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Cell cycle arrest and senescence as a consequence of CDK4/6 kinase inhibition

Zástava buněčného cyklu a senescence jako důsledek inhibice CDK4/6 kinázy

Bachelor's thesis

Supervisor: MUDr. Libor Macůrek, Ph.D.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu. Při úpravě jazykové a stylistické stránky textu jsem využila asistenci umělé inteligence, která mi pomohla s drobnými úpravami struktury vět, aniž by ovlivnila obsah či odbornou správnost práce.

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Abstract

The dysregulation of the cell cycle is one of the hallmarks of cancer. CDK4/6 kinases play an essential role in the G1/S phase progression, and CDK4/6 inhibitors can block this progression. This inhibition effectively suppresses tumor growth, especially in HR+ HER2- breast cancer. However, beyond their cytostatic effects, these inhibitors can also promote cellular senescence, a state of irreversible cell cycle arrest associated with metabolic and epigenetic changes. The induction of senescence is influenced by many factors, such as the p53 and p21 status, oncogene signaling, and replication stress. This thesis explores the effect of CDK4/6 inhibition in cell cycle arrest and senescence, the connection between cell cycle arrest and cell overgrowth, discussing its implications for cancer therapy, resistance mechanisms, and potential combination treatments to enhance the treatment efficacy.

Key words: CDK4/6, CDK4/6 inhibitors, cell cycle, G1 arrest, senescence, cell growth, breast cancer, p53, p21

Abstrakt

Deregulace buněčného cyklu je jedním z charakteristických znaků rakoviny. CDK4/6 kinázy hrají důležitou roli v progresi G1/S fáze, a CDK4/6 inhibitory mají schopnost tuto progresi blokovat. Tato inhibice efektivně potlačuje růst nádoru, zvláště u HR+ HER2 – rakoviny prsu. Ovšem, kromě jejich cytostatických efektů mohou tyto inhibitory taky způsobovat buněčnou senescenci, což je stav nevratné zástavy buněčného cyklu, spojený s metabolickými a epigenetickými změnami. Tato bakalářská práce zkoumá úlohu CDK4/6 inhibice při zástavě buněčného cyklu a senescenci, souvislosti mezi zástavou buněčného cyklu a přerůstáním buňky, diskutuje jejich terapeutický význam při léčbě rakoviny, mechanismy vedoucí ke vzniku resistence a potenciální kombinační terapie, které mají za cíl zvýšit efektivitu léčby.

Klíčová slova: CDK4/6, CDK4/6 inhibitory, buněčný cyklus, zástava buněčného cyklu v G1 fázi, senescence, buněčný růst, rakovina prsu, p53, p21

List of Abbreviations

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1	**MHC**	Major histocompatibility complex
53BP1	p53-binding protein 1	**MKK3/6**	MAP kinase kinase 3/6
Akt	Ak strain transforming	**MKKK**	Mitogen Activated Protein (MAP) kinase kinase kinase
APC	Anaphase promoting complex	**MMP**	Matrix metalloproteinase
AR	Aldose reductase	**MPF**	Maturation promoting factor
ATP	Adenosine triphosphate	**mTOR**	Mammalian target of rapamycin
ATRX	Alpha-thalassemia/mental retardation, X-linked	**mTORC1/2**	Mammalian target of rapamycin complex 1/2
BGT1	Na(+)/Cl(-) betaine/GABA transporter	**MYB**	MYB genes
CAK	Cyclin-dependent protein kinase-activating kinase	**MYC**	Myelocytomatosis
CAMKII	Ca2+/calmodulin-dependent protein kinase II	**NADPH**	Nikotinamidadenindinukleotidphosphate
CCL2	Chemokine (C-C motif) ligand 2	**NF-1**	Neurofibromin 1
Cdc25B	Cell division cycle 25B	**NF-B**	Nuclear factor kappa-light-chain-enhancer of activated B cells
Cdc25C	Cell division cycle 25C	**NFAT5**	Nuclear factor of activated T cells 5
Cdc6	Cell division cycle 6	**NSCLC**	Non-small cell lung cancer
CDK1	Cyclin dependent kinase 1	**P14ARF**	p14 alternative reading frame
CDK2	Cyclin dependent kinase 2	**P38MAPK**	p38 mitogen-activated protein kinase
CDK4	Cyclin dependent kinase 4	**PASP**	p53-associated secretory phenotype
CDK6	Cyclin dependent kinase 6	**PCNA**	Proliferating cell nuclear antigen
CDK7	Cyclin dependent kinase 7	**PDK1**	Phosphoinositide-dependent protein kinase1

CDK9	Cyclin dependent kinase 9	**PIK3R4**	Phosphoinositide-3-Kinase Regulatory Subunit 4
CDKN2A	Cyclin dependent kinase inhibitor 2A	**PNPLA6**	Phospholipase-neuropathy target esterase
CENPA/B	Centromere protein A/B	**PTEN**	Phosphatase and tensin homolog
CTD1	Chromatin licensing and DNA replication factor 1	**Raf**	Rapidly accelerated fibrosarcoma
CXCL	Chemokine (C-X-C motif) ligand	**Ras**	Rat sarcoma
DNA Pol	DNA Polymerase	**Rb**	Retinoblastoma protein
DP	Dimerization partner	**RET**	Rearranged during transfection
DREAM	Dimerization partner, RB-like, E2F and multi-vulval class B	**RFC1-5**	Replication factor C subunit 1-5
ECM29	Proteasome-associated protein ECM29 homolog	**RIF1**	Replication Timing Regulatory Factor 1
EGF	Epidermal growth factor	**ROS**	Reactive oxygen species
eIF4E	Eukaryotic translation initiation factor 4E	**RPL9**	Ribosomal Protein L9
Erap1	Endoplasmic reticulum aminopeptidase 1	**RRM1**	Ribonucleoside-diphosphate reductase large subunit
FAT1	Protocadherin FAT1	**S6K**	Ribosomal protein S6 kinase
FDA	U.S. Food and Drug Administration	**SAHF**	Senescence-associated heterochromatic foci
FGFR1	Fibroblast growth factor receptor 1	**SASP**	Senescence-associated secretory phenotype
FOXM1	Forkhead box M1	**Smad3**	Mothers against decapentaplegic homolog 3
GOF	Gain of function	**SMIT**	sodium/myo-inositol cotransporter
GSK3	Glycogen synthase kinase-3	**SORD**	Sorbitol dehydrogenase
HER2-	Human epidermal receptor 2 -	**STAT3**	Signal transducer and activator of transcription 3
HLA-A/B/C	Human leukocyte antigen A/B/C	**Tapbp**	TAP-associated glycoprotein

HSC's	Hematopoetic stem cells	**TauT**	Taurine transporter
IL-6,8	Interleukin 6, 8	**TCF/LEF**	T-cell factor/Lymphoid enhancer-binding factor
INK4	Inhibitors of CDK4	**TGF-β**	Tumor growth factor β
KIP/CIP	Kinase inhibitory protein /CDK interacting protein	**TNF**	Tumor necrosis factor
KO	Knock-out	**Treg**	T-regulatory cells
LOF	Loss of function	**TSC2**	Tuberous sclerosis complex 2
LRP	Lipoprotein receptor	**VEGF**	Vascular endothelial growth factor
MAPKAP-K2	MAP kinase-activated protein kinase 2	**XRCC2**	X-ray repair cross complementing 2
MCM2/10	Protein MCM2/10 homolog	**YAP**	Yes-associated protein 1
MDM2	Mouse double minute homolog 2		

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1. Introduction

The cell cycle is a series of different stages in the life of the cell, that are crucial to maintain the integrity, to grow, replicate its DNA and to divide. Cell cycle is divided into 4 phases: G1, S, G2, and M. Each of these cell cycle phases are strictly regulated and controlled by cyclin dependent kinases (CDK's), together with their activators, cyclins. The levels of cyclins fluctuate during the cell cycle, whereas the levels of CDK's remain the same. The early G1 phase is being regulated by cyclin D - CDK4/6, driving the initiation of S phase gene expression. In the late G1, the role is being overtaken by cyclin E - CDK2, condemning the cell into S phase. In this subsequent S phase, cyclin A and CDK2 play a role, particularly in DNA replication. G2 phase is driven by cyclin A - CDK1, which drive the cell towards mitosis. In the G2/M phase transition, cyclin B – CDK1 initiate mitotic entry. Cyclin B is then degraded in the metaphase with the help of anaphase-promoting complex (APC/C), an E3 ubiquitin ligase, marking cyclin B for destruction in the proteasome. After completing mitosis and cytokinesis, the cell cycle starts again from the beginning.

If the balance of some CDK's, cyclins get disrupted, cell may end up in a G0 state, where the cell cycle progression is restricted, or even commit themselves for apoptosis, if the defects are too severe. However, if the detection and restriction mechanisms fail, the cell can escape all safeguard systems and proliferate uncontrollably, and consequently, acquire even more mutations in the genome and become cancerous. Therefore, many efforts have begun to develop drugs, that block the cell cycle progression of these cancer cells.

CDK4/6 inhibitors are relatively recently discovered molecules nowadays being used in breast cancer treatment. CDK4/6 inhibitors, such as palbociclib, ribociclib, abemaciclib and others, block CDK4/6 activity. CDK4/6 inhibitors work as ATP-binding competitors and their activity leads initially to G1 arrest, but also to senescence, quiescence, or cell death, depending on the conditions inside of the cell.

In this work, I will describe the molecular mechanisms leading to the progression from G1 to S phase, mainly focusing on the CDK4/6 pathway. I will also discuss the dysregulation of the cell cycle, that leads to cancer phenotype and summarize main findings about CDK4/6 inhibitors, their effect on the cell cycle, and their importance in causing either G1 arrest, or senescence, as well as their relation to cell-overgrowth. Lastly, I would like to introduce some therapeutic implications of CDK4/6 inhibitor-associated phenotype and propose some future direction in this field of study.

2. General Overview of CDK4/6

2.1. Structure, Domains and Function of CDK4/6

Cyclin-dependent kinase 4 (CDK4) is an enzyme encoded by the *CDK4* gene. This gene is located on the 12q14.1 in humans and the translated protein product is 303 amino acids long (Mitchell *et al.*, 1995). Similarly, CDK6 is encoded by the *CDK6* gene on 7q21.2 chromosome locus. CDK4 and CDK6 consist of an N-terminus, that contains a 5 stranded β-sheet and an αC-helix, whereas the C-terminal domain has an α-helical structure (Day *et al.*, 2009). These motifs are crucial for cyclin D binding. Additionally, a cleft is formed out of the N-terminal domain and the C-terminal domain. This cleft then serves as ATP-binding site, critical for the enzymatic activity of CDK4/6 (Matsushime *et al*.,1992; Russo *et al*., 1998).

After the formation of the complex CDK4/6-cyclin D, this complex acquires the catalytic activity and ability to phosphorylate various substrates, as it belongs to the Ser/Thr kinase family (reviewed in Sherr and Roberts, 1995). However, not only cyclin D binding is required for the activation of CDK4/6. There is also a Thr172 inside of the T-loop of CDK4 and a Thr177 in CDK6, that need to be phosphorylated by the CAK complex (Cyclin-dependent protein kinase-activating kinase), to fully activate CDK4/6 in complex with cyclin D (Kaldis *et al.*, 1998; Bockstaele *et al.,* 2009).

One of the main downstream targets of CDK4/6 is the Retinoblastoma protein (Rb), which has been identified as a critical inhibitor of the cell cycle progression (Knudsen and Wang, 1997). Rb is inactivated upon hyperphosphorylation, which leads to its conformational change. In this newly acquired conformation, Rb is not sufficient in inhibiting E2F transcription factor family, allowing it to initiate the S phase gene expression (Chellappan *et al.*, 1991). Interestingly, CDK4 and CDK6 differ in the way they phosphorylate the Rb protein. CDK4 tends to phosphorylate Thr826 on Rb, while CDK6 preferentially targets Thr821. The whole structure of the Rb protein, and not only specific amino acids are being recognized by CDK4/6, this means, that not only cyclin D in complex with CDK4/6, but also CDK4/6 alone can have the ability to recognize their substrate. However, without cyclin D, CDK4/6 remain inactive, as its presence is essential for their activation. (Takaki *et al.*, 2005).

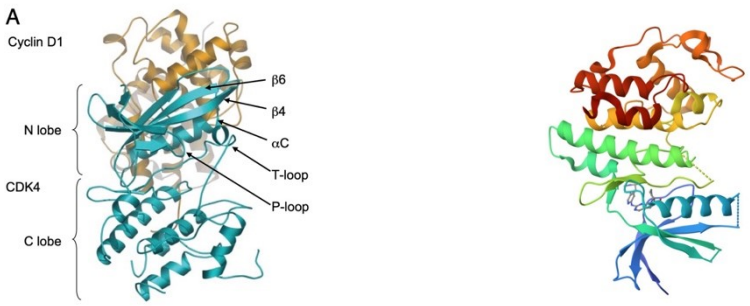


Figure 1. Structure of CDK4 (left) (blue)/Cyclin D1 (yellow) complex and CDK6 (right). T-loop is critical for CDK4/6 activation, as it blocks the ATP-binding site in inactive state, it undergoes a conformational change as type D cyclin binds to CDK4/6.

P-loop is positioning the ATP bound in CDK4/6 in the right way and helps thus to facilitate the phosphorylation. Adapted from Day *et al*., 2009; https://www.rcsb.org/structure/5l2t

2.2. Role of CDK4/6 in the Cell Cycle Progression

As already mentioned, CDK4/6 require the binding of type D cyclins for activation and translocation from the cytoplasm into the nucleus, to induce the transition through the G1 cell cycle phase. Type D cyclins are encoded by three different genes: *CCND1*, *CCND2* and *CCND3*. They are being transcriptionally regulated by many transcription factors and pathways, as shown in Figure 2. Namely, it is the AP-1, that is activated downstream of MAPK/ERK signaling, or the STAT3/5, which are a part of the JAK/STAT pathway. NF-κB or Estrogen/Progesterone/Androgen receptors may also indirectly activate cyclin D expression. The Wnt pathway is also contributing to cyclin D expression. Upon Wnt-dependent Frizzled and LRP (lipoprotein receptor) activation, the degradation complex kinases are recruited to the plasmatic membrane and phosphorylate LRP. This recruits Dishevelled protein to the cytoplasmatic site, and the destruction complex gets inactivated. Therefore, β-catenin isn't degraded anymore by the proteasome and translocases to the nucleus, where it inhibits the transcription repressor TCF/LEF (T-cell factor/Lymphoid enhancer-binding factor), which promotes the expression of cyclin D, and many other genes (reviewed in Pawlonka, Rak, Ambroziak, 2021).

After their expression, type D cyclins get competent in binding CDK4/6. This process is also partly facilitated by the inhibition of GSK3β (Glycogen synthase kinase-3β) through PI3K-AKT or Wnt/β-Catenin pathway, which, in unblocked stage, phosphorylates Thr286 in cyclin D1, and marks it for polyubiquitination and subsequent degradation in the proteasome (Diehl, Cheng, Roussel, 1998). Once stabilized, the cyclin D-CDK4/6 complex then translocates into the nucleus, where it undergoes a series of phosphorylation by CAK complex (Kato *et al.*, 1994).

In early stages of the G1 phase, cyclin D-CDK4/6 complex targets many substrates in the nucleus, among them the Rb protein. The phosphorylation of Rb results in its inactivation and this frees E2F1/E2F2/E2F3 transcription factors from its inhibitory binding (Weintraub, Prater, Dean, 1992). E2F can thus drive the expression of genes that commit the cell into S phase, such as for example cyclin E, that cooperates with CDK2, required for the late G1/S phase entry (Ohtani, DeGregori, Nevins, 1995).

CDK4/6 also stabilize and activate FOXM1 by phosphorylating it on more than one amino acid site (Anders *et al.,* 2011). FOXM1 is a transcription factor, known to have a serious impact on more than 18 genes involved in G1/S transition, such as cyclin E2, MYB (transcription factor), MCM2 (a helicase subunit), MCM10 (activates MCM2-7 helicase required for replication), and CDT1 (involved in initiation of DNA replication), XRCC2 (regulation of DNA repair), Cdc25B (a phosphatase involved in cell cycle progression), Aurora B kinase (a protein involved in the attachment of the mitotic spindle to the centromere), survivin (a protein involved in mitosis and inhibition of apoptosis), centromere protein A (CENPA, a histone H3 variant), centromere protein B (CENPB, a DNA binding

protein important in the assembly of centromere), etc. (Wang *et al.*, 2005). Thanks to its features, FOXM1 is involved in the protection from senescence and apoptosis in cancer cells upon CDK4/6-dependent phosphorylation (Anders *et al.* 2011).

Another important downstream target of CDK4/6 is the protein Smad3 (Mothers against decapentaplegic homolog 3). Smad3 works as a tumor-suppressor in non-transformed cells, and is inhibited after CDK4-dependent phosphorylation, resulting an enhanced cell proliferation in TGF-β-driven cancers (Matsuura *et al.*, 2004). Beyond its tumor-suppressive role, Smad3 has been implicated in tumor progression. TGF-β-induced Smad3 activity has been associated with breast cancer bone metastasis, through the regulation of angiogenesis and epithelial-mesenchymal transition (Petersen *et al.*, 2010). A critical regulator of Smad3 function in this context is FOXM1 (Forkhead box M1). FOXM1 can increase the ability of breast cancer cells to metastize, by stabilizing the Smad3/Smad4 complexes in the nucleus, thereby maintaining TGF-β signaling and promoting cell invasion (Xue *et al.*, 2014).

Among others, CDK4/6 also phosphorylates tuberous sclerosis complex 2 (TSC2) on Ser1217 and Ser1452. TSC2 gets inactivated by phosphorylation. As a result, inactivation of TSC2 by CDK4/6 relieves the inhibition on mTORC1, thereby promoting mTORC1 activation. Activated mTORC1 enhances cell growth and metabolic activity, contributing to the proliferative and anabolic effects associated with CDK4/6 signaling (Romero-Pozuelo *et al.*, 2020). All of these, along with numerous other effects mediated by the CDK4/6–cyclin D complex, contribute to cell cycle progression. However, overexpression of CDK4/6 or cyclin D, or enhanced CDK4/6 activity, can drive tumorigenesis by disrupting normal cell cycle control.

Recently, it has been shown, that CDK4/6 may not play a role just in the G1 phase of the cell cycle, but also in other phases. All cells possess cell cycle exit molecular clock and mitogenic clock. The length of each of these can determine the cell fate upon loss of mitogenic signaling, normally convicting the cell to mitosis. Even after the R-point (restriction point), some cells can exit the cell cycle into a G0-like state, and afterwards, they tend to profile themselves as senescent. Many factors may determine this commitment. It has been shown that the distance from the mitotic initiation can play a role – the closer to the mitotic entry, the less likely the cell is to stop and exit the cycle, as it needs some time to show a delayed response to the loss of mitogenic signaling. Moreover, the exit is driven by the loss of cyclin A2-CDK2 activity after loss of mitogenic signaling and CDK4/6 has been found to drive cyclin A2 expression in S/G2 phase. The CDK4/6 activity is thus crucial for the resumption of the cell cycle not only in G1 phase, but also in other cell cycle phases (Cornwell *et al.*, 2023).

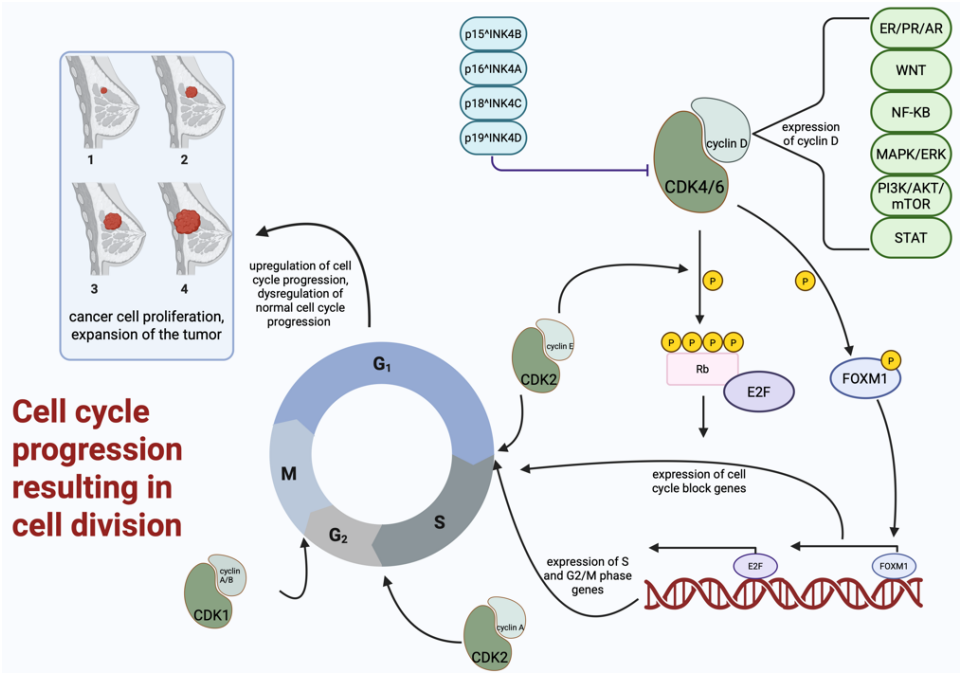


Figure 2. Cell cycle with detail on G1/S phase progression. Cyclin D levels are regulated by various pathways (ER/PR/AR, NF-κB, MAPK, PI3K/Akt/mTOR, STAT). CDK4/6 in complex with cyclin D phosphorylate Rb, driving the expression of E2F-dependent G1/S phase genes, and FOXM1, that induces the expression of genes inhibiting cell cycle arrest. After finishing the cycle, cell divides. If the dysregulation of some of the components of this machinery appears, tumor expansion may occur. Created in https://BioRender.com, adapted from a review Xu *et al.*, 2017

2.3. Downstream Effects of the E2F Transcription Factor Family

There are currently 6 known types of E2F. E2F1, E2F2 and E2F3, which activate their target genes, E2F4, E2F5 and E2F6, having rather repressive effect, and lastly E2F7 and E2F8, with a yet not well understood function. (reviewed in Attwooll *et al.,* 2004).

Focusing on the first class, E2F1, E2F2, and E2F3 play a key role in the regulation of genes involved in cell cycle progression. They stimulate the expression of various proteins, involved in S-phase progression, most importantly of cyclin A2, and cyclin E1, which drive the G1/S transition. The activity of CDK2 depends on both of these cyclins (Schulze *et al.*, 1995; Geng *et al.*, 2001). Besides, E2F1-3 regulate the expression of genes required for DNA replication, such as DNA Pol ε, DNA Pol α primase, or PCNA (Proliferating cell nuclear antigen) (Tommasi, Pfeifer, 1999; Nishikawa *et al.*, 2001).

Interestingly, the E2F family can not only enhance cell cycle progression, but also block it, when necessary. Another important protein of E2F1 is p53, the most famous tumor-suppressor, disrupted in a range of cancer cells. E2F1 indirectly regulates p53, by promoting the expression of p14^ARF, a protein that inhibits MDM2. Higher levels of active E2F1 thus lead to the stabilization of p53, as MDM2 can no longer target p53 for degradation, and the favors shift towards the p53-driven phenotype, such as apoptosis and cell cycle arrest (Wu, Levine, 1994; Bates *et*

al., 1998). Additionally, the interaction of E2F1 with p53 has also been found to downregulate the expression of VEGF (Vascular endothelial growth factor) (Qin *et al.*, 2006).

E2F1 also takes part in metabolic reprogramming in stressed cells. Under stressful conditions, E2F1 drives the upregulation of fructose-2,6-bisphosphatase expression, an enzyme stimulating the glycolytic pathway. This causes the cell to switch from oxidative phosphorylation to glycolysis (Blanchet *et al.*, 2011). Interestingly, this metabolic shift towards glycolysis is one of the hallmarks of cancer and is part of the Warburg effect, in which cells preferentially rely on glycolysis for energy production even in the presence of oxygen. As glycolysis leads to the accumulation of lactic acid, stressed cells will become more acidic, particularly if the stress conditions also involve low oxygen levels. In these conditions, the hypoxic environment, combined with increased glycolytic activity, causes acidification of the cell and its microenvironment.

3. G1 Arrest

3.1. Molecular Basics of the G1 Arrest

There are several established ways for inducing G1 arrest in cells, but this thesis will primarily focus on the disruption of CDK4/6 activity. CDK4/6 inhibitors target and inhibit cyclin-dependent kinases 4 and 6, along with other associated kinases, ultimately leading to a prevention of cell cycle progression. This process is commonly referred to as G1 arrest. To fully understand the significance and consequences of CDK4/6 inhibition, it is crucial to consider the underlying molecular events that occur during this disruption. Specifically, when CDK4/6 is inhibited, the Rb protein remains in its hypophosphorylated state, which prevents it from dissociating from the E2F transcription factors. This failure to release E2F blocks the expression of genes required for S phase progression, including cyclin E. Cyclin E, bound to CDK2, is essential for the late G1/S transition, driving the cell past the critical checkpoint into DNA replication (Ohtsubo *et al.,* 1995). Thus, the inhibition of CDK4/6 disrupts a key regulatory mechanism that normally facilitates the transition from G1 to S phase, leading to a cell cycle arrest at the G1 phase.

3.2. Endogenous Inhibitors of CDK's

Two main families have been found to inhibit CDK4/6 activity in-vivo, called the KIP/CIP and INK4 inhibitor family. The KIP/CIP family blocks many kinds of CDK´s, not just CDK4/6 specifically. Proteins of this family are called p21, p27 and p57. They do inhibit CDK's required for the cell cycle progression either by direct detention, or by the inhibition of CAK complex (reviewed in Csergeová, Krbušek, Janoštiak, 2024). When overexpressed, they can

enforce the cell into a G1 arrest. (Reynisdóttir *et al.,* 1995). In other words, these proteins are major physiological contributors to the G1 arrest phenotype, and thus tumor-suppressors.

Specifically, both CDK4 and CDK6 are being selectively inhibited by the members of the INK4 family, which include p15 (INK4B), p16 (INK4A), p18 (INK4C), and p19 (INK4D) (reviewed in Cánepa *et al.,* 2007). Among the INK4 family members, p16^INK4A has been most extensively studied. It plays a critical role in tumor suppression and is frequently deleted, silenced, or mutated in a wide range of human cancers (Serrano et al., 1993). p16^INK4A is also involved in the process of cellular senescence, where the upregulation of p16 during senescence prevents malignant transformation by ensuring, that aberrant cells do not continue to divide.

3.3. CDK4/6 inhibitors and G1 Arrest

3.3.1. Duration of the CDK4/6 inhibition Causes Replication Stress

According to the findings of Crozier *et al.*, long-term CDK4/6 inhibition by palbociclib results in the downregulation of the MCM2-7 complex, required for the DNA origin licensing and the formation of a helicase complex, but also the expression of PCNA, RFC1-5, RRM1, RB1, RPL9 and other factors required for DNA replication, as well as protein synthesis and cell cycle progression (Focher *et al.*, 1989; Evrin *et al.*, 2009; Cheng *et al.*, 2021; Crozier *et al.*, 2022). This depletion of replisome components causes replication stress, and activation of the p53-p21 pathway. Both of these conditions can inhibit long-term proliferation during S phase or even force the cell to withdraw from the cycle, however, cancer cells have found mechanisms to circumvent this hindrance and are able to proliferate even under such genotoxic stress, so, although the proliferation is slowed down, a permanent cell cycle arrest doesn't occur in these cancer cells. Breast cancer cells dominantly produce micronuclei, that form when just a few chromosomes fail to segregate correctly. In cancer cell lines, this defective cell cycle progression results in the accumulation of genetic defects, that may be present during either continuous or intermittent drug exposure, and that can lead to apoptosis, or other types of cell death (Crozier *et al.*, 2022).

Interestingly, Crozier *et al.*, observed, that after a 4- or 7-day treatment with CDK4/6 inhibitors, cells which succeeded to enter S and G2 phase stop before entering M phase and return into G1 phase (Crozier *et al.*, 2022). This phenomenon, where the cells exit from the G2 directly into G1 arrest, is p53 and p21 dependent and can lead to senescence (Bunz *et al.*, 1998; Krenning *et al.*, 2014). The loss of p53 (in p53-KO cells) allows cells to escape the initial G1 arrest, as there is no way to activate p21, the downstream target of p53, required for p21-driven senescence. The activity of p21 depends on the amount of activated p53. If p53 levels are high, p21 will act as a CDK inhibitor and hold cells in an arrested state (El-Deiry *et al.*, 1993). If not, cells will continue in cell cycle progression. Interestingly, p53 loss doesn't have any striking effect on sustaining long-term proliferation, which means, that other cellular mechanisms may play a role in the maintenance of cell cycle arrest.

After CDK4/6 inhibition, p53 plays a crucial role in enforcing cell cycle withdrawal. However, even in p53-deficient (p53-KO) cell lines, prolonged CDK4/6 inhibition still suppresses tumor growth. In the absence of functional p53, cells continue to cycle unchecked, ultimately undergoing catastrophic mitosis, resulting in severe DNA damage, incompletely replicated chromosome segregation and replication stress. All these conditions are incompatible with the survival of the cell.

In summary, long-term CDK4/6 inhibitor treatment disrupts cell viability either through p53-dependent cell cycle arrest, which halts proliferation, or through p53-independent pathways, resulting in catastrophic mitosis, excessive DNA damage, and finally cell death. The cellular response to defective DNA replication strongly depends on p53 status (Crozier *et al.,* 2022).

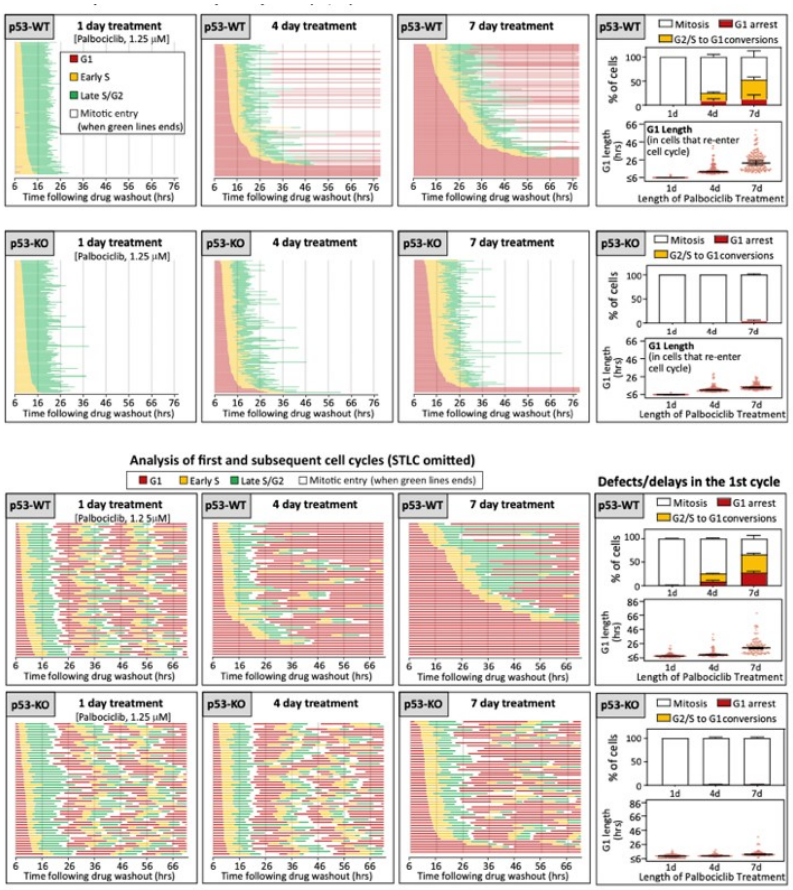


Figure 3. The cell cycle arrest induction is p53-dependent. In p53-WT cells, p53 and p21-dependent G1 arrest occurs after short-term treatment with CDK4/6 inhibitors. Long-term treatment with CDK4/6 inhibitors results in the downregulation of replisome components and persistent E2F repression, which may further elevate p21 levels and condemn the cells into senescence. On the other hand, in p53-KO cells CDK4/6 inhibitors induce catastrophic mitosis, DNA damage, etc., which can block tumor growth. CDK4/6 inhibition will thus induce tumor regression independently of the p53 status, however, if p53 is lost, cancer cells may develop certain ways to continue proliferating despite therapy and acquire resistance to drugs. The graph shows individual cells after drug washout (either p53-WT, or p53-KO) occurring in different phases of the cell cycle depending on the duration of palbociclib (1.25 μM) treatment (1, 4, or 7 days). G1 (red), early S (yellow), late S/G2 (green), mitotic entry (white). Cell cycle phase and G1 duration were tracked using FUCCI reporters (mKO-Cdt1). Adapted from Crozier *et al.*, 2022.

3.3.2. Recovery or Commitment to Senescence after G1 Arrest Rescue

Many factors determine the fate of cells following CDK4/6 inhibition, whether they will enter a reversible G1 arrest, quiescence, permanent cell cycle arrest, senescence, apoptosis, or a drug-resistant state. A functional Rb protein is required for the commitment to G1 arrest or senescence upon CDK4/6 inhibition, as it is the major downstream target of CDK4/6 (reviewed in Sherr and Roberts, 1999).

The commitment to either G1 arrest or senescence following CDK4/6 inhibition has long been thought to depend primarily on the dose and duration of treatment: lower doses typically induce a reversible G1 arrest, while higher doses and prolonged exposure are more likely to trigger senescence (Vijayaraghavan *et al.,* 2017). However, recent research suggests that additional factors contribute to the cell's fate. One such factor is the formation of a DREAM complex, that condemns the cell to quiescent state (G0 phase) by repressing the expression of many E2F-dependent genes taking part in the cell cycle (Sandoval *et al.*, 2006). DREAM complex includes p130, the repressor (E2F4 or E2F5), a dimerization partner DP1 or DP2 and lastly MuvB (Pilkinton *et al.*, 2007). Both DREAM and Rb block E2F and each of them is more abundant at different stages of the cell cycle (Litovchick *et al.*, 2007). Active CDK4/6 phosphorylates the DREAM complex, thereby inactivating it and ensuring that E2F gets released. Upon CDK4/6 inhibition, the DREAM complex remains active, and a quiescent phenotype prevails (Schade *et al.*, 2019). Therefore, the outcome of whether the cell will continue in the cell cycle, or enter a senescent state are not not only determined by the duration of the treatment, but also by additional factors, such as the status of the DREAM complex, or the activity of growth-promoting pathways, which will be further discussed in Chapter 6.

4. Senescence

4.1. Senescence Hallmarks

Senescence is a state of permanent cell cycle arrest, where there remains the ability of the cells to stay metabolically active and grow. In non-transformed cells, this state is reached after threshold telomeric shortening, and such senescent type is referred as replicative senescence. Senescence can also be induced upon excessive DNA damage, stress response, protein degradation, or in case of an activated oncogene (Hayflick and Moorhead, 1961; Bartkova *et al.*, 2006; reviewed in Mikuła-Pietrasik *et al.,* 2020). This is called premature cell senescence. It has been found out, that a protein from the KIP/CIP inhibitor family called p21 plays a crucial role in the induction and maintenance of senescence. p21 is being regulated by the p53 protein, the major tumor suppressor of human cells, by p53's direct binding to the promoter of p21 gene (El-Deiry *et al.*, 1993). This protein has been identified as a major inhibitor of all members of the CDK/cyclin family and can therefore block cell cycle progression (Xiong *et al.*, 1993). p21 can for example act on active CDK2 or CDK4/6, preventing it from phosphorylating the Rb protein and forcing the cell to halt the progression in G1 phase (Brugarolas *et al.*, 1999). Another protein, p16, has also been

found to mediate cell cycle arrest, leading to senescence by acting on CDK4/6 and blocking Rb phosphorylation (Fåhraeus *et al.*, 1996).

Oncogene-induced senescence (OIS) is a mechanism by which a cell can protect itself from malignant transformation and the development of cancer. Activation and overexpression of a protooncogene *Ras* can also induce cellular senescence in cells which acquired mutations in this gene. *Ras* overexpression activates tumor suppressor pathways, leading to activation of p53 and p16^INK4A/ARF, another crucial factor from the INK4 family, further promoting senescence (Figure 4.). This effect was shown in human primary lung fibroblasts, and mammary epithelial cells, but also in other cell lines (Serrano *et al.*, 1997; Sarkisian *et al.*, 2007). There are also other genes, by which either a gain of function mutation (GOF), loss of function mutation (LOF), or overexpression results in an induction of senescence. For example, B-Raf (Raf kinase) and Akt (Akt serine-threonine kinase) can induce senescence if they acquire a GOF mutation (Miyauchi *et al.*, 2004; Michaloglou *et al.*, 2005). Overexpression of E2F1, CDC6, or cyclin D1/E also leads to senescence (Lucibello *et al.*, 1993; Dimri *et al.*, 2000; Ueda *et al.*, 2016). LOF mutations of tumor suppressors like *PTEN* can paradoxically lead to senescence, but this effect is highly dependent on the presence of functional p53. For example, acute PTEN inactivation induces a p53-dependent senescence (Chen *et al.,* 2005). Similarly, NF1 loss activates RAS and induces senescence, dependent on p53 (Courtois-Cox *et al.*, 2006). It is also important to mention, that not all cell lines are sensitive for oncogene-induced senescence. According to Collado *et al*., senescent cells exist in premalignant tumors but not in malignant ones. This observation may be explained by the fact, that malignant cancer cells have developed certain ways, how to overcome senescence, presumably by the loss of p53 or p16^INK4A/ARF (Collado *et al.*, 2005).

The appearance of senescent cells differs from that of normal cells. Senescent cells exhibit a distinct senescence-associated secretory phenotype (SASP). These cells undergo alterations at both macromolecular and micromolecular level. One of them is the variation in morphology of the cell - it increases its volume and alternates its shape (Hayflick, Moorhead, 1961). Senescent cells also modify their gene expression, most importantly by activating the transcription factor family NF-κB, resulting in the secretion of pro-inflammatory cytokines (IL-6, IL-8, TNF-α, CCL2), growth factors (VEGF, EGF), and proteases, such as MMP (Matrix metalloproteinase), which can promote epithelial-mesenchymal transition (Coppé *et al.*, 2006; Chien *et al.*, 2011). This NF-κB-dependent secretion enhances the invasivity of surrounding tumor cells. Cytokines, such as IL-6 and IL-8 are known to induce inflammation in the site of secretion, which can also improve cancer cell proliferation, promote epithelial-mesenchymal transition, or inactivate NK cells via the STAT3 pathway (Wu *et al.*, 2019). Therefore, active NF-κB gene expression may result in a less favorable patient's outcome.

The status of p53 plays a crucial role in determining whether the SASP promotes or suppresses cancer cell proliferation. The loss of p53 has been evaluated to enhance the pro-inflammatory environment, accelerating cancer cell growth. Notably, p53 loss is a common occurrence in cancer cell lines (Coppé *et al.*, 2008). As all these findings suggest, SASP may have a dual effect on the elimination of cancer cells (Krtolica *et al.*, 2001).

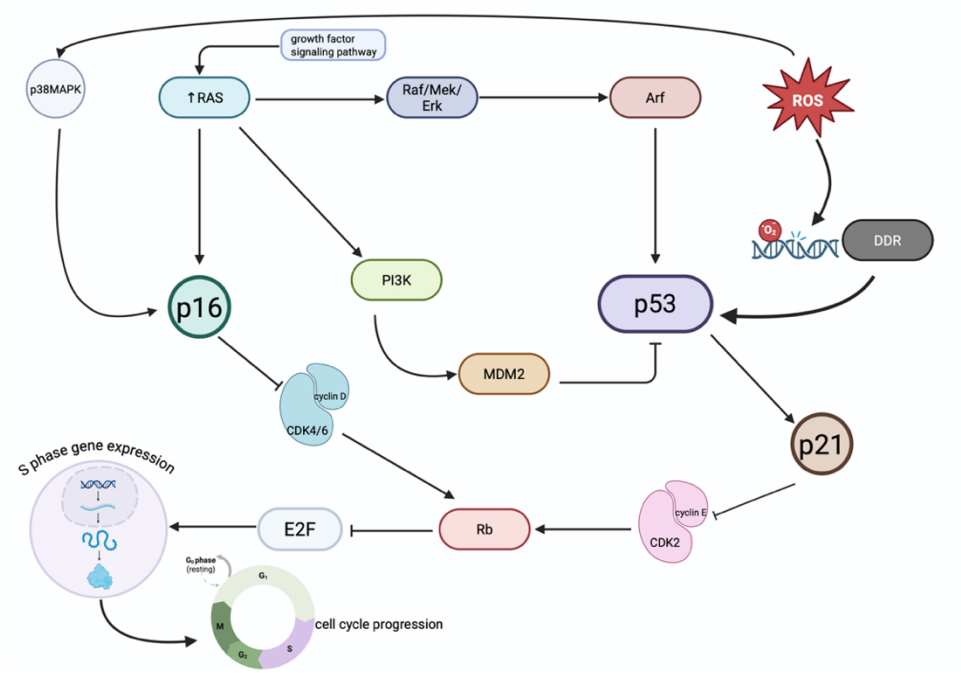


Figure 4. Pathways inducing senescence. ROS induce senescence through DNA damage response pathway, resulting in activation of p53 and its downstream effector p21, which inhibits CDK2-cyclin E, preventing the phosphorylation of Rb in late G1 and therefore also the E2F-dependent gene expression. Simultaneously, ROS activates p38MAPK (p38 mitogen-activated protein kinase), leading to the activation of p16, an inhibitor of CDK4/6 - cyclin E, promoting stronger G1 arrest. An excessive mitogenic signaling, often seen in some types of cancer hyperactivates Ras and this promotes oncogene-induced senescence via the Raf/Mek/Erk pathway activation. Erk then enhances the expression of Arf, an inhibitor of MDM2. Another MDM2 inhibitor regulated by Ras is Pi3K. Ras dependent-activation of p16^INK4A also contributed to senescence, as p16 serves as an inhibitor of CDK4/6-cyclin D complexes. Created in https://BioRender.com

4.2. CDK4/6 Inhibitors as Senescence and PASP Inducing Agents

After long-term inhibition by palbociclib in melanoma cancer cells, phosphorylation of mTOR decreases, which further leads to its inactivation and loss of its expression. Inhibition of the mTOR1 complex plays a crucial role in senescence induction. Inactivation of mTOR leads to decreased phosphorylation of mTOR downstream targets, including S6K, 4EBP1 and, in some cases, Akt and GSK3b. This disruption contributes to growth suppression and reinforces senescence (Yoshida *et al.*, 2016). In summary, CDK4/6 inhibitors are responsible for downregulation of mTOR, as also confirmed by others (Romero-Pozuelo *et al.,* 2020). However, resistance to CDK4/6 inhibitors can emerge, directed by the reactivation of mTOR through the induction of Raptor in some cancer cells, leading to uncontrolled proliferation. Therefore, the combinational therapy that targets both CDK4/6 as well as mTOR may prevent cancer cell acquired resistance to the treatment (Yoshida *et al.*, 2016).

Another target of CDK4/6 inhibitors that promotes senescence is ATRX, a chromatin-remodeling enzyme that takes part in genomic stability and chromatin organization. CDK4/6 inhibition induces a G1 arrest and leads to ATRX

overexpression. ATRX has been shown to downregulate MDM2 turnover by remodeling its chromatin. Under normal conditions, ATRX is downregulated, resulting in increased MDM2 levels, which in turn promote the destabilization and degradation of p53 and cell cycle progression, as well as senescence prevention. In quiescent cells, CDK4/6 inhibition and the subsequent upregulation of ATRX is required for the p53-dependent senescence induction. Furthermore, according to this study, ATRX is also required for the formation and maintenance of SAHF, further supporting senescence (Kovatcheva *et al.*, 2015, 2017).

Additionally, blocking the direct target of CDK4/6, FOXM1, is another way by which CDK4/6 inhibitors possibly induce senescence. As discussed above, FOXM1 is a transcription factor that increases the expression of many genes involved in the G1/S transition and inhibition of cell cycle arrest. By inhibiting CDK4/6, FOXM1 remains in an inactive state and senescence can occur (Anders *et al.* 2011).

Some studies have reported conflicting findings regarding the extent of the SASP phenotype, depending on the specific senescence-inducing agents or cellular states. Wang *et al.* showed that the CDK4/6 inhibitor driven senescence is p53 dependent and doesn't result in the production of NF-κB pro-inflammatory agents, which does however not apply to the chemotherapy or radiation therapy-induced senescence (Wang *et al.*, 2020, 2022). NF-κB family are transcription factors, that drive the expression of genes involved the induction and maintenance of SASP (Chien *et al.*, 2011). This downregulation of NF-κB during the treatment with CDK4/6 inhibitors may be advantageous for tumor clearance, as the pro-inflammatory phenotype intensified by chemotherapy and radiation therapy can otherwise promote tumor proliferation and invasion in the patient's tissue. Wang *et al.* decided to call this p53-dependent secretory phenotype PASP (p53-associated secretory phenotype), as further highlighted in Figure 5. (Wang *et al.*, 2020). A study from Lee *et al.* further supported this statement by showing that cells, whose senescent phenotype got induced by CDK4/6 inhibitors reported lower expression of SASP pro-inflammatory molecules (such as CXCL-1, CXCL-8, IL-6, TGF-β), in comparison to the SASP of those cells, whose senescent phenotype got induced by DNA damaging agents (Lee *et al.*, 2024). Concurrently, senescence induction by p16^INK4A expression has been found to result in practically none SASP expression, and this led the scientists to the conclusion, that not every senescence is capable of inducing inflammation (Coppé *et al.*, 2011). However, according to Guan *et al.*, prolonged CDK4/6 inhibitor treatment of fibroblasts induces senescence and a strong SASP phenotype in a DNA damage-independent manner, promoting tumor growth by suppressing antitumor immune responses and recruiting pro-tumorigenic immune cells, which contradicts the statements of Lee and Wang *et al.* (Guan *et al.*, 2017). The variation between studies suggests, that the level of SASP activation depends on the cell type, activity of p53, the senescence inducer and whether DNA damage occurs, or is not present.

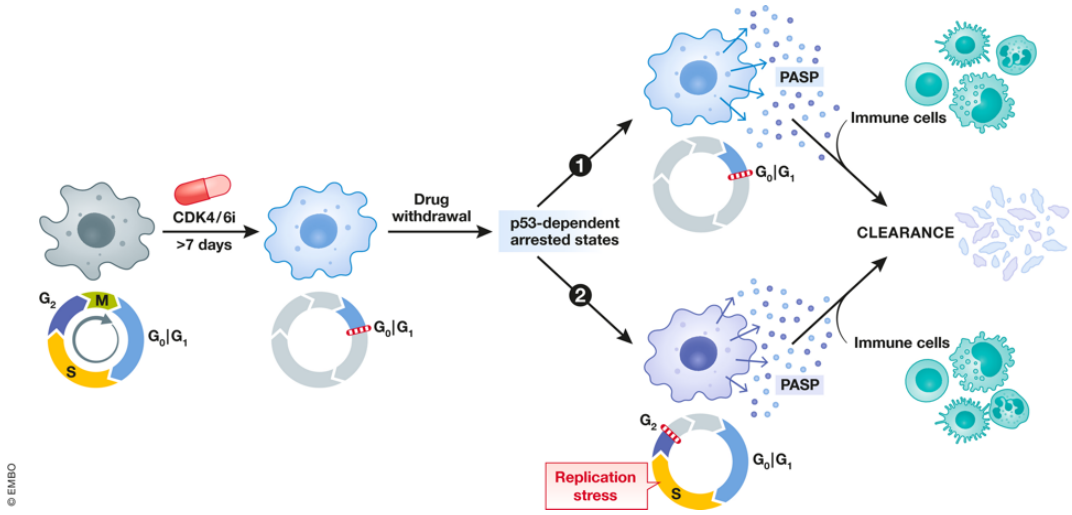


Figure 5. The cell fate upon CDK4/6 inhibition in the context of secretory phenotype. Long-term CDK4/6 inhibitor treatment leads to p53-dependent senescence (G0/G1) and replisome downregulation, profiling itself as senescence. Some cells are capable of S phase re-entry, but are unable to finish the cycle, as the defects due to replication stress and unlicensed replication origins are too severe. These cells end up in a subsequent G2 arrest. Both of these types of CDK4/6 inhibited cells express PASP, a p53-dependent secretory phenotype like SASP, but unlikely to SASP, PASP is more efficient in the signalization and promotion of the tumor cell clearance by the immune system. Adapted from Barr, McClelland, 2022, based on the findings of Crozier *et al.*, 2022; Wang *et al.*, 2020.

5. CDK4/6 inhibitor Interference in Metabolic Pathways

Cyclin dependent kinases also play an important role in the regulation of the metabolic pathways. For instance, upon normal conditions, the CDK4/cyclin D3 complex promotes cell cycle progression by upregulating anaerobic glycolysis and simultaneously downregulating fatty acid oxidation via AMPKα2 phosphorylation (Lopez-Mejia *et al.*, 2017). On the contrary, CDK6/cyclin D3 complex has been shown to cause the direct opposite by phosphorylating 6-phosphophructokinase (3. enzyme of the glycolysis) and pyruvate kinase M2 (10. enzyme of the glycolysis, leading to their inhibition. As a result, glycolytic intermediates are redirected towards the pentose phosphate pathway and the serine pathway. Both pathways produce glutathione and NADPH, essential molecules that function as antioxidants. These antioxidants can reduce reactive oxygen species (ROS), which are known to induce DNA damage, protein and lipid oxidation and can thus activate the DNA damage response pathway, by activating p53, which is likely to lead to either senescence, or apoptosis. Therefore, the upregulation mutations of CDK6 provides an advantage for cancer cells, trying to avoid as much DNA damage as possible during their rapid division and upregulated metabolism, generating elevated levels of ROS in comparison to normal cells, as shown in Figure 6. (Wang *et al.*, 2017).

CDK4/6 inhibition by palbociclib increases the rate of oxidative phosphorylation in mitochondria and the glucose and glutamine turnover, as well as Krebs cycle activity in pancreatic ductal adenocarcinoma. Upon CDK4/6

inhibition, cancer cell metabolism shifts towards oxidative phosphorylation and anaplerotic reactions (reactions, that replenish the intermediates of the Krebs cycle). This indicates, that even under CDK4/6 inhibitor treatment, cancer cells still maintain an increased metabolism. Furthermore, CDK4/6 inhibition leads to a higher activation state of the mTOR pathway (Franco *et al.*, 2016). This effect on cancer cells has been confirmed on melanomas, where CDK4/6 inhibitors not only increased glycolytic activity, but also enhanced fatty acid metabolism (Santiappillai *et al.*, 2021). One proposed mechanism underlying this metabolic shift is the accumulation of the MYC transcription factor in the nucleus following CDK4/6 inhibition. MYC promotes glycolysis by upregulating the expression of glycolytic genes and activates mTOR signaling by directly inhibiting TSC2, a negative regulator of mTOR (Ravitz *et al.*, 2007; Tarrado-Castellarnau *et al.*, 2017). However, Yoshida *et al.*, reported, that long-term CDK4/6 inhibition decreases the mTOR1 signaling activity through dephosphorylation, suggesting that while short-term CDK4/6 inhibition promotes MYC-driven metabolic activation and mTOR1 activation, long-term inhibition may lead to the suppression of mTOR1, potentially resulting in resistance, quiescence, or other survival pathways (Yoshida *et al.*, 2016).

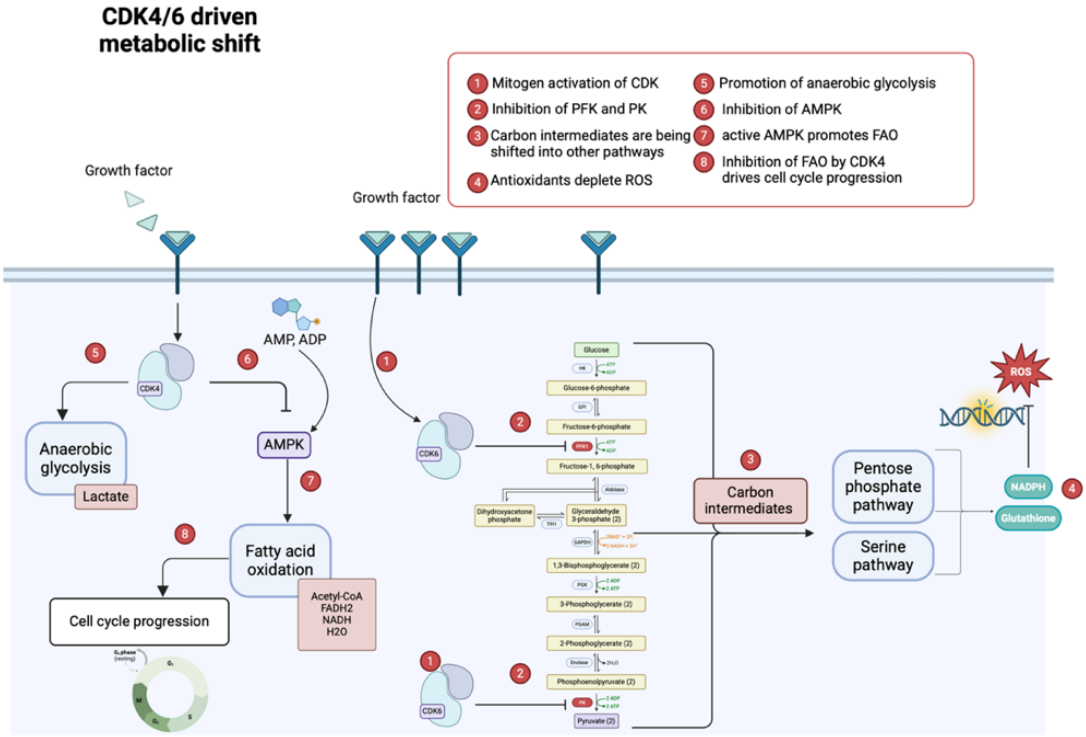


Figure 6. CDK4/6 - cyclin D driven variations in the metabolic pathway. CDK6 inhibits PFK and PK, redirecting carbon intermediates towards the PPP or Serine pathway. The elevated production of Glutathione and NADPH can serve as antioxidative protection against DNA damage inducing agents (right side of the picture). CDK4 promotes anaerobic glycolysis and simultaneously inhibits AMPK, resulting in downregulated FAO (left side of the picture). Created in https://BioRender.com

6. Growth Pathways and the Consequences of Cell Enlargement

6.1. The Role of mTOR and p38MAPK Growth Pathways

Cells tend to increase in size and volume during G1 arrest caused by CDK4/6 inhibition, or other agents blocking cell cycle progression. While this inhibition can suppress tumor growth, alternative growth-promoting pathways may remain active or become reactivated in response to the treatment. One such pathway is mTOR signaling pathway, which, depending on the cancer type, may be indirectly inhibited or remain largely unaffected (Demidenko, Blagosklonny, 2008). The mTOR pathway plays a role in lipid and nucleotide synthesis, rRNA expression, mitochondrial DNA amounts and the expression of genes required for oxidative phosphorylation (Mayer *et al.*, 2004; Cunningham *et al.*, 2007; Kantidakis *et al.*, 2010). mTOR exists in two distinct complexes, mTORC1 and mTORC2, each of which possesses unique regulatory roles in cell growth and metabolism. For example, mTOR1 regulates cell growth by controlling the activity of key downstream effectors, such as S6K1 and 4EBP1/eIF4E. These proteins play an essential role in translation and gene expression initiation, both of which are crucial for cellular expansion and proliferation (Fingar, *et al.*, 2002). In cancer, mTOR2 was shown to promote vascularization, a system that the cancer cells use to deliver nutrients, required for uncontrolled growth (Farhan *et al.*, 2006).

Another important protein in the regulation of cell size is the p38MAPK, which interfaces cell cycle progression with cell growth. The p38MAPK selectively blocks cell cycle progression in cells, that did yet not attain threshold size. Smaller cells show upregulated p38MAPK signaling, thereby promoting the duration of G1 phase (Liu *et al.*, 2018). Moreover, the research group of Tan *et al.*, observed, that p38MAPK and CDK4 work together, as shown in Figure 7. (Tan *et al.*, 2021). p38MAPK acts as a sensor of small cells and withholds them in G1 phase, whereas CDK4 activity sets the threshold size required for the cell cycle progression. This was demonstrated on the fact, that CDK4 inhibition by palbociclib resulted in unrestricted cell growth. Consistently with previous statements, since CDK4/6 - cyclin D complex targets some proteins of the metabolic pathway, it may also contribute to the regulation of the cell size. CDK4/6 inhibition-mediated cell cycle arrest can lead to cellular overgrowth, not only due to metabolic and transcriptional reprogramming, but also because it interferes with the CDK4 (and maybe CDK6) mediated cell size threshold setpoint (Tan *et al.*, 2021).

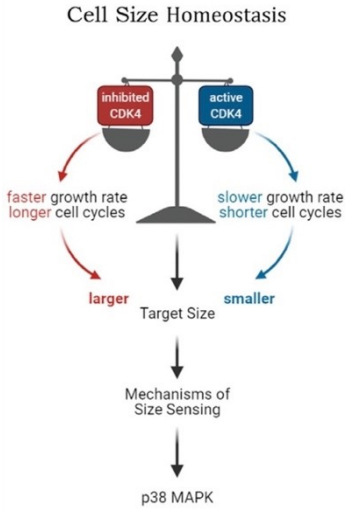


Figure 7. Cell size is controlled by p38MAPK levels, whereas the threshold size setpoint is being determined by CDK4. If CDK4 is active, cells grow slower and have shorter cell cycles. This accelerated division does not give them time to grow and as a result, they are smaller. CDK4 raises the threshold and allows smaller cells to divide. However, if they do not have the required target size determined by CDK4, p38MAPK gets activated and withholds these cells in G1 arrest, until they reach required size. On the other hand, reduced activity of CDK4 results in faster growth rate and longer cell cycle, which makes the cells to grow larger in size. This also means, that CDK4 is somehow involved in the modulation in p38MAPK sensitivity to cell size. Adapted from Tan *et al.*, 2021.

6.2. Mechanisms of Cell Growth During CDK4/6 Inhibition

In the last few years, it has been shown, that cell overgrowth may not be a consequence of senescence, but rather a cause of it. CDK4/6 inhibitor-dependent senescence is closely linked to cell overgrowth during G0/G1 arrest (Korothkina *et al.*, 2010). During prolonged G1 arrest induced by CDK4/6 inhibition, cells exhibit continued growth, a process that is both PI3K/mTOR and oncogene dependent (Foy *et al*., 2023). mTOR plays a dual role in this context; it promotes p21 accumulation, further supporting the G1 arrest, while simultaneously driving cell growth and metabolic processes. This abnormal cell growth has got a significant consequence on the fate of the cell. These findings suggest that while the inhibition of CDK4/6 can initially halt cell cycle progression, the subsequent cellular adaptations, particularly those involving mTOR signaling, can lead to cytoplasmatic dilution, senescence induction, or mitotic catastrophe. The status of mTOR is therefore in a large extend determining the fate of the cell (Foy *et al*., 2023).

In summary, CDK4/6 inhibition does not only result in direct cell cycle arrest, but also indirectly triggers complex cellular mechanisms, influencing cell size, metabolism, and genomic stability. Further research will be required to evaluate both the potential and disadvantages of these drugs.

6.3. Potential Causal Link Between Cell Enlargement and Senescence

6.3.1. p38MAPK Role in the Response to Cell Enlargement

Cell overgrowth, rather than the duration of CDK4/6 inhibition, promotes the acquisition of a permanent growth arrest. As a result of impaired G1 arrest and cell growth, an accumulation of protein and RNA levels in the cell occurs, which increases the osmotic pressure inside of the cell. By attempting to balance the osmolarity differences between the internal and external environment, the cell opens channels that allow water to flow in,

causing osmotic stress inside of the cell (Crozier *et al.,* 2023). This excessive growth causes osmotic stress, which activates the OSM scaffold protein, that forms a complex with Rac GTPase, MEKK3 and MKK3 kinases and as a complex, these proteins activate p38MAPK (Uhlik *et al.*, 2003). Active p38MAPK than acts on MAPKAP-K2, a kinase that further phosphorylates Cdc25B and Cdc25C. These two phosphatases subsequently bind to 14-3-3 protein. This interaction will cause the sequestration of Cdc25B and Cdc25C into cytoplasm and by this delocalization, Cdc25B/C will lose its function and will no longer dephosphorylate CDK1/cyclin B complex, which is essential for its activity. Therefore, the activation of p38MAPK will result in a G2 phase delay (Cueda *et al.,* 2007). Not only G2 phase delay, but also the delay in G1 phase is being caused by the activation of p38MAPK. According to Casanovas *et al.,* p38MAPK also downregulates the levels of cyclin D by its direct phosphorylation on Thr283, marking it for the destruction in the proteasome (Casanovas *et al.*, 2004).

Shortly after hyperosmotic stress occurs, the levels of the transcription factor NFAT5 (Nuclear factor of activated T cells) mRNA rise (Crozier *et al.,* 2023). The direct phosphorylation of NFAT5 by p38MAPK contributes to its dimerization and activation. NFAT5 is then further driving the expression of genes involved in osmotic regulation, such as *AR* (aldose reductase), *SORD* (sorbitol dehydrogenase), *PNPLA6* (phospholipase-neuropathy target esterase, *BGT1* (Na+/Cl-betaine/GABA transporter), *SMIT* (sodium/myo-inositol cotransporter), or *tauT* (taurine transporter) (Gómez *et al.*, 2000, Crozier *et al.,* 2023). Each of these gene products contains a regulatory subunit with an osmotic response element, that binds to its targets. Interestingly, NFAT5 has also been found to interact with RNA helicase, promoting its inactivation and therefore further supporting the role of p38MAPK in cell cycle delay (reviewed in Burg *et al.* 2008). Not only p38MAPK, but also other factors in the cell are responsible for the reversion of the osmotic pressure back to the physiological levels. For example, actin-myosin cortex rounds the shape of the cells, and the osmotic pressure has been found to act as a driver of this reorganization (Steward *et al.,* 2019).

Upon cell overgrowth driven by CDK4/6 inhibition, p38MAPK activates and stabilizes p53 by its direct phosphorylation on Ser33. P38MAPK also contributes to MDM2 degradation (Kishi *et al.*, 2001). Both conditions lead to the elevation of p21 levels. High p21 levels maintain in the cell even after drug washout. The size of the cell can thus determine the fate of the cell – bigger cells will have higher amounts of p21 and will thus be more sensitive to senescence induction. Some of these aberrantly large cells will indeed enter senescence. However, cells with medium or small size after CDK4/6 inhibition will be able to continue in the cell cycle upon its re-entry, because the amounts of p21 didn't reach the threshold. Additionally, p21 gets degraded after cells enter S phase, so it may seem, that cells that managed to continue in the cell cycle after CDK4/6 inhibitor drug washout, will continue to cycle just like before the inhibition. However, the opposite is true. Those smaller cells, that managed to re-enter the cell cycle after CDK4/6 inhibitor washout, are still too large for normal progression and accumulate replication stress and DNA damage due to unlicensed replication forks and the overall decrease in replisome components. This induces a second wave of p21 expression after S phase and leads to permanent cell cycle withdrawal. In summary, cell overgrowth is a secondary effect of CDK4/6 inhibition and not its direct consequence (Crozier *et al.*, 2023).

6.3.2. DNA Damage and Repair Defects in Enlarged Cells

A study by Manohar *et al.,* demonstrates that excessively enlarged cells upregulate p21 levels (Manohar *et al.*, 2023). After CDK4/6 inhibitor drug washout, some cells will remain in a senescent state. However, a larger fraction will be able to continue in the cell cycle. These cells will however accumulate DNA defects, which will result in a mitotic catastrophe. Manohar *et al.*, focused on the reason, why these cells acquire so many DNA replication defects and found conflicting results to Crozier *et al.* (Crozier *et al.*, 2023; Manohar *et al.*, 2023). According to them, the defects do not arise because of unlicensed replication forks, as Crozier *et al.*, claims, but because of dysfunctional DNA repair mechanisms. They found out, that enlarged cells fail to robustly stabilize p53 in response to damage and that 53BP1 (p53-binding protein 1) and RIF1 (Replication Timing Regulatory Factor 1), two proteins involved in DSB's repair, especially NHEJ (non-homologous end joining) fail to be recruited to DNA damage sites. These proteins are also recruited to stalled replication forks, and their deficiency may cause further DNA damage. Therefore, not only DNA stability, but also DNA damage repair pathways are intact in aberrantly large cells, causing mitotic catastrophes. Large cells are less capable of managing stress effectively, probably also due to the dilution of DNA damage signaling response dilution as highlighted in Figure 8. Another crucial finding from Manohar *et al.* is, that the cell cycle progression following the release from the cell cycle arrest can be rescued, if the growth of the cell gets reduced, or if p53 fails to upregulate p21 levels (Manohar *et al.*, 2023). This may also overcome the senescence induction. Although this finding does not have a significant impact in terms of therapeutic effect, it further supports the discovery of Manohar *et al.*, as well as other research groups (Manohar *et al.*, 2023).

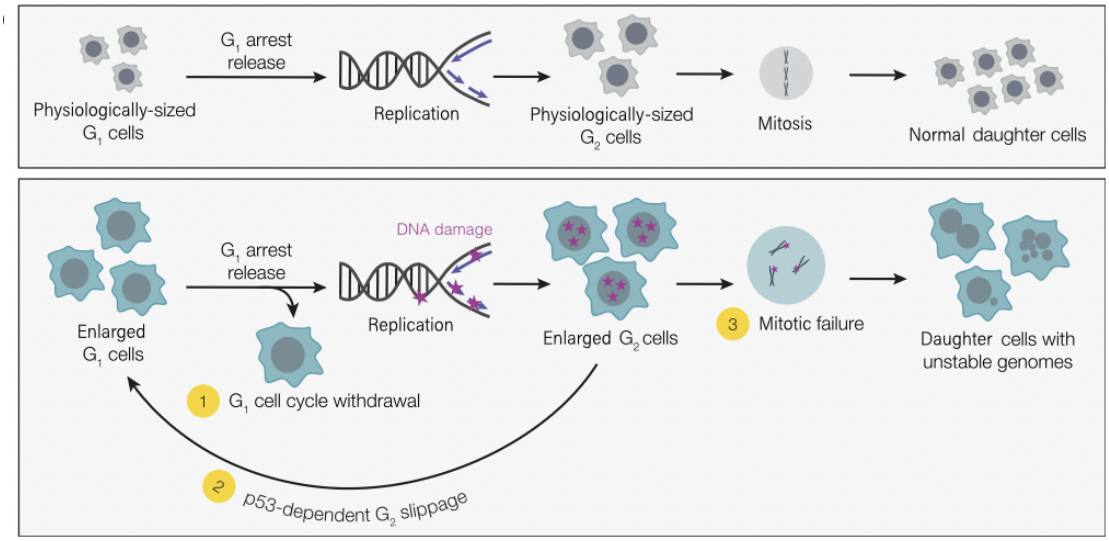


Figure 8. The behavior of normal, or too large cells after the release from G1 arrest. Normal cells (in the upper half of the picture) undergo flawless DNA replication and can continue throughout the cell cycle and complete mitosis. On the other hand, cells that are enlarged after G1 arrest release, undergo a defective DNA replication due to various factors, which forces these cells to either activate the p53 stress response pathway and return to G1 arrest, or to continue in the cycle and undergo catastrophic mitosis. Adapted from a review Manohar *et al.*, 2024.

6.3.3. The Relationship Between Cell Size and Ribosome Biogenesis

Other proposed mechanism underlying the connection between cell size and senescence induction is the stress response, which is caused by unbalanced DNA:cytoplasm ratio in the favors of cytoplasm. This ratio seems to be very important. It determines the range of cell size, in which the protein and RNA synthesis can occur. If cells are too large, the amount of DNA is not satisfactory enough to cover the needs of enlarged cell. And similarly, if rDNA amounts are too insufficient, the amounts of rRNA will also be insufficient and unassembled ribosomal proteins are going to accumulate in the cytoplasm. Therefore, transcriptional and translational activity will be lacking, causing cytoplasmatic dilution. One possible explanation for cell cycle delay, or even senescence induction may be the impairment of cell cycle regulators, such as CLN2 (cyclin D and E), or CLB2 (cyclin A and B), analogues of mammalian cyclins, occurring in yeasts. Presumably, the amount of these factors may be limiting for an aberrantly large cell, causing delays (Neurohr *et al.*, 2019). This observation can also be supported by the fact, that cells, that are physiologically large in organisms, such as hepatocytes, are polyploid and their nucleus size increases proportionally to the size of the cytoplasm (Wu *et al*., 2022). However, it is yet not known how other cell types, such as neurons, resolve these volume imbalances.

6.3.4. Cell Size Impairs the Cell Viability through Intracellular Protein Content Instability

The amounts of intracellular components have been found to scale differently depending on the cell size. Therefore, based on knowledge gained from others who claim that cells have a certain threshold size, excessive large cells will very likely contain the amounts of intracellular proteins, that are far from the optimum, required for normal cell function and cell cycle progression. Lanz *et al.,* observed, that the changes in protein concentration are directly proportional to the cell size and not to the duration of G1 phase and that both transcriptional and post-transcriptional mechanisms play a role (Lanz *et al.*, 2022). The amounts of individual proteins may vary which will affect their function. For example, the decrease in the amounts of proteins important for the cell cycle progression may slow down the process. Interestingly, in larger cells, stable proteins are more likely to accumulate. While protein synthesis is influenced by cell size, with larger cells having a greater capacity for biosynthesis, protein degradation mechanisms remain relatively constant and are not scaled to match the increased size. As a result, the imbalance between increased protein production and unchanged degradation leads to the accumulation of stable proteins in larger cells (Lanz *et al.*, 2022). The effect of such accumulation has yet not been accurately described, however, but there is a strong correlation with the discoveries of Crozier *et al.* (Crozier *et al.*, 2023).

However, regarding the activity of CDK4/6, a study by Miettinen *et al*., shows, that palbociclib treatment increases proteasome activity independently of the ubiquitin pathway in breast cancer cell lines, such as MCF7. Palbociclib activates 20S proteasome indirectly by thermal stabilization and simultaneously prevents the association of proteasome with ECM29, a scaffold protein inhibiting proteasomal activity. This disruption leads to enhanced proteasomal activity and further contributes to senescence. Moreover, lower levels of ECM29 mRNA

have been shown to result in a better survival rate of breast cancer patients (Miettinen *et al.*, 2018). Additionally, the proteasome machinery is a downstream target of the mTOR pathway, therefore there is a slight possibility, that the mTOR pathway may be affected directly, as shown by Franco *et al.*, as well as indirectly by the CDK4/6 inhibition (Franco *et al.*, 2016; Miettinen *et al.*, 2018). Therefore, it seems unlikely that the senescence observed following CDK4/6 inhibition is primarily driven by this type of mechanism.

According to Lengefeld *et al.,* who investigated hematopoietic stem cells (HSC's), maintaining a small cell size is essential for optimal cellular function (Lengefeld *et al.*, 2021). Treatment with palbociclib results in cell cycle arrest in G1 phase, during which cells continue to grow in size. This enlargement correlates with an increased accumulation of DNA damage, that was also observed by others. However, palbociclib treatment itself does not directly induce such damage. Presumably, this DNA damage could have occurred in these cells due to defects, that accumulated during their previous divisions, however, the exact reason remains unknown. The study also proposes that cells may have an upper size limit, beyond which the DNA:cytoplasm ratio becomes imbalanced. This imbalance could impair biosynthetic homeostasis and lead to the accumulation of both stable and unstable proteins (Lengefeld *et al.*, 2021). I propose that, in certain contexts, such unstable proteins may misfold and aggregate, potentially forming oligomers or fibrils similar to those observed in neurodegenerative diseases such as Alzheimer's. Therefore, the size of the cells may not only have a direct impact on the cell itself, but also on the viability of the whole organism. This notion is further supported by the observation that enlarged cells with active p38MAPK signaling exhibit increased expression of SASP. This upregulation is driven by enhanced NF-κB transcriptional activity downstream of p38 MAPK activation (Freund *et al.*, 2011). As previously discussed, NF-κB promotes the expression of pro-inflammatory cytokines, which can have direct effects on neighboring cells, potentially altering their behavior and contributing to a pro-inflammatory tissue environment.

6.3.5. Mitochondrial Dysfunction

Further evidence also links the function of mitochondria to the size of the cell, and subsequently, to the senescence induction. The organelle content scales proportionally to the cell size, bigger cells will therefore have higher amounts of mitochondria. However, larger volume does not always mean greater functionality. Scientists showed that mitochondria in enlarged cells have a less effective membrane potential and display a decrease in OXPHOS (oxidative phosphorylation) metabolism. This reduces cellular viability, as the ratio of NAD+/NADH, as well as the amounts of ATP are imbalanced. Additionally, the levels of ROS are elevated in bigger cells. As already mentioned in Chapter 4.1., ROS do elevate p53 levels, as well as the levels of its downstream target p21, which contributes to senescence induction. However, it is highly improbable, that just ROS are capable of the senescence induction. Most likely, other additional factors would have to support the effect caused by ROS and other organelles will also have an impaired function due to cell enlargement (Miettinen, Björklund, 2016).

It should also be emphasized that the metabolic activity of mitochondria has been found to influence the growth rate of the cell and have an impact on the progression of the cell cycle, presumably via the CDK and E2F-dependent mechanism (Homem *et al.*, 2014). If this is the case, then cell overgrowth caused by CDK4/6 inhibition would likely be dependent on mitochondrial activity, as mitochondrial metabolic control appears to be crucial for regulating cell growth and progression through the cell cycle via CDK4/6 and E2F machinery. In other words, mitochondria will continue to provide the cell with energy and materials, even if the cell cycle is blocked.

7. Therapeutic Strategies and Challenges

7.1 CDK4/6 Inhibitors in Cancer Treatment, their Selectivity and Mechanism of Action

Early studies in genetically modified mice revealed that CDK4 activity loss significantly reduced tumor formation, particularly in models driven by oncogenes like Ras and HER2/neu. Importantly, cyclin D1-mutant mice remained viable, suggesting that CDK4/6-cyclin D inhibition could be selectively targeted in cancer without harming normal tissues (Landis *et al.*, 2006; Yu *et al.*, 2001, 2006). Based on this observation, small molecule inhibitors have been developed, to inhibit CDK4 as well as its close homolog CDK6. This strategy led to a new class of drugs that now play an important role in the treatment of HR+ breast cancer.

Nowadays, three CDK4/6 inhibitors of the third generation have been approved by the FDA (U.S. Food and Drug Administration), namely palbociclib (PD0332991), abemaciclib (LY2835219) and ribociclib (LEE011). Recently, new drugs have been synthetized, such as trilaciclib (G1T28) and lerociclib (G1T38), but they won't be discussed in this thesis. These drugs are currently used to treat HR+, HER2- breast cancer, but recent research has established their high potential for the treatment of other diseases. The importance of CDK4/6 inhibition lies in their ability to induce cell cycle arrest and to stop further cancer proliferation. However, the efficacy and survival rate differ in various studies and is still a subject of ongoing research (Goetz *et al.*, 2017; Turner *et al.*, 2018).

All these inhibitors interfere with the CDK4/6 kinase activity by binding to the ATP-binding groove. (Fry *et al.*, 2004). Palbociclib selectively binds to CDK4/6 – cyclin D1 complexes, abemaciclib inhibits both CDK4/6-cyclin1/2/3, ribociclib inhibits CDK4/cyclin D1 and CDK6/cyclin D3 complexes (Sumi *et al.*, 2015). Other targets have also been recorded, as highlighted in Figure 9. and 10. The IC50 for CDK4 and CDK6 differ depending on the drug. Palbociclib has IC50 value for CDK4 11nM, and for CDK6 16nM. The IC50 of ribociclib is 10nM for CDK4 and 39nM for CDK6. The IC50 value of abemaciclib is 2nM for CDK4 and 10nM for CDK6. Abemaciclib has been shown to have the highest affinity to its target enzymes, however, it is important to point out, that it has also the most potent activity against other proteins, which can affect its selectivity. According to these findings, ribociclib has the highest selectivity for CDK4/6, in contrast, abemaciclib is quite unselective (reviewed in Braal *et al.*, 2021). The primary purpose of CDK4/6 inhibitors is to disrupt the CDK/Rb/E2F pathway, thereby blocking the G1/S transition and establishing G1 arrest, or senescence in breast cancer cells of patients, as well as in other cancer cell lines, which is

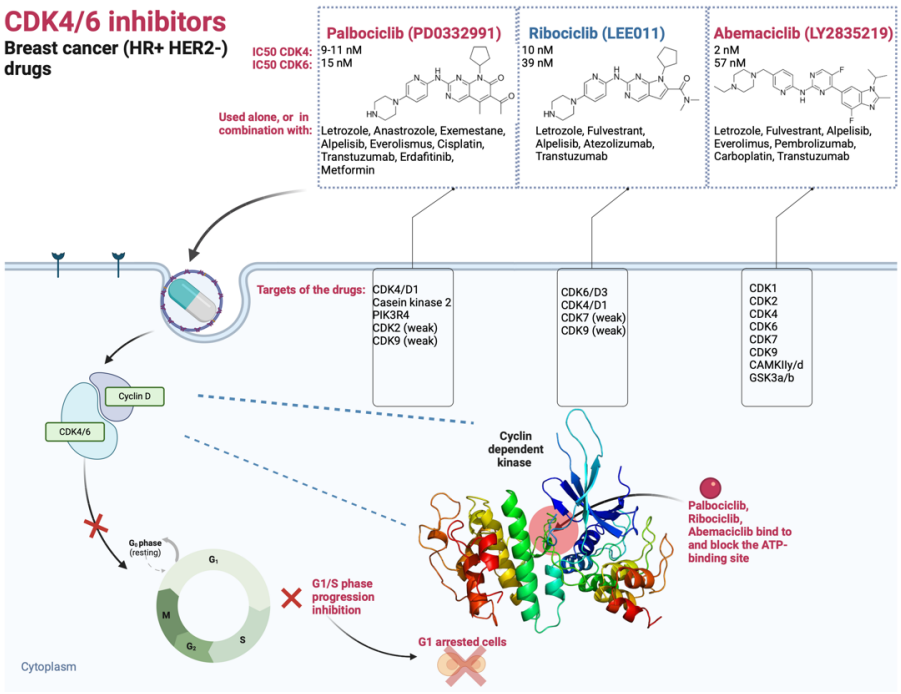
still a subject of further research. Interestingly, a study from Pennycook, Barr, found out, that p21 or p27 do not contribute to the induction of G1 arrest upon CDK4/6 inhibition. This means, that CDK4/6 inhibitors are sufficient to induce G1 arrest without requiring physiological CDK inhibitors, when administered at high doses (Pennycook, Barr, 2021).

Molecular pathways of kinases which are targeted by palbociclib, ribociclib and abemaciclib. The grading of affinity between CDKis and each target kinase is arbitrary expressed by -, no affinity; +, presence of affinity; ++, high affinity; +++, very high affinity.

Targeted Kinase	Pathophysiological Activities of Targeted Kinases	Affinity of Palbociclib	Affinity of Ribociclib	Affinity of Abemaciclib
CDK1	It is mainly involved in controlling the transition from G2 to M phase of cell-cycle	-	-	+
CDK2	It selectively orchestrates processes of phase S, binding Cyclin E, and not Cyclin D as for the other CDKs	-	-	+
CDK4	It inhibits members of the retinoblastoma (RB) protein family including RB1 and regulate the cell-cycle during G(1)/S transition [34]	++	+++	+++
CDK6	It inhibits members of the retinoblastoma (RB) protein family including RB1 and regulate the cell-cycle during G(1)/S transition [34]	++	++	+
CDK7	It regulates the initiation of transcription through phosphorylation of the heptad repeats that comprise the C-terminal tail of RNA polymerase II (CTD)	-	-	+
CDK9	It regulates the release from promoter proximal arrest of transcription through phosphorylation of the heptad repeats that comprise the C-terminal tail of RNA polymerase II (CTD)	-	-	++
GSK3 α/β	It promotes the synthesis of pro-inflammatory IL-6 and the expression of oncogenic genes	-	-	+
CAMKII α/β/γ	It is involved in apoptosis and autophagy in cancer cells	-	-	+
DYRK	It regulates some proteins controlling the cell cycle	-	-	+
PIM protein kinase	It is an oncogenic protein which is frequently amplified in cancer	-	-	+
HIPK	It promotes JAK/STAT signaling	-	-	+
CAMK families	They are enzymes overexpressed in several cancer types	-	-	+

CDK—Cyclin Dependent Kinase; GSK3 α/β—glycogen synthase kinase 3α/β; CAMKII α/β/γ—calmodulin-dependent protein kinase II α/β/γ; DYRK—dual-specificity tyrosine phosphorylation-regulated kinase; HIPK—homeodomain-interacting protein kinase; CAMK—Ca^{2+}/calmodulin-stimulated protein kinase; JAK—Janus Kinase; STAT—Signal Transducer and Activator of Transcription.

Figure 9. Binding capacity of Palbociclib, Ribocicib and Abemaciclib to other kinases and their affinity rates. Adapted from Roncato *et al.*, 2020



10. Structure, IC50 and targets of palbociclib, ribociclib and abemaciclib, and their mechanism of action. Created in https://BioRender.com

7.2. Mechanisms of Resistance to CDK4/6 Inhibition

Many changes of the gene expression, or alteration of other mechanisms have been reported to lead to CDK4/6 inhibitor acquired resistance in metastatic HR+ HER2- breast cancer.

Loss of an *RB1* gene or the amplification of the *CCNE1* and *CCNE2* gene are the main conditions leading to CDK4/6 inhibitors acquired resistance (Condorelli *et al*., 2018). This upregulation of cyclin E2 results in an excessive interaction with CDK2 upon CDK4/6 inhibition. CDK2/cyclin E1/2 enhanced activity can bypass the CDK4/6 inhibition and, as well as the loss of *RB1* gene, results in G1/S phase progression and resistance to CDK4/6 (Guarducci *et al.,* 2018). Screening cancer patients for *CCNE1/2* amplification and *RB1* gene status has been proposed as a strategy to improve personalized therapy with CDK4/6 inhibitors (Herrera-Abreu *et al.,* 2016). In addition to *RB1* loss, elevated p16^INK4A expression has also been identified as a potential biomarker of intrinsic resistance to CDK4/6 inhibition, further supporting the value of molecular profiling in therapeutic decision-making (Dean *et al.,* 2012; Palafox *et al.,* 2022). Interestingly, it has also been discovered, that the *CCNE1* gene amplification results in the downregulation of HLA-A, HLA-B, HLA-C genes, that code for MHC complexes. Thus, the MHC class I-dependent antigen presentation is lacking in those cells, and these cells can escape the immune system (Goel *et al.*, 2017).

GOF mutations have also been found to promote cancer cell resistance not only to CDK4/6 inhibitors, but also to endocrine therapy. Cyclin D (*CCND1*) overexpression has been observed in some breast tumors (Hanf *et al.,* 2025). The upregulation of the *CCND1* gene is a consequence of the amplification of the fibroblast growth factor receptor 1 (FGFR1), which is a regulator of signaling pathways promoting growth, proliferation, migration, or prolonged survival (Turner *et al.*, 2010). The amount of the *CCND1* gene is directly proportional to the patient's outcome (Formisano *et al.*, 2019). As the cyclin D upregulation remains PI3K dependent, there is an assumption, that the combinational therapy of CDK4/6 inhibitors together with PI3K/mTOR inhibitors could restore this resistance (Parrales *et al.*, 2011). Additionally, mTOR overexpression was observed in melanoma cancer cell lines with acquired resistance (Yoshida *et al.*, 2016). However, PI3K is frequently mutated in ER+ breast cancers and was found to be an ineffective marker for screening, therefore the choice whether to use this combinational treatment in personalized therapy will still require further research (Cancer Genome Atlas Network, 2012; Wander *et al.*, 2020). Recently it has also been shown that HR+ cancer cell lines, that overexpress *RET* proto-oncogene, acquire resistance to CDK4/6 inhibitors and fulvestrant (an estrogen receptor inhibitor) (Kindt *et al.*, 2025).

The amplification of CDK6 has also been observed in multiple cell lines resistant to CDK4/6 inhibitors. Interestingly, CDK6 was found to possess some unique functions in comparison to CDK4. It has been shown, that CDK6 can work in complex with other proteins as a transcription factor, and, independently of its kinase activity, binds to specific promoter regions of angiogenesis genes, such as VEGF-A (Vascular endothelial growth factor A), and enhance the expression of those genes (Kollmann *et al.*, 2013). As broadly known, angiogenesis is one of the key hallmarks of cancer, therefore the amplification or overexpression of a gene coding for CDK6 could thus potentially lead to a more invasive and aggressive form of cancer (Hanahan, Weinberg, 2000). Higher doses of

CDK4/6 inhibitors might be efficient enough to block the G1/S transition in this case, however, the amount of the dose has yet not been determined and the amplification of CDK4 hasn't been observed, thus target-specific CDK6 inhibitors could be more effective in this case. Combination with endocrine therapy seems to be unsuitable, as *CDK6* gene amplification also results in the resistance to fulvestrant (Alves *et al.*, 2016; Sledge *et al.*, 2020).

The overexpression of PDK1, an activator of Akt-driven survival and growth pathway has also been found to contribute to the resistance and cell cycle progression (Jansen *et al.*, 2017). This type of resistance could be reversed by PDK1 inhibitors (Garrido-Castro *et al.,* 2025). The mutations in *Ras*, *Akt* and *AURKA* were also found to strengthen the resistance to CDK4/6 inhibitors (Wander *et al.*, 2020). Another study revealed the role of *PTEN* loss in both primary and acquired resistance to ribociclib and fulvestrant. *PTEN* deficiency leads to hyperactivation of the PI3K/AKT pathway, resulting in AKT-mediated phosphorylation and cytoplasmic mislocalization of p27, which prevents it from inhibiting CDK2 in the nucleus. *PTEN* deficiency is however only effective, if a mutation in PI3K simultaneously occurs. Moreover, the inactivation of Rb, resulting in the G1/S phase progression is still required. This resistance was found to be reversed by a combinational treatment of CDK4/6 inhibitors and Akt inhibitors (Costa *et al.*, 2020).

According to other studies, the LOF mutation of *FAT1* tumor-suppressor gene is another potential mechanism resulting in acquired resistance to CDK4/6 inhibitors. Although it is not a very common mutation, it significantly enhances the resistance of its carriers, if biallelic. In normal conditions, FAT1 is thought to indirectly suppress the expression of CDK6 by activating the Hippo growth-restricting pathway. The Hippo pathway then inactivates two transcription factors, YAP and TAZ, responsible for the upregulation of *CDK6* gene expression. But after *FAT1* deleterious mutation, YAP and TAZ remain active and CDK6 can be overexpressed, and this causes resistance to CDK4/6 inhibitors (Li *et al.*, 2018). It is rather unlikely, that higher doses of CDK4/6 inhibitors would overcome this type of resistance, but the screening for *FAT1* mutation in breast cancer patients could lead to a more effective adjusted treatment.

Additionally, a small molecule CDKN2A (Cyclin-dependent kinase inhibitor 2A), which can inhibit CDK4/6 by its direct binding, have been found to promote the resistance to CDK4/6 inhibitors, if overexpressed in the tissue. The screening for CDKN2A could therefore also determine the patient's response to CDK4/6 inhibition (Green *et al.*, 2019).

Numerous other mutations have also been found to contribute to the acquired resistance to CDK4/6 inhibitors; however, due to space constraints, they will not be discussed in this thesis.

8. Therapeutic Implications

8.1. Therapeutic Implications of Inducing G1 Arrest

The synchronization of cancer cells in G1 arrest is one of the main potentials of CDK4/6 inhibition. These cells can then be then better targeted by other anti-cancer drugs, that specifically target certain cells in a particular cell cycle stage. Additionally, the enforced G1 arrest slows down the rate, at which cancer cell divide, giving specialists more time to come up with an appropriate treatment, which is necessary for personalized therapy. There have been some ongoing trials and research combining CDK4/6 inhibitors with PI3K/mTOR inhibitors, aromatase inhibitors, or IL6/STAT3 inhibitors (Finn *et al.,* 2015, 2016; Michaloglou *et al.,* 2018; Kettner, *et al*., 2019).

One of the major advantages of CDK4/6 inhibitors is their high selectivity for cancer cells, while exhibiting relatively low toxicity towards normal cells. This selectivity can be attributed to several factors. First, cancer cells exhibit an increased dependency on E2F transcription factors, as elevated levels of E2F-dependent proteins are required to support the rapid proliferation characteristic of malignant cells. To be able to proliferate and divide rapidly, they require excessive amounts of cyclin D, CDK4/6, CDK2, cyclin E, and other cell cycle promoting factors, in comparison with normal cells.

Secondly, oncogenes play a critical role in regulating cell cycle progression and cellular growth. Under normal conditions, oncogenes drive both proliferation and growth; however, when CDK4/6 inhibitors block cell cycle progression, their only remaining effect is growth stimulation. Hyperactive oncogenic signaling (such as Ras or Myc) can trigger oncogene-induced senescence via the activation of tumor-suppressors p53 and Rb through the upregulation of CDK inhibitors such as p21 and p16. Elevated levels of CDK inhibitors leads to cell cycle arrest. During this arrest, continued growth driven by oncogene activation causes cellular overgrowth and stress, reinforcing senescence. Tumors with dysfunctional Rb, low p16 levels, and excessive mitogenic signaling become highly dependent on CDK4/6 for cell cycle progression and are thus selectively vulnerable to CDK4/6 inhibitors. In contrast, tumors with high p16 or loss of Rb tend to be resistant. Lastly, cancer cells are much more prone to cell overgrowth upon CDK4/6 inhibition, because they have lost the ability of contact inhibition and have become independent of growth factors. Both of these conditions further support and increase the probability and rate of cell overgrowth (Foy *et al.*, 2023).

8.2. Therapeutic Implications of Inducing Senescence

It seems to be more favorable to induce senescence in cancer cells rather than causing a temporary G1 cell cycle arrest, as senescent cells exhibit a distinct and recognizable phenotype that sets them apart more clearly from non-transformed, healthy cells. In contrast to G1-arrested cells, which can appear like normal quiescent cells and

may eventually resume proliferation, senescent cells enter a state of irreversible growth arrest and exhibit distinct changes in shape, gene activity, and SASP (Yoshida *et al.*, 2016). Additionally, the presence of senescent cells at the site of tumor post-chemotherapy treatment has been linked to a favorable outcome, suggesting that some cancer cells may also undergo senescence after chemotherapy. In case of an activated oncogene, cells may undergo senescence to reverse these oncogenic effects and prevent cancer genesis (Haugstetter *et al.,* 2010). However, the cell fate after CDK4/6 inhibition is unfortunately variable, depending on many yet not fully understood factors. It will be a subject of further research to determine, which conditions inside of the cell must be fulfilled, to induce senescence rather than G1 arrest.

A considerable advantage of CDK4/6 inhibition inducing senescence is the modulation of immune system response, resulting in a more favorable outcome for breast cancer patients. A relatively recent study from Goel *et al.*, found out, that abemaciclib treated cancer cells tend to upregulate their expression of MHCI molecules, as well as peptide cleavage proteins (Erap1), TAP-associated glycoprotein (Tapbp) and peptide transporter proteins (Tap1 and Tap2), all required for correct MHCI presentation, thus recruiting more CD8+ cytotoxic T cells to the site of the tumor. Additionally, CDK4/6 inhibitors reduce the numbers of Treg cells in the site of tumor, resulting in a more pro-inflammatory environment (Goel, *et al.*, 2017). This might be explained by the fact, that Treg cells express higher levels of Rb protein, in comparison to CD8+ cytotoxic T cells, and are thus more sensitive to CDK4/6 inhibition (Heng *et al.*, 2008).

Since it remains uncertain whether the secretory phenotype of CDK4/6-inhibited senescent cells will be p53-dependent (PASP), which could contribute to tumor clearance, or p53-independent (SASP), which may have the opposite effect due to its pro-inflammatory phenotype, it is desirable to eliminate senescent cells following CDK4/6 inhibition. Several studies have proposed that the SASP supports tumor recurrence, as already discussed above (Lee *et al.*, 1999). A promising and novel treatment over the last years have been the senolytics. Combinational therapy of CDK4/6 inhibitors together with senolytic agents provides big potential and is a promising strategy in cancer treatment. Senolytics are synthetic small molecule inhibitors (but also naturally occurring molecules), that selectively target and clear senescent cells. Senescent tumor cells, particularly those exhibiting a strong SASP, undergo metabolic reprogramming, which increases their sensitivity to apoptosis induction driven by metabolic stress. This is consistent with the fact, that CDK4/6 inhibitor-induced senescence may enhance the efficacy of subsequent senolytic treatment (Dörr *et al.*, 2013; Wang *et al.*, 2017). However, there are still many hindrances, such as the right timing and dosage, or the fact, that the senolytic drugs also eliminate senescent cells beneficial for the clearance of tumor cells, such as PASP cells. However, there are not many studies examining the effect of CDK4/6 inhibition together with senolytic drugs on the cancer viability, as senolytics were primarily not meant for cancer treatment, but rather other age-related diseases (Kennedy *et al.*, 2014). Therefore, further research will be necessary, to fully understand the beneficial role of senolytics in cancer therapy.

9. Future Directions of CDK4/6 Inhibition

In the upcoming years, it will be important to further examine the downstream targets of CDK4/6, as well as the ones of CDK4/6 inhibitors, to determine the tumor types sensitive to the treatment. Other tumor types, such as non-small cell lung cancer (NSCLC), glioblastomas, colorectal cancer, melanomas, multiple myelomas, leukemias, or ovarian cancer, have also been found to potentially benefit from CDK4/6 inhibitor therapy, as these cancer types also depend on CDK4/6 (Patnaik *et al.,* 2016). Another important goal is going to be the determination of the right amount and dosage schedule of these drugs. Personalized therapy has become very popular and sought in recent years, thus the screening of patient's cancer cells, or cDNA, might help with the right amount and duration of CDK4/6 inhibitor-driven inhibition. This could enhance the efficacy of the treatment. Moreover, clinical trials will need to be performed on newly developed CDK4/6 drugs, such as trilaciclib (G1T28) and lerociclib (G1T38). It will also be important to find a way to bypass acquired resistance to CDK4/6 inhibitors, which is a common problem not only with CDK4/6 inhibitors, but also other anti-cancer drugs. CDK4/6 acquired resistance could be prevented by CDK2 inhibition, because CDK2 is another cyclin-dependent kinase, that is often upregulated in cancer cells with acquired resistance to CDK4/6 inhibition, but also in various ways, that are nowadays being examined.

Regarding research, a more comprehensive understanding of the impact of CDK4/6 inhibition on various growth-promoting pathways would provide valuable insights into its biological effects, particularly in the context of cell growth and senescence induction. Investigating the activity status of key signaling pathways, such as Wnt, PI3K/AKT, MAPK/ERK, Notch, and others, is crucial to determine, how these pathways respond to CDK4/6 inhibition. Specifically, identifying which pathways remain unaffected, which are upregulated, and which are downregulated, will help to clarify the mechanisms by which CDK4/6 inhibitors influence the fate of the cell.

Understanding the proteomic imbalances that arise during cell overgrowth following CDK4/6 inhibition will also provide valuable insights. Enlarged cells experience changes in the concentrations of intracellular stable and unstable proteins, which may impair cell functions required for its viability, such as DNA replication, DNA repair pathways and the ability to reenter the cell cycle (Neurohr *et al.*, 2019; Crozier *et al.*, 2022). Identifying specific proteins that are most sensitive to these imbalances in size could reveal the exact mechanism behind senescence induction. Additionally, it will be important to explore cell type-specific responses to CDK4/6 inhibition, as different tumor types or normal tissues may vary in their sensitivity to overgrowth and senescence. Additionally, although it has been proposed that cells in G1 phase have impaired activation of the NHEJ DNA repair pathway, it is still unknown whether this issue persists in a cell that continues the cell cycle, and whether this contributes to the inability of these enlarged cells to fix the damage caused by replication stress (Manohar *et al.*, 2023). Finally, the role of metabolic reprogramming during CDK4/6 inhibitor-induced arrest remains largely unexplored. Changes in glycolysis, oxidative phosphorylation, and nutrient sensing pathways may influence cell size regulation and senescence induction.

10. Conclusion

Cancer is these days the second most common cause of death, which points out the necessity to examine it deeper and develop anticancer drugs. Breast cancer remains the most abundant type of cancer among women, and the development of CDK4/6 inhibitors offers a new significant potential and revolutionizes breast cancer therapy. Several single or combinational treatments with CDK4/6 inhibitors are nowadays undergoing clinical trials and seem very promising.

CDK4/6 inhibitors affect not only cell cycle progression, but also cell growth, metabolism, secretory phenotype, gene expression, chromatin remodeling and other factors contributing to either cancer proliferation, or regression. Most certainly, not all of them have been discovered yet.

Cell enlargement is not only a consequence of senescence but can act as a driver of it. Persistent growth during prolonged G1 arrest—such as that induced by CDK4/6 inhibitors—can result in excessive cell size, particularly when mTOR signaling remains active. This abnormal growth leads to cytoplasmic dilution, proteome changes, and impaired organelle function. All these conditions can activate stress-response pathways such as p53/p21, further reinforcing growth arrest and promoting a senescent phenotype.

Furthermore, large cells exhibit replication stress, the accumulation of DNA damage, and impaired repair mechanisms (e.g., defective NHEJ signaling), which worsens genomic stability. Stable proteins tend to accumulate due to reduced degradation efficiency, while unstable proteins become diluted. These effects further compromise cell viability and functionality and can also lead to senescence.

Overall, the interplay between cell size, metabolic and gene expression activity, and signaling pathways such as mTOR, p38MAPK, and NF-κB define the cellular homeostasis status. These insights show that cell size can act as a regulatory feature determining cellular fate.

In conclusion, CDK4/6 inhibitors represent a powerful new strategy in cancer therapy, not only by halting cell division but also by inducing long-term cellular senescence via cell growth and cell cycle impairment that can influence treatment outcomes. A better understanding of the interplay between cell cycle arrest, senescence, and resistance will be essential for optimizing their use and improving therapeutic strategies in oncology.

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