion of the soluble P-cadherin form into the conditioned media. Moreover, the conditioned media of depleted P-cadherin cells also reduced the capacity of breast cancer cells to resist

anoikis. Those results reinforce the role of cell-cell communication within tumor microenvironment, demonstrating that tumor heterogeneity is a key factor in promoting cancer progression.
Furthermore, we explored the relationship between the co-expression Plk4 and P-cadherin as having a potential prognostic value in breast cancer. Therefore, we decided to analyze the mRNA of breast cancer patients in the publicly available dataset from Kaplan Meier plotter. We observed that patients co-expressing both high Plk4 and high P-cadherin shows a worse prognosis, demonstrating a decreased overall survival and disease-free survival. Because CA is a consequence of overexpressed Plk4, we wondered whether CA could also be associated with high P-cadherin expression. We took advantage of a small breast cancer cohort (n=51) used in our previous studies(de Almeida et al., 2019; Marteil et al., 2018). Importantly, this breast cancer tumor series is also well characterized for different biomarkers, including the ones that are clinically used for breast cancer prognosis and treatment(Paredes et al., 2005; Sousa et al., 2010; Vieira et al., 2017). Information was available for classical breast cancer prognostic markers, such as tumor size, lymph node invasion and histological grade, as well as for the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), cytokeratin 5 (CK5), cytokeratin 14 (CK14), epidermal growth factor receptor (EGFR), E-cadherin, P-cadherin, CD49f (or integrin a6) and vimentin. Based on immunofluorescence staining of two centriolar markers (GT335 and pericentrin), we defined tumors with >13.3% of cells with more than 4 centrioles as positive for CA, a threshold that was determined considering the mean of CA observed in all tumor samples evaluated. In accordance to what has been previously described(de Almeida et al., 2019), we found that CA was correlated with more aggressive breast tumors, such as basal-like subtype (100%, *p=0,00002*), and high-grade tumors (64,5%, *p=0,004*) **(Table 5.1)**. Interestingly, as mentioned, P-cadherin has also been described as a poor prognostic biomarker in basal-like breast tumors, and correlated with histological

grade, being essentially present in high-grade tumors(Paredes et al., 2002, 2005; Turashvili et al., 2011) and associated with poor patient survival. In accordance, we also found that CA is correlated with a worse disease-free survival (DFS, *p=0.003*) **(Figure 5.1A)** and overall survival (OS, *p=0.005*) **(Figure 5.1B)**, as revealed in the Kaplan-Meier plots.

Concerning the molecular markers, we found that the expression of ER was inversely correlated with CA (*p=0,001*), but no significant correlation was found in the analysis of PR and HER2 expression. No correlation was also observed in the analysis of basal markers, such as CK5, CK14 and EGFR. However, and in line with our *in vitro* results, CA was strongly correlated with P-cadherin expression (*p=0,003*), as well as with Vimentin (*p=0,033*) and CD49f (*p=0,015*), but not with E-cadherin expression **(Figure 5.1C)**.

All of these points validate our findings concerning the association between Plk4 (and also CA indirectly) and P-cadherin, in tumor aggressiveness. Nevertheless, some questions remain open and need further exploration. Do all cells have an inherit capacity to block *anoikis*? Or is there a developed mechanism of *anoikis* evasion? What is the exact molecular mechanism involved in the process of *anoikis* resistance induced by Plk4 overexpression? What benefits or problems can this bring in cancer therapies based on Plk4-inhibition? Answers to all of these open questions will give new insights and will help to understand the impact of Plk4 deregulation in tumor microenvironment, contributing to tumor initiation and/or progression.

	No amplification n=27		Amplification n=24		
	Frequency	Percentage (%)	Frequency	Percentage (%)	P-value
Tumor size					
T1: <2cm (15)	11	73,3%	4	26,7%	0,199
T2: <2-5cm(22)	10	45,5%	12	54,5%	
T3: >5cm (11)	5	45,5%	6	54,5%	
Lymph Node Invasion					
negative (12)	8	66,7%	4	33,30%	0,506
positive (30)	16	53,3%	14	46,7%	
Histological Grade					
I (9)	6	66,7%	3	33,3%	**0,004**
II (11)	10	90,9%	1	9,1%	
III (31)	11	35,5%	20	64,5%	
Molecular subtypes					
Luminal A	23	79,3%	6	20,7%	**0,00002**
Luminal B	0	0,0%	3	100,0%	
HER2	2	66,7%	1	33,3%	
Basal	0	0,0%	13	100,0%	
Not classified	2	66,7%	1	33,3%	

Table 5.1: Centrosome amplification is associated with more aggressive and invasive breast tumors.

Association of CA with different breast cancer molecular subtypes and with clinico-pathological features. CA is significantly associated with Basal-like tumors, and increased tumor stage, suggesting its role in more aggressive tumors. No association was observed with tumor size and lymph node invasion. X^2 test and contingency tables were performed and *P* values <0.05 were considered statistically significant.

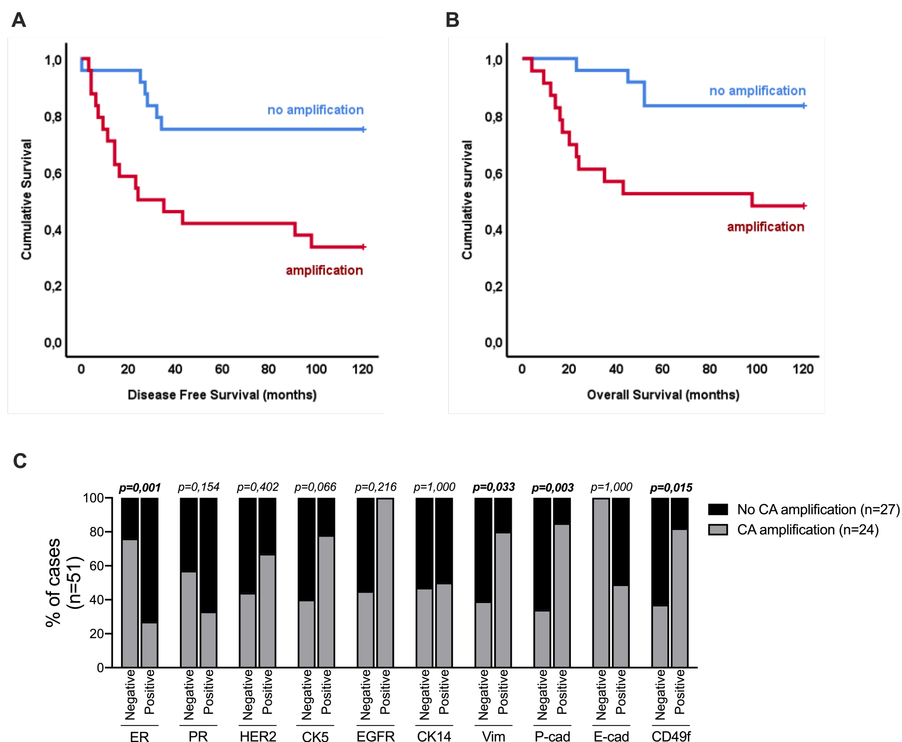

Figure 5.1. Centrosome amplification is associated with more aggressive and invasive breast tumors and significantly correlated with tumors with high P-cadherin expression.

A) CA showed a significant correlation with a worse DFS (p=0.003) and OS (p=0.005), as revealed in Kaplan-Meier plots for patients with tumors with CA versus patients with tumors without centrosome amplification. Univariate survival curves were estimated with Kaplan-Meier and compared using the log-rank test. *P* values <0.05 were considered statistically significant.

B) Association of CA (considered as positive when tumors showed >13.3% of cells with more than 4 centrioles) in the primary breast carcinomas, and with breast cancer molecular markers: Estrogen Receptor (ER), Progesterone Receptor (PR), human epithermal growth factor receptor 2 (HER2), Cytokeratin 5 (CK5), epidermal growth factor receptor (EGFR), Cytokeratin 14 (CK14), and E-cadherin, Vimentin, P-cadherin, E-cadherin, and CD49f. ER was inversely correlated with CA, whereas Vimentin and CD49f expression was positively correlated with tumors with CA. P-cadherin expression showed to be significantly associated with tumors with centrosome amplification. No correlation was observed with other markers, such as PR, HER2, CK5, EGFR, CK14, and E-cadherin. Statistical analysis was performed by SPSS statistics (SPSS Inc., USA). X^2 test and contingency tables were performed to determine associations between groups and results where P values were <0.05 were considered statistically significant.

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