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Beyond cell cycle regulation: The pleiotropic function of CDK4 in cancer

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SUMMARY

CDK4, along with its regulatory subunit, cyclin D, drives the transition from G1 to S phase, during which DNA replication and metabolic activation occur. In this canonical pathway, CDK4 is essentially a transcriptional regulator that acts through phosphorylation of retinoblastoma protein (RB) and subsequent activation of the transcription factor E2F, ultimately triggering the expression of genes involved in DNA synthesis and cell cycle progression to S phase. In this review, we focus on the newly reported functions of CDK4, which go beyond direct regulation of the cell cycle. In particular, we describe the extranuclear roles of CDK4, including its roles in the regulation of metabolism, cell fate, cell dynamics and the tumor microenvironment. We describe direct phosphorylation targets of CDK4 and decipher how CDK4 influences these physiological processes in the context of cancer.

1. Introduction

Cyclin-dependent kinase 4 (CDK4) belongs to the cyclin-dependent kinase family. This catalytic subunit binds the regulatory subunit cyclin D, forming the active CDK4-cyclin D holoenzyme. Canonically, this complex plays a crucial role in regulating the cell cycle [11]. The CDK4/6-cyclinD complex accumulates in the nucleus, where it phosphorylates nuclear proteins, including retinoblastoma protein (RB), p107, and p130 [13,50]. CDK4 promotes the transition from G1 to S phase of the cell cycle by RB phosphorylation, which allows release of the transcription factor E2F and subsequent E2F target gene transcription.

As it canonically drives cell cycle progression, CDK4 is largely deregulated in many cancers, and its contribution to tumorigenesis has been depicted in two recent reviews [36,43]. Altogether, the literature has increasingly drawn the attention of clinicians to CDK4/6 inhibitors (CDK4/6is) as valuable antitumoral therapeutic tools [68]. Among such inhibitors, palbolciclib, ribociclib and abemaciclib, which target the ATP-binding domains of CDK4/6, are the most thoroughly tested CDK4/6is in active and recruiting clinical trials accounting for over 50 tumor types, including triple-negative breast, colon, liver, glioblastoma, uterine and ovarian cancers [36]. These three inhibitors have been approved for HR+ , HER2- advanced breast cancers [46,66]. Therefore, an increasing number of studies have deciphered the effects of inhibitors of both CDK4/6, making it difficult to address the specific function of CDK4 or CDK6. Notably, CDK4 and CDK6 have redundant functions, as shown by their roles in not only canonical RB phosphorylation but also FOXM1 phosphorylation [6]. In this review, we address the role of CDK4

Abbreviations: ACC, Acetyl-CoA Carboxylase; ACLY, ATP Citrate Lyase; AMPK, AMP-Activated Protein Kinase; CDK, Cyclin-Dependent Kinase; CDK4/6i, Cyclin-Dependent Kinases 4/6 Inhibitors; EGFR, Epidermal Growth Factor Receptor; ELOVL, Elongation Of Very Long Chain Fatty Acids Protein; EMT, Epithelial-Mesenchymal Transition; FA, Fatty Acids; FASN, Fatty Acid Synthase; FBP1, Fructose-Bisphosphatase 1; FLCN, Folliculin; FLNA, Filamin A; FOXM1, Forkhead Box M1; GCN5, General Control of Amino Acid Synthesis Protein 5-Like; GSK3, Glycogen Synthase Kinase 3; LPA, Lysophosphatidic Acid; MAGED1, MAGE Family Member D1; MAPT, Microtubule-Associated Protein Tau; MARCKS, Myristoylated Alanine Rich Protein Kinase C Substrate; MCP1, Monocyte Chemotactic and Activating Factor; MEP50, Methylosome Protein 50; mTOR, Mechanistic Target Of Rapamycin Kinase; NAFLD, Non-Alcoholic Fatty Liver Disease; NF-kB, Nuclear Factor Kappa B; NLS, Nuclear Localization Signal; NRF1, Nuclear Respiratory Factor 1; OXPHOS, Oxidative Phosphorylation; PAK1, P21 Activated Kinase 1; PDH, Pyruvate Dehydrogenase; PDK, Pyruvate Dehydrogenase Kinase; PDX, Patient-Derived Xenograft; PGC1, Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha; PI3K, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase; PRMT5, Protein Arginine Methyltransferase 5; PXN, Paxillin; RB, Retinoblastoma Protein; SCD, Stearoyl-CoA Desaturase; SMYD2, SET and MYND Domain Containing 2; SPOP, Speckle Type BTB/POZ Protein; SREBP, Sterol Regulatory Element-Binding Protein; TFEB, Transcription Factor EB; TNBC, Triple-Negative Breast Cancer; USP51, Ubiquitin-Specific Peptidase 51; ZEB1, Zinc Finger E-Box-Binding Homeobox 1.

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as determined through genetic and pharmacological studies, making as clear as possible where CDK4-specific data are lacking if needed.

Beyond CDK4 substrates involved in its strict cell cycle-related functions, accumulated evidence has shown the use of other CDK4 substrates in addition to RB. For instance, CDK4 can directly phosphorylate c-Jun, leading to activation of the transcription factor AP-1 [121,133]. Linked to chromatin organization, the CDK4-cyclin D1 complex also phosphorylates MEP50, a coregulatory factor of protein arginine-methyltransferase 5 (PRMT5) [3], which is a determinant for suppressing the expression of p53 target genes, including

Table 1
Cellular localization of CDK4.

Protein	Localization	Origin	Biological function	Techniques used	Cell line	Reference
CDK4 and Cyclin D1	Cytosolic	Endogenous	G1/S progression	Co-IP, Cell fractionation, IHC, WB	SNU449	[67]
CDK4-Cyclin D1 complex	Nuclear	Endogenous	RB phosphorylation	Co-IP, Cell fractionation, IHC, WB	SNU449	[67]
CDK4- Cyclin D2 complex	Nuclear membrane	Exogenous, Endogenous	RB phosphorylation and DNA synthesis	IF	HeLa, NIH3T3	[131]
CDK4- Cyclin D2 complex	Nuclear	Exogenous, Endogenous	RB phosphorylation and DNA synthesis	IF	HeLa,NIH3T3	[131]
CDK4 and Cyclin D2	Cytosolic	Exogenous, Endogenous	G1/S progression	IF	HeLa,NIH3T3	[131]
CDK4-Cyclin D1 complex	Cytosolic	Exogenous	G1/S progression	IP, WB, IF	NIH3T3	[31]
CDK4-Cyclin D1 complex	Nuclear	Endogenous	RB phosphorylation	IP, WB, IF	NIH3T3	[31]
CDK4 and Cyclin D1	Cytosolic	Endogenous	CDK4 subunits are inactive	IP, WB, IF	NIH3T3	[31]
CDK4 and Cyclin D1	Cytosolic	Endogenous	G1/S progression	Co-IP, Cell fractionation, IF, WB	Cardiomyocytes	[111]
CDK4-Cyclin D1 complex	Nuclear	Cyclin D1 mutant (D1NLS- Exogenous, CDK4- Endogenous)	RB phosphorylation	Co-IP, Cell fractionation, IF, WB	Cardiomyocytes	[111]
Cyclin D1	Nucleus- G1 phase	Endogenous	-	Co-IP, Cell fractionation, IF, WB, Synchronization	NIH3T3	[4]
Cyclin D1	Cytosolic- S, G2 phase	Endogenous	-	Co-IP, Cell fractionation, IF, WB, Synchronization	NIH3T3	[4]
Cyclin D1	Nuclear	Exogenous	-	Co-IP, Cell fractionation, IF, WB, Synchronization	NIH3T3	[4]
CDK4-Cyclin D1 complex	Nuclear	Endogenous	RB phosphorylation	Co-IP, Cell fractionation, IF, WB, Synchronization	NIH3T3	[4]
CDK4-Cyclin D1- T286A complex	Nuclear	Cyclin D1 mutant-Exogenous, CDK4-Endogenous	Function downstream of RB	Co-IP, Cell fractionation, IF, WB, Synchronization	NIH3T3	[4]
CDK4	Nuclear	Endogenous	-	IF	HeLa	[22]
Cyclin D1	Cytosolic and relocated to nucleus for CDK4 activation	Endogenous	-	IF	HeLa	[22]
CDK4-Cyclin D1 complex	Nucleus (gradually along cell cycle phases)	Exogenous, Endogenous	Cell cycle progression	IF	HeLa	[22]
CDK4-Cyclin D1 complex	Nuclear	Exogenous, Endogenous	-	Co-IP, IF, WB	Murine fibroblast	[15]
CDK4-Cyclin D1 complex	Nuclear	Endogenous	RB phosphorylation	IHC	Rat liver tissue	[61]
Cyclin D3 (In quiescent phase)	Cytosolic	Endogenous	-	IHC	Rat liver tissue	[61]
CDK4 (In quiescent phase)	Cytosolic	Endogenous	-	IHC	Rat liver tissue	[61]
Cyclin D1 (In quiescent phase)	Nuclear	Endogenous	-	IHC	Rat liver tissue	[61]
Cyclin D3 (5 h after PH)	Nuclear and cytosolic	Endogenous	-	IHC	Rat liver tissue	[61]
CDK4 (5 h after PH)	Nuclear and cytosolic	Endogenous	-	IHC	Rat liver tissue	[61]
Cyclin D1 (5 H AFTER PH)	Nuclear	Endogenous	-	IHC	Rat liver tissue	[61]
CDK4 and Cyclin D1	Nuclear	Endogenous	-	IF	NIH3T3	[124]
CDK4-Cyclin D1 Complex	Nuclear	Endogenous	-	IF	NIH3T3	[124]
CDK4-Cyclin D1 Complex	Nuclear	Exogenous	-	IF	U2OS, SAOS-2	[72]
Cyclin D1	Nuclear and plasma membrane	Endogenous	-	IF	MRC-5	[23]
Cyclin D1	Outer Mitochondrial Membrane	Exogenous, Endogenous	-	IF	Ramos human Burkitt lymphoma cell line, Human MM U266	[113]

antiproliferative and proapoptotic genes [76]. Furthermore, and in addition to its nuclear localization, CDK4 and associated cyclin D proteins have also been shown to be present in extranuclear compartments [11]. For instance, cyclin D1 is actively synthesized and located in extranuclear locations in postmitotic neurons [109], cardiomyocytes [111] and hepatocytes [60]. CDK4 is found not only in the cytoplasm but also in mitochondria and on the plasma membrane of various cell types (summarized in Table 1), suggesting its involvement in various biological processes within the cell.

The nuclear-cytoplasmic localization of CDK4 is primarily regulated by its binding partners. Cyclin D1 acts as a notable shuttle protein for CDK4 between the nucleus and cytoplasm [111,131]. Cyclin D1 is found in the cytosol but is also associated with the outer mitochondrial membrane [113] and/or at the plasma membrane [147] and can mediate the extranuclear activation of CDK4. The ectopic expression of a nucleus-targeted variant of cyclin D1, but not wild-type cyclin D1, promotes nuclear CDK4 localization and subsequent re-entry of cardiomyocytes into the cell cycle [111]. Nuclear translocation of the CDK4-cyclin D1 complex has also been observed in vivo during rat liver regeneration [61] and estrogen-induced proliferation during mitogenic processes [118]. The nuclear to cytoplasmic redistribution of cyclin D1 during S phase of the cell cycle is also stimulated by glycogen synthase kinase 3 beta (GSK-3β)-dependent phosphorylation of cyclin D1 at threonine 286 [4,31]. However, it remains unclear how this phosphorylation controls the redistribution of CDK4. Finally, $p21^{CIP1}$ and $p27^{KIP1}$, two KIP/CIP inhibitors of CDK2-containing complexes that act as assembly factors for CDK4/6-cyclin D [24], regulate the intracellular localization of CDK4, as the deletion of their nuclear localization signal (NLS) relocalized CDK4 to the cytoplasm [16,72,99].

In addition to the cell cycle-related nuclear function of CDK4, its presence in the extranuclear space [25] suggests additional functions of CDK4 in other biological pathways. Many recent studies have reported non-cell cycle-associated activities for the CDK4-cyclin D complex that may be linked with their cytoplasmic localization. For instance, CDK4 has been shown to regulate glycolysis [38], mitochondrial biogenesis [57,119], lysosomal functions [82] and cytoskeleton-associated functions [19,75]. The main subject of this review is not the role of CDK4 in the regulation of the cell cycle, which is reviewed elsewhere. Instead, we focus on the function of CDK4 in the regulation of these extra-cell cycle events that support tumorigenesis, including but not limited to the control of metabolism, cell fate, cellular dynamics and immune function.

2. Metabolic regulation in cancer cells: the thrifty regulator CDK4

Since the first observation of Otto Warburg [132], it is well known that cancer cells have a distinct metabolic profile that involves increased glucose uptake and utilization, increased fatty acid (FA) synthesis, modifications in amino acid-related metabolic pathways, and increased lactate production [122]. Indeed, it is currently accepted that specific metabolic changes depend on the cancer type, stage, and even environment. In this review, we discuss three major metabolic pathways, glycolysis, lipid biology, and mitochondrial OXPHOS, in which CDK4 has a significantly reported function. An important hallmark of cancer cells is the need for increased macromolecular biosynthesis. Cancer cells also need, however, energy-producing pathways. In view of its functions in several metabolic processes, as described below, we propose that CDK4 regulates metabolism, preserving the energy needed for biosynthetic processes.

2.1. The glycolytic pathway

In the glycolytic pathway, glucose is converted into pyruvate, generating ATP and NADH in the process. In normal cells, this pathway operates in the presence of oxygen and is followed by oxidative phosphorylation (OXPHOS) in the mitochondria. In contrast, in many cancer cells, even in the presence of oxygen, pyruvate is converted into lactate in the so-called Warburg effect [28]. By relying on glycolysis for ATP production, cancer cells can rapidly generate energy and macromolecules, which are needed for cell growth and proliferation. Additionally, the glycolytic pathway produces intermediates that can be used for anabolic processes such as the biosynthesis of lipids, nucleotides, and amino acids [122]. The upregulation of glycolytic enzymes and downregulation of OXPHOS have been observed in many types of cancer. Metabolic reprogramming confers a survival advantage to cancer cells, enabling them to rapidly proliferate and resist apoptosis. Targeting the glycolytic pathway and general metabolic changes in cancer has emerged as a promising strategy for cancer treatment [115].

CDK4 contributes to metabolic rewiring in cancer cells through the regulation of glycolysis. The CDK4/6i palbociclib stabilizes FBP1 by repressing MAGED1 expression in pancreatic duct adenocarcinoma [142], and in malignant pleural mesothelioma cells, inhibition of CDK4/6 decreased both glycolysis and mitochondrial respiration alone or in combination with PI3K/mTOR inhibitors [17]. Other combination treatments, such as cotreatment with PI3K/AKT/mTOR inhibitors, were efficient in decreasing tumor growth in TNBC cells. Interestingly, this effect was dependent on the impairment of glucose metabolism [26]. In the same sense, the inhibition of both EGFR and CDK4/6 delayed the progression of head and neck squamous cell carcinoma by inducing metabolic rewiring, including rewiring of the glycolytic pathway [21]. Moreover, the treatment of liver cancer cells with a combination of palbociclib and the tyrosine kinase inhibitor regorafenib showed enhanced antitumor effects and decreased overall glucose metabolism in these cells [32].

Little is known, however, about the molecular mechanisms underlying the participation of CDK4 in regulation of the glycolytic pathway, and its direct targets have not been described. However, it is possible that CDK4 indirectly affects the glycolytic pathway by regulating transcription factors or other proteins involved in glucose metabolism. This is the case for the E2F transcription factors. Through the canonical pathway, CDK4 activates the transcriptional activity of E2F1, which regulates the expression of pyruvate dehydrogenase kinase 1 and 3 (PDK1,3) (Fig. 1). PDK negatively regulates pyruvate dehydrogenase (PDH), which catalyzes the conversion of pyruvate to acetyl-CoA, and thus redirects pyruvate for lactate production, enhancing glycolysis [130]. Extensive further studies are needed to precisely identify whether the regulation of glycolysis by CDK4 is RB-dependent and, if so, what other putative substrates may participate in this regulation.

2.2. Lipid metabolism

In addition to the Warburg effect [132], cancer cells exhibit changes in lipid metabolism, including increased FA synthesis and utilization, to not only store energy but also form membrane structures that are essential for cell division and growth (reviewed in [108]).

De novo lipid biosynthesis is of the utmost importance for cancer cells because it contributes to their proliferation, survival, and dissemination. This process includes the synthesis of new membranes with specific lipidic compositions that facilitate the formation of lipid rafts for increased signaling of cell growth receptors [110]. Lipid synthesis also generates lipid intermediates such as malonyl-CoA, which is involved in the transcriptional regulation of growth factor receptors [94]. In addition, circulating lipids, such as lysophosphatidic acid (LPA), impact cancer cell growth, migration, and invasion [59,120]. Enhanced lipogenesis in cancer cells is proposed to be needed to balance redox potential via the utilization of NADPH and to produce specific lipids that regulate the activity of various oncogenes, such as PI3K/AKT [123]. Posttranslational modification with lipid moieties is also a key process that regulates the functions of various oncoproteins, such as the RAS, or the WNT pathway [37].

The participation of CDK4 in the control of lipid metabolism was first proven by the finding that the CDK4-cyclin D3 complex controls the

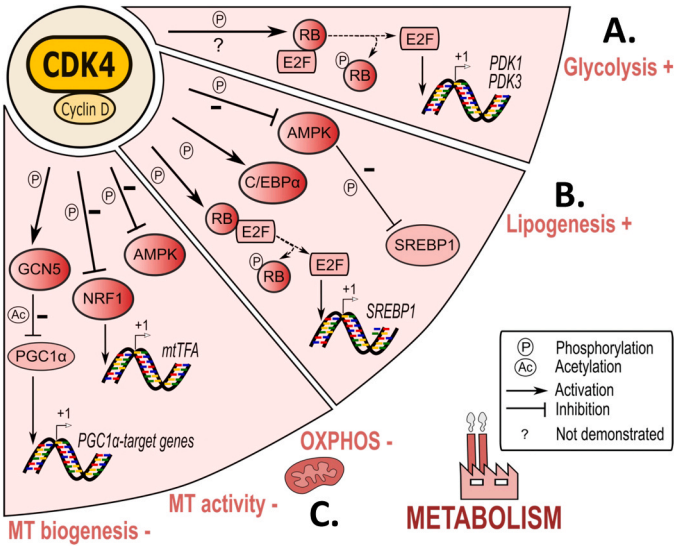

Fig. 1. CDK4 modulates cancer cell metabolism. CDK4 is involved in at least three major metabolic pathways determinant for cancer growth. **A.** Through RB-dependent manner, it may regulate glycolysis enhancing *PDK1– 3* expression. **B.** Regulation of lipids metabolism by CDK4 is also partly RB-dependent through the regulation of *SREBP1* gene. CDK4-CyclinD phosphorylates and inactivates AMPK, stimulating SREBP1. In parallel, CDK4-CyclinD phosphorylates also C/EBPa to promote global lipogenesis in cancer cells. **C.** CDK4 represses mitochondrial functions and oxidative phosphorylation (OXPHOS) through inhibition of AMPK, NRF1 transcription factor, and GCN5-mediated acetylation of PGC1a. CDK4 is a general anabolic promoter and a catabolic suppressor through both RB-dependent and RB-independent mechanisms.

activity of peroxisome proliferator-activated receptor gamma (PPARγ) in adipocytes and regulates the adipogenic program and therefore lipogenesis [2,103]. Interestingly, dysregulation of CDK4 has been implicated in various metabolic disorders, including obesity [58,86]. In obesity, CDK4 activation has been shown to lead to insulin resistance and decreased insulin sensitivity [40]. Strikingly, a CDK4 mutation, *CDK4* IVS4-nt40AA, was found to be correlated with obesity-related cancers [85], suggesting that some cancer-inducing effects of CDK4 are mediated by the regulation of lipid metabolism. AMP-dependent protein kinase (AMPK) is a negative regulator of lipid synthesis through the phosphorylation and inhibition of ACC, the enzyme that catalyzes the conversion of acetyl-CoA into malonyl CoA. AMPK also phosphorylates SREBP and SREBP-regulatory proteins to decrease SREBP activity [48,77]. The result is an overall decrease in the expression of genes involved in lipid synthesis. Interestingly, CDK4 phosphorylated and inhibited the activity of AMPK in transformed mouse embryonic fibroblasts and muscle cells (Fig. 1) [80]. Additionally, in pancreatic cancer cells, CDK4/6i treatment increased AMPK activity and reduced FA synthesis in these cancer cells [98]. Another study, however, reported that the CDK4/6i palbociclib also inhibited hepatocellular carcinoma through the activation of AMPK but did so in a CDK4/6-independent manner [55]. More evidence of the participation of CDK4 in lipogenesis comes from an elegant study from Jin et al., who demonstrated that CDK4 phosphorylates C/EBPα and facilitates the development of hepatic steatosis and hepatocellular carcinoma (Fig. 1). The authors also showed in this study that CDK4/6i treatment prevents fat accumulation [64].

At odds with these observations, it was also reported that the long-term inhibition of CDK4/6 resulted in activation of the PI3K/AKT pathway, creating therapeutic resistance [144,38,5,54]. Indeed, cotreatment with a CDK4/6i and PI3K/AKT inhibitors restored therapeutic sensitivity [125]. The induction of AKT signaling typically impacts lipid biology, notably increasing lipid synthesis [70]. This paradox could be partly explained by changes in lipid metabolism that are promoted by CDK4 deletion or inhibition, which could underlie the activation of the PI3K/AKT pathway as a compensatory pathway. Similarly, even if CDK4 inhibition decreases proliferation, it also activates the PI3K/AKT pathway, which also regulates the proliferation of cancer cells. Whether the PI3K/AKT pathway is dominant remains a critical issue to be addressed in future investigations.

CDK4 also regulates lipid metabolism through the canonical RB-E2F1 pathway. When CDK4-cyclin D phosphorylates RB, it releases the transcription factor E2F1, which regulates the expression of genes involved in lipid synthesis. E2F1 KO mice exhibited deregulated lipogenesis in the liver, which is the major lipogenic tissue [30], and E2F1 KO mice were protected from diet-induced liver steatosis. Most relevant for cancer metabolism was the finding that both E2F1 and E2F2 repress expression of the carnitoyl transferase 2 gene, which is part of the FA oxidation pathway, and therefore promote nonalcoholic fatty liver disease (NAFLD), a precursor of hepatocarcinoma [44]. More specifically, E2F1 also regulates the expression of *SREBP1* and induces lipogenesis in clear cell renal cell carcinoma [105]. In summary, CDK4 participates directly and indirectly in the regulation of FA synthesis in normal and cancerous cells through at least three complementary mechanisms involving AMPK, C/EBPα and the canonical RB-E2F pathway (Fig. 1).

2.3. CDK4 and mitochondria

Mitochondria are a hub where metabolic pathways converge and, as such, are deregulated in cancer. Remodeling of mitochondrial biology, in one sense or another, is essential for the survival, proliferation, and dissemination of cancer cells. Because distinct tumor types and stages are characterized by specific metabolic alterations, it cannot be expected that changes in mitochondrial activity are as homogeneous as first described by Otto Warburg [132]. Eventually, mitochondrial respiration defects were found not to cause the Warburg effect (reviewed in [150]. Mitochondria, in addition to providing energy to cancer cells [112], mostly contribute to the production of oncometabolites and biosynthetic substrates [10,87]. Moreover, mitochondria are also a source of ROS and modulate calcium signaling [97]. The generation of ROS is indeed needed for K-RAS to transform cells [135]. The OXPHOS activity of mitochondria has been found to be either increased or decreased in cancer cells. On the one hand, cancers with low expression levels of mitochondrial genes involved in OXPHOS exhibit a worse clinical prognosis, suggesting that the inhibition of OXPHOS genes is a hallmark of cancer progression, independent of cancer type [41]. On the other hand, OXPHOS is not only increased in some cancers but also considered a therapeutic target [137,34,8].

Direct participation of CDK4 in oxidative metabolism was first proven in cells devoid of CDK4 that showed an increased oxidative phenotype, including augmented oxygen consumption in response to FA. This was attributed, at least partially, to the inhibitory effects of CDK4 on AMPK activity. Accordingly, mice deficient in CDK4 had a marked oxidative phenotype, indicated by increased endurance and muscle activity [80]. Another direct target of CDK4-cyclin D1 in the control of OXPHOS is nuclear respiratory factor 1 (NRF1), which regulates the expression of nuclear-encoded mitochondrial genes. CDK4-cyclin D1 phosphorylates and inhibits NRF1 activity; therefore, cyclin D1-deficient cells showed increased mitochondrial size and activity [101,129]. CDK4-cyclin D1 also phosphorylates the acetyl transferase GCN5 and therefore modulates the activity of the peroxisome proliferator gamma coactivator (PGC1α). Although a study performed in hepatocytes showed a decrease in PGC1α-mediated gluconeogenesis upon inhibition of CDK4 or deletion of cyclin D1, the authors suggested that the increased PGC1α activity could also result in augmented mitochondrial activity [74].

In cancer cells and in patient-derived xenograft (PDX) mice, cotreatment with CDK4/6 and OXPHOS inhibitors had a synergistic antitumor effect [34]. Although not specifically discussed, the synergistic effect of the CDK4/6i palbociclib is consistent with the effect of

this drug and with observations in CDK4 depletion models. In this scenario, CDK4 depletion increased OXPHOS in cancer cells, which consequently became sensitive to OXPHOS inhibition [34]. More evidence of the inhibition of OXPHOS by CDK4 inhibitors comes from a study in uveal melanoma. In this cancer, CDK4 inhibition in combination with MEK inhibition upregulated OXPHOS [114], so the effect of CDK4 inhibition could not be distinguished from that of MEK inhibition in this study. Similar to the effects of OXPHOS inhibition in TNBC cells [34], this class of inhibitors also synergized with CDK4/6is and MEK inhibitors to decrease tumor growth. Other examples of the stimulatory effects of CDK4/6is on oxidative metabolism include effects observed in chondroma [93] and pancreatic cancer [38].

Current knowledge about the function of CDK4 in controlling oxidative metabolism and mitochondrial activity supports the hypothesis that CDK4 prevents catabolic processes to preserve energy for biosynthesis in cancer cells, including mitochondrial biogenesis, at least through GCN5 and NRF1 phosphorylation (Fig. 1).

Overall, CDK4 is an important metabolic regulator that modulates lipid biogenesis-related mitochondrial functions and, to a lesser extent, glycolysis. Taken together, these data clearly indicate that CDK4 favors anabolic processes and limits catabolic processes.

3. Cell fate (apoptosis, senescence, autophagy)

Cancer cell fate has been intimately linked to the aggressiveness and prognosis of cancers through the regulation of not only cellular proliferation but also cell death, cellular senescence and autophagy [49]. Interestingly, growing evidence has emerged to decipher the role of CDK4 in these different cancer cell fates.

3.1. CDK4 and cellular senescence

Pharmacological inhibition of both CDK4 and CDK6 was shown to not only stop the cell cycle in normal and cancerous cells but also induce cellular senescence [127,128,141,82,95]. Cellular senescence is characterized by not only stable proliferation arrest but also a specific secretome, termed the senescence-associated secretory phenotype (SASP), and increased activity of senescence-associated β-galactosidase (SA β-gal) [45,53]. The senescent state is triggered by numerous stressors, including aberrant oncogene activation, oxidative stress and mitochondrial dysfunction [45,53]. Senescent cells accumulate over time in multiple tissues [52,63] and drive age-related pathologies through chronic inflammation and tissue dyshomeostasis [51]. Beyond transient cell cycle arrest, CDK4/6 inhibition also elicits cellular senescence in these normal and cancerous contexts, as evidenced by additional hallmarks of cellular senescence, such as increased SA β-gal activity and/or SASP acquisition [127,128,141,82,95].

Mechanistically, cellular senescence is induced by two main pathways, the $p16^{INK4A}$/RB and ARF/p53/$p21^{CIP1}$ pathways [45]. Within the cell, $p16^{INK4A}$ is a canonical inhibitor of both CDK4 and CDK6 and one of the most robust markers of cellular senescence, explaining why pharmacological CDK4/6 inhibition triggers cellular senescence. Early studies have depicted the specific role of CDK4, independent of CDK6, in regulating replicative and oncogene-induced senescence [89,151] in an ARF/p53-independent way. Furthermore, CDK4 specifically allows escape from chemotherapy-induced senescence through regulation of methylase EZH2 (Le Duff et al., 2018) via potential direct phosphorylation, as EZH2 is a CDK4 substrate (Müller et al., 2020).

More recently, and due to the growing interest in CDK4/6is in cancer treatment, CDK4/6i-induced senescence has been better characterized, relying on direct downstream RB in melanoma and breast cancer cells [133,141]. In this RB-dependent fashion, CDK4/6 inhibition reprograms the enhancer landscape by stimulating AP-1 transcriptional activity [133], which constitutes a canonical transcriptional program in senescent cells [83]. Other additional RB-independent mechanisms have also been suggested to participate in the induction of CDK4/6i-induced senescence. For instance, the transcription factor Forkhead Box M1 (FOXM1) was found to be a critical phosphorylation target of both CDK4/6, allowing its later stabilization [6] (Fig. 2). Lack of this stabilizing phosphorylation leads to FOXM1 degradation with a concomitant increase in the levels of ROS. In this context, ROS generation was proposed to be a priming mechanism for senescence induction [6]. Last, but not least, the AKT/mTOR pathway was also found to contribute to full establishment of the senescence phenotype in the various contexts of CDK4-induced senescence [119,12,82], but the exact mechanism by which CDK4 regulate AKT has not yet been elucidated (Fig. 2).

In cancer, cellular senescence acts as a first initial antitumoral barrier to cellular transformation. Nevertheless, further acquisition of the SASP by senescent cells results in a long-term proinflammatory and protumoral effect [45]. For instance, chemo- and radiotherapy-induced

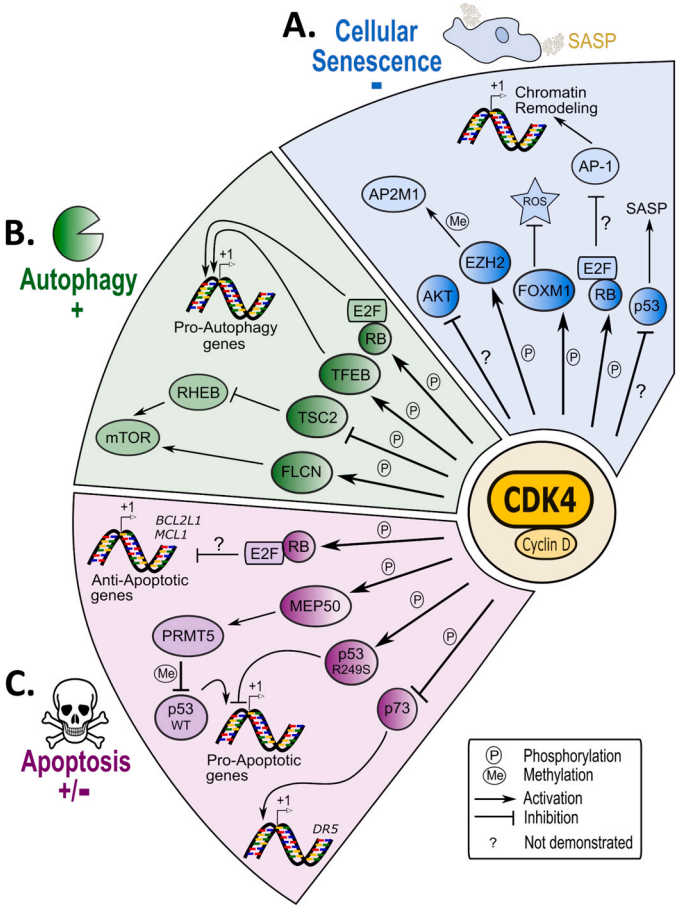

Fig. 2. CDK4 participates in the regulation of cancer cell fate. Beyond cell cycle, CDK4 controls some aspects of cell fate through cellular senescence, autophagy and apoptosis. **A.** CDK4-CyclinD limits cellular senescence via reducing activity of the transcription factor AP-1 and subsequent chromatin accessibility in a RB-dependent manner, dampening ROS generation through FOXM1-stabilizing phosphorylation, inhibiting AKT pathway through an unknown mechanism, promoting senescence escape through EZH2 phosphorylation and subsequent AP2M1-methylation. Finally, senescence-associated secretory phenotype (SASP) is limited by CDK4 via the repression of p53 with not yet clear elucidated mechanism. **B.** CDK4-CyclinD stimulates autophagy-associated transcriptional program through RB- and TFEBphosphorylations. CDK4-CyclinD phosphorylates both FLCN and TSC2 to induce overall increased mTOR activity. **C.** Dual role of CDK4 in apoptosis. In the one hand, CDK4-CyclinD has RB-dependent pro-apoptotic roles, repressing expression of *BCL2L1* and *MCL1* genes. In the other hand, CDK4-CyclinD is more anti-apoptotic, phosphorylating MEP50, p53-R249S- and p73, to hamper transcription of proapoptotic genes, including *DR5*. CDK4-mediated phosphorylation of MEP50 activates the PRMT5 methylase to suppress p53 WT transcriptional activity.

senescence has been proposed to be at the origin of cancer relapse, notably through SASP activity [29]. Notably, nontumor cells undergoing CDK4/6i-induced senescence lack many of the NF-κB-driven proinflammatory components of the SASP, and such senescence drives a p53-dependent SASP [128]. Mechanistically, this repression of p53 by CDK4/CDK6 may occur through the direct phosphorylation of MEP50, a coregulatory factor of protein arginine-methyltransferase 5 (PRMT5), the latter of which is crucial to methylate and inactivate p53 [76,106]. From a clinical point of view and in contrast to classical chemo- and radiotherapy-induced senescence, CDK4/6i-induced senescence induces a reduced tumorigenic proinflammatory p53 secretome that could be considered in future clinical investigations. Interestingly, CDK4/6i-treated breast cancer cells also secreted the chemokines CCL5 and CXCL10, facilitating intratumoral T-cell infiltration and adoptive T-cell therapy [119]. Taken together, these data clearly indicate that CDK4/6is impact cell secretion, notably through the SASP, and favor an antitumoral microenvironment.

In a clinical context, the induction of senescence through CDK4/6i treatment has also been proposed as a therapeutic strategy that can synergize with senolytics, i.e., drugs that specifically kill senescent cells, thus enhancing the antitumoral efficiency of CDK4/6i-based therapies in multiple contexts [126].

While several reports implicate CDK4/6is in establishing senescence, genetic deletion of solely CDK4 was not fully sufficient to recapitulate the CDK4/6i-senescence phenotype [82], underlying the overlapping targets of both CDK4 and CDK6 in the context of senescence [6]. Hence, the particular mechanisms underlying their specific contributions to the senescence phenotype remain elusive, indicating that extensive specific genetic studies should be carried out to decipher the relative contributions of CDK4 and CDK6 to cellular senescence.

3.2. CDK4, apoptosis, and autophagy

The role of CDK4 in regulating apoptosis is still under debate. Initially, many studies reported the induction of apoptosis through the use of CDK4/6is [107,116], which may rely on the potential anti-apoptotic role of CDK4. This proapoptotic effect is context-dependent and may be partly the result of nonspecific cytotoxicity and the use of CDK4/6is at supramicromolar doses (>10 μM) [68,107]. Nevertheless, if not able to primarily induce apoptosis, the inhibition of CDK4 alone may sensitize breast cancer cells to certain types of apoptosis, such as irradiation-induced apoptosis [47]. Indeed, numerous independent mechanistic studies have noted the presence of crosstalk between CDK4 and p53 family members, which could further explain some of the antiapoptotic effects of CDK4. Indeed, CDK4 phosphorylates MEP50, a coregulatory factor of PRMT5, which is crucial to methylate p53 and dampen the expression of proapoptotic p53 target genes in lymphomagenesis [106,3,76] (Fig. 2). In hepatocellular carcinoma, CDK4 can phosphorylate and activate the gain of function (GOF) p53 mutant p53-R249S [78], leading to the repression of proapoptotic p53-regulated genes (Fig. 2). Finally, CDK4 mediates phosphorylation of the p53 family member p73 at threonine 86 to sequester p73 in the cytoplasm. CDK4/6i treatment resulted in p73 dephosphorylation and nuclear translocation, which triggered the transcription of activated death receptor 5 (DR5), an important component of the extrinsic apoptotic pathway [117] (Fig. 2).

Dividing cells are usually more chemosensitive than arrested cells. Considering the mechanism of CDK4/6is in stopping the cell cycle, it has also been hypothesized that concomitant CDK4/6 inhibition may antagonize the cytotoxic effects of chemotherapeutic agents in tumor treatment. Indeed, early in vitro studies displayed protection from doxorubicin-, paclitaxel- or carboplatin-mediated cytotoxicity and the associated cell death of RB-proficient cells upon CDK4/6 inhibition in TNBC and ovarian cancer cells [27,69,84]. Mechanistically, CDK4/6i treatment also has been shown to lead to apoptosis evasion through the upregulation of genes encoding antiapoptotic members of the Bcl-2 protein family, namely, *BCL2L1* (encoding Bcl-xL) and *MCL1* [133], in breast cancer, suggesting a proapoptotic role for CDK4 (Fig. 2). Remarkably, under pathological conditions in the kidney, CDK4/6 inhibition has also been shown to promote cell survival [33,90,91], specifically dampening caspase 3/7 activation upon treatment with nephrotoxins such as cisplatin or etoposide [33]. Accordingly, CDK4/6i treatment reduced the apoptosis of normal intestinal cells upon radiation-induced injury [134] but also caused hematological toxicity, improving lung cancer patients' tolerability of chemotherapy, as evidenced by myelopreservation within multiple hematopoietic lineages [136]. Importantly, the apparent antagonism or synergism of CDK4/6is with chemotherapeutic drugs can be explained by the order in which the compounds are added, which could explain the opposing anti- and proapoptotic effects observed in these previous studies [102]. Indeed, when CDK4/6i treatment precedes chemotherapeutic agent treatment, antagonism is predominant, while when chemotherapeutic agent treatment precedes CDK4/6i treatment, synergism is frequently observed. The specific contributions of CDK4 and CDK6 remain uninvestigated. Future studies to understand the role of CDK4 in apoptosis and, more broadly, cell death are needed, which will clarify the antagonistic and synergetic effects that have been described in the recent literature.

Multiple cell cycle regulators were found to influence the autophagy machinery, an important catabolic process that promotes the recycling of endogenous components following exposure to various stressors. More specifically, the role of CDK4 in autophagy has also been studied with either CDK4/6i treatment or specific genetic CDK4 deletion. Accordingly, CDK4 and CDK6 were found to promote the expression of key autophagy genes through the RB-E2F axis. Indeed, E2F target loci include many autophagy-encoding genes, namely, *BNIP3*, *GABARAP*, *UVRAG*, *ULK1/ATG1*, *ATG5*, *ATG9A*, *ATG12*, and *MAP1LC3B* [149]. In an RB-independent fashion, CDK4 also regulates autophagy through the modulation of at least three complement phospho-targets, namely, TSC2, folliculin (FLCN) and TFEB/TFE3. First, CDK4/CDK6-cyclin D inhibits TSC2 through serine 1217 and serine 1452 phosphorylation, resulting in the activation of mTORC1 [100]. Of note, as mTOR regulates functions other than autophagy in the cell, CDK4/6-mediated regulation of TSC2 also enhances protein synthesis [100]. Second, CDK4-cyclin D also phosphorylates FLCN, which facilitates its later recruitment to the lysosomal surface upon amino acid deprivation [82]. Finally, CDK4 (and CDK6) limits lysosome biogenesis by TFEB/TFE3 phosphorylation [140]. Altogether, these data highlight the importance of CDK4 in modulating autophagy through not only activated mTOR but also dampened lysosome biogenesis (Fig. 2). Altogether, these studies have noted the roles of CDK4 and CDK6 in regulating autophagic processes through transcriptional and posttranslational regulation, reinforcing their redundant functions, especially in this context.

Overall, the majority of studies using CDK4/6is have unraveled the roles of CDK4 and CDK6 not only in the cell cycle regulation of cancer cells but also in other associated cell fates, such as cellular senescence, autophagy and apoptosis. Only a few specific genetic studies have deciphered how CDK4 primes CDK6 function (Table 2), highlighting the need for further specific genetic studies in the future to decipher their specific and overlapping mechanisms.

4. CDK4 in cancer cell dynamics and the cytoskeleton

The cytoskeleton is a highly dynamic network of filamentous proteins that connects every area of both normal and cancerous cells in a 3D framework. This dynamic property involves the complex interplay between the cell cytoskeleton and other cellular components, such as organelles, membranes, and membrane-associated proteins. Through the formation of actin, microtubules and intermediate filaments, cytoskeleton proteins regulate various cellular processes, such as cell migration, division and signaling, sustaining tumorigenesis. Together, the three filament types form a dynamic and interconnected network that

Table 2
List of RB-independent CDK4 phospho-substrates.

Protein	Phospho-Site or Region	CDK4/6 Specificity		Consequence of Phosphorylation for the Targeted Protein	Biological-Associated Functions	Reference
		CDK4	CDK6			
MEP50	Thr-5, Ser-264, Ser-306	+	N/A	Activation	**Cell Cycle**	[3]
SMAD3	Thr-178, Ser-203, Ser-207, Ser-212, Thr-8	+	N/A	Inactivation		(Liu, 2006)
p107	Thr-369, Ser-640, Ser-964, Ser-975	+	N/A	Inactivation		(Leng et al., 2002)
p130	Ser-672	+	N/A	N/A		(Schade et al., 2019)
p73	Thr-86	+	+	Cytoplasmic retention	**Apoptosis**	[117]
p53-R249S	Ser-249	+	-	Activation		[78]
SPOP	Ser-6	+	-	Stabilization	**Immune System**	[143]
NRF1	Ser-47	+	N/A	Inhibition	**Metabolism**	[129]
C/EBPα	Ser-193	+	N/A	N/A		[64]
AMPK	Thr-85, Ser-176, Ser-345, Ser-377	+	N/A	Inactivation		[80]
GCN5	Thr-272, Ser-372	+	N/A	Activation		[74]
FLCN	Ser-62, Ser-73, Ser-571	+	N/A	N/A	**Autophagy**	[82]
TSC2	Ser-1217, Ser-1452	+	+	Inactivation		[100]
TFEB	Ser-142, Ser-211	+	+	Inactivation		[140]
FOXM1	Ser-4, Ser-35, Thr-611, Thr-620, Thr-627	+	+	Stabilization	**Cellular Senescence**	[6] (VanArsdale et al., 2015)
EZH2	Thr-345	+	+	Activation		(Müller et al., 2020)
FLNA	*Ser-2152, *Ser-2523, *Ser-1459	+	N/A	N/A	**Cellular Dynamics**	[147]
SHARPIN	Ser-146	+	N/A	ARP2/3 interaction		[19]
PXN	Ser-83	+	N/A	Activation		(Fuste et al., 2016) [88]
MARCKS	*Ser-27, *Thr-150	+	N/A	N/A		(Manenti et al., 1999)
MAPT	N/A	+	N/A	N/A		(Schmetsdorf et al., 2009)
SMYD2	N/A	+	+	Activation		[75]
USP51	Ser-26	+	+	Activation		[142,145,146]
DUB3	Ser-41	+	+	Activation		[79]

provides structural support and mechanical stability to cancer cells.

During cancer progression, cell dynamics are crucial during metastasis, a multistep process involving epithelial-mesenchymal transition (EMT), migration, invasion, and resistance to multiple stressors (mechanical and metabolic) (Van Zijl et al., 2011). In the context of cancer, multiple findings have shown the participation of the CDK4-cyclin D complex in various models of migration (Vlad-Fiegen et al., 2012), invasion [19] and, more globally, metastasis [148]. Remarkably, an increasing number of mechanistic studies suggest that CDK4-cyclin D may participate in these processes by interacting with or phosphorylating cytoskeleton-regulated [14] or EMT-regulated proteins [79,142,145,146].

Remarkably, increased expression of CDK4 in osteosarcoma cells is correlated with metastatic potential and poor prognosis in osteosarcoma, while inhibition of CDK4 significantly decreased cell migration, indicating that CDK4 is a potential therapeutic target for osteosarcoma [148]. Recent research suggests a link between CDK4 activity and cytoskeleton dynamics, as various cytoskeleton proteins are directly or indirectly phosphorylated by CDK4 [14].

4.1. CDK4 and actin dynamics

The actin filament network and its interaction with actin-binding proteins (ABPs) are determinants of migration, invasion and metastasis and require the coordination of cytoskeleton dynamics, organization, and signal transduction (Izdebska et al., 2020).

Early evidence of the involvement of the CDK4-cyclin D1 complex came from numerous studies in cyclin D1-deficient models. Cyclin D1 has been shown to be a key determinant of the migration of various normal cell types, such as human fibroblasts [7], mouse embryonic fibroblasts (MEFs) (Li et al., 2006), human macrophages (Neumeister et al., 2003), and human aortic smooth muscle cells [71]. Cyclin D1 deficiency in MEFs is, for instance, associated with increased Rho GTP and Rho activated kinase II (ROCKII) activity and the phosphorylation of various actin-related proteins, such as LIM kinase, cofilin-ser3 (an ABP) and myosin light chain 2 (an actin-binding motor protein) (Li et al., 2006). While CDK4 activity was not thoroughly explored in these studies, we can speculate that the absence of cyclin D1 may decrease the kinase activity of CDK4, accounting for some of the effects observed in these cells.

In the context of cancer, CKD4 or cyclin D1 expression levels were found to correlate with migration and, more broadly, metastasis in at least two different metastatic cancer contexts. Indeed, CDK4 and cyclin D1 increased expression in human osteosarcoma and TNBC, respectively, correlating with metastatic potential and poor prognosis [147,148]. Furthermore, cyclin D1 overexpression also correlates with the dissemination of glioblastoma [20]. Beyond correlative analysis, some of these studies have revealed functional data. For instance, reducing cyclin D1 expression or the activity of the CDK4/6-cyclin D1 complex resulted in decreased motility in invasive TNBC [147] or cell migration in osteosarcoma [148]. Altogether, these studies have noted the potential interplay between CDK4 and cyclin D and some players in cancer cell migration.

Mechanistically, a few studies have shown how CDK4 and/or cyclin D may impact migration, notably through actin reorganization (Vlad-Fiegen et al., 2012), [147,148,19,88]. First, cyclin D1 binds the cytoplasmic membrane (Alhaja et al., 2004), (Nebot-Cegarra & Domenech-Mateu, 1989) and regulates cytoskeleton-associated proteins, including protein kinase C and casein kinase substrate in neurons 2 (PACSIN II) (Meng et al., 2011), filamin A (FLNA) ([147]b) and paxillin (PXN) (Fusté et al., 2016) [88]. More precisely, PACSIN II binds both cyclins D1 and D2 and represses cellular migration through modulation or the actin-related protein 2/3 (ARP2/3) complex (Meng et al., 2011). Moreover, cyclin D1 also binds the ABP FLNA, mediating CDK4-cyclin D1 phosphorylation [147]. Finally, CDK4-cyclin D1 regulates phosphorylation of the membrane-associated protein PXN, potentially through the RAC1 axis (Fuste et al., 2016), which is crucial for actin rearrangement [23]. Nevertheless, whether PXN is a direct target of

CDK4 remains to be critically addressed. In addition, another recent study showed that CDK4, this time with cyclin D3, phosphorylated SHARPIN in vitro and interacted with the ARP2/3 complex, ultimately resulting in increased lamellipodia formation, cell invasion and subsequent metastasis [19]. Finally, CDK4 can phosphorylate another actin filament-crosslinking protein, namely, myristoylated alanine-rich-C-kinase substrate (MARCKS) (Manenti et al., 1999), a known regulator of the actin cytoskeleton, cell motility and adhesion (Chen et al., 2021b). How CDK4 directly impacts these outcomes through MARCKS in cancer cells remains unknown and will need further investigation. Finally, cyclin D1 mediates specific WNT-induced migration in colorectal and cervical carcinoma cells (Vlad-Fiegen et al., 2012). Indeed, cyclin D1 was found to be a WNT target gene that is critical for cell migration, affecting actin cytoskeleton polymerization/depolymerization and destabilizing adherent junctions through an as-of-yet unknown mechanism (Vlad-Fiegen et al., 2012).

In conclusion, these findings underscore the significance of CDK4 in the migration and invasion of various cancer cell lines through the phosphorylation of numerous actin-related proteins, including FLNA, SHARPIN, PXN and MARCKS (Fig. 3). Mounting evidence suggests that the pharmacological inhibition of CDK4/6-cyclin D1 may find a place in the therapeutic arsenal to not only counteract tumor cell proliferation but also curtail invasive capacity.

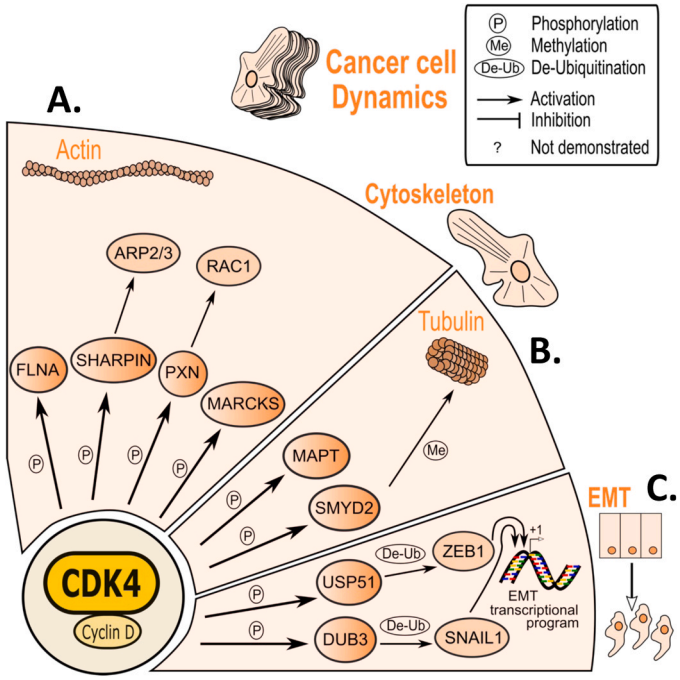

Fig. 3. CDK4 regulates cancer cellular dynamics processes: CDK4 is interacting and phosphorylating many cytoskeleton proteins. **A.** In context of actin organization influencing cell migration and adhesion, CDK4 mediates the phosphorylation of Filamin A (FLNA), SHANK-Associated RH Domiain Interacting Protein (SHARPIN), Paxillin (PXN), and Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS). Phosphorylation of SHARPIN facilitates its interaction with the ARP2/3 complex, a key regulator of actin filament nucleation and branching, contributing to cytoskeletal remodelling. Phosphorylation of PXN results in Rac1 activation which is crucial for actin filament reorganisation and cell motility. **B.** CDK4 influences microtubules oranization through at least two mechanisms. It includes the phosphorylation of Microtubule-Associated Protein Tau (MAPT) and SMYD2, involved in cell division and mitotic function. More precisely, SMYD2 phosphorylation leads to its activation and subsequent methylation of a-tubulin. **C.** CDK4's role in Epithelial-Mesenchymal Transition (EMT). Two independent and complementary deubiquitination mechanisms are governed by CDK4 (and CDK6). CDK4/6-dependent phosphorylations of two deubiquitinases USP51 and DUB3, are essential for the deubiquitination and stabilisation of master EMT transcription factors, namely and respectively ZEB1 and SNAIL1. It results in an enhanced EMT-associated transcriptional program.

4.2. CDK4 and tubulin dynamics

Beyond the role of CDK4 in regulating actin dynamics, migration and invasion, CDK4 may also influence tubulin dynamics. Microtubules are composed of alpha, beta, and gamma-tubulin and microtubule-associated proteins, which together play a crucial role in cell motility, division, and chromosome segregation.

Evidence shows that CDK4 and/or cyclin D may play a role in regulating microtubule stability and associated cell motility. Indeed, any disruption in CDK4 or the complex results in mitotic defects and chromosomal instability. The dysregulation of CDK4 expression in mouse keratinocytes can lead to chromosomal instability and, in certain instances, the development of cancer. This dysregulation is attributed to the binding of CDK4 to the promoter region of genes related to chromosomal segregation, such as Aurora-B (Aurkb) and Centromere Protein P (CENP-P) [73]. In a specific cancer context, long-term CDK4/6 inhibition acted to enhance mitotic errors, including micronuclei and mis-segregation errors, leading to genomic instability in ER+ breast cancer cells (Soria-Bretones et al., 2022).

From a mechanistic point of view, some studies have depicted how CDK4 may indirectly impact microtubule dynamics. First, CDK4, as a proline-directed protein kinase or by analogy with CDK1, can phosphorylate microtubule-associated protein tau (MAPT) on the SP and TP motifs in a proline-rich region (Schmetsdorf et al., 2009). Second, CDK4 phosphorylates SMYD2, a protein methyltransferase that modulates microtubule methylation and subsequent polymerization/depolymerization during the cell cycle [75]. Third, SIRT2 (a member of the SIRTUIN family of NAD+-dependent deacetylases that targets alpha-tubulin) was also found to be phosphorylated by the CDK4-cyclin D3 complex to regulate the effect on cell motility modulated by microtubule dynamics (Pandithage et al., 2008). Finally, CDK4 interacts with $p27^{KIP1}$ in sarcoma [24], which later binds the microtubule-destabilizing protein Stathmin (microtubule cytoskeleton-regulating protein) (Baldassarre et al., 2005). However, the precise role of this interaction, as well as subsequent outcomes, will require future investigations.

Taken together, multiple findings suggest a role for CDK4 in regulating the activity of certain microtubule-associated proteins, such as MAPT and SMYD2, through direct phosphorylation (Fig. 3). The implications of these regulatory processes on microtubule biology and the exact mechanism through which CDK4 is involved are topics that require further investigation.

4.3. CDK4 and EMT

EMT is a biological process that disperses cells in embryos, forming mesenchymal cells in injured tissues and initiating the invasive and metastatic behavior of epithelial cancers. This process occurs when epithelial cells lose cell—cell adhesion capacity and transform into mesenchymal cells, detaching from the basal membrane and acquiring a migratory phenotype (Kalluri & Weinberg, 2009). EMT is an early fundamental process that occurs during tumor progression and the early metastasis cascade.

Some reports provide evidence that CDK4 controls some aspects of EMT, but this role remains debatable, and CDK4 could have dual roles in this process. Indeed, an early study showed that in pancreatic cancer cells, anti-CDK4/6 therapy could induce EMT, enhancing cancer cell invasion mechanistically by activating Smad-dependent TGF-beta signaling (F. Liu & Korc, 2012). However, two more recent studies have emphasized the important role of CDK4 in enhancing the EMT transcriptional program. Two independent and complementary deubiquitinate-related mechanisms were found to stabilize two major EMT transcription factors. Indeed, the deubiquitinases DUB3 and USP51

are two bona fide targets of both CDK4 and CDK6. Their CDK4/6-dependent phosphorylation is necessary to deubiquitinate and stabilize the EMT transcription factors SNAIL1 [79] and ZEB1 [146], respectively, in breast cancer cell lines. Remarkably, mesenchymal cells from lung tumors with high ZEB1 were particularly sensitive to CDK4/6 inhibition (Padhye et al., 2021), reinforcing the importance of CDK4 in ZEB1-mediated EMT. Altogether, CDK4 and CDK6 may both drive activation of the EMT transcriptional program to promote breast cancer metastasis. However, research and clinical trials to explore the potential role of CDK4/6 in various other cancer types to understand the molecular mechanism underlying EMT are ongoing.

While CDK4/6is have shown promising results with certain cancer treatments, the development of resistance to these inhibitors, particularly in the context of EMT, poses a significant challenge; therefore, it is critical to understand the mechanism of acquired resistance. One study addressed this critical issue and showed that palbociclib-resistant cells exhibit IL-6/STAT3-mediated upregulation of the EMT and B-CSC-L pathways (Kettner et al., 2019). Correlative but independent downregulation of the DNA repair pathway was also identified, suggesting the synergetic effect of targeting IL6/STAT3 and DNA repair pathways to overcome CDK4/6i resistance (Kettner et al., 2019). Whether such IL-6/STAT3-mediated EMT is dependent on CDK4 and/or CDK6 and not due to parallel compensation will need to be further investigated.

Overall, while two precise mechanistic studies have depicted CDK4 as crucial for stabilization of the EMT transcription factors SNAIL1 and ZEB1 (Fig. 3), the relationship between CDK4 and EMT remains highly complex, and the role of CDKs in EMT may not be as direct as that of other signaling pathways and transcription factors that explicitly regulate EMT.

In summary, evidence from the literature shows that the phosphorylation of cytoskeleton-associated proteins by CDK4 alters their activity and function (Fig. 3) and impact cancer cell dynamics at least through cytoskeleton remodeling and EMT decisions. Nevertheless, the role of CDK4 in the regulation of the cytoskeleton remains an emerging area of research. Hence, further investigations are needed to recapitulate the molecular mechanism and physiological implications of CDK4-mediated regulation of the cytoskeleton and its potential impact on metastatic cancers.

5. CDK4 in the tumor microenvironment

5.1. CDK4 and angiogenesis

Angiogenesis is a process that involves the growth of new blood vessels from preexisting vessels. It plays a crucial role in tumor progression and metastasis by supplying oxygen and nutrients to support tumor growth. Tumor growth and metastasis depend on angiogenesis, so this process is an important factor in the progression of cancer (Nishida et al., 2006). While the factors that govern this process are frequently produced by the tumor, their expression and the mechanisms governing angiogenesis-related proliferation are intertwined with the cell cycle (Baker et al., 2010).

There is some evidence that CDK4 may be partly involved in the control of angiogenesis through the use of the Min mouse system, which harbors a mutation in the APC gene and is thus predisposed to the formation of intestinal adenoma. When $Apc^{+/Min}$ mice were crossed with Ink4a/arf-/- mice, the offspring exhibited increased colorectal tumor angiogenesis, indicating that the dysregulation of CDK4 (due to the loss of Ink4a-mediated suppression) may contribute to enhanced angiogenesis [42]. In line with this initial evidence, another study showed increased angiogenesis in colorectal tumors from the offspring of $Apc^{+/Min}$ mice crossed with $CDK4^{R24C/R24C}$ mice, which expressed a form of CDK4 insensitive to INK4A [1]. These reports provided the initial evidence of the direct involvement of CDK in in vivo angiogenesis and emphasized its potential significance as a drug target for reducing or preventing angiogenesis in intestinal tumor development [1,42]. Mechanistically, one study suggested that CDK4 enhances angiogenesis *by* increasing E2F1 target genes [1].

CDK4 was recently suggested to have an indirect effect, as CDK6 was found to regulate tumor angiogenesis in melanoma in a CDK4-dependent manner (Kollmann et al., 2019). Indeed, CDK6 enhances the transcription of the angiogenic factor VEGF-A through cooperation with c-Jun (a vesicular endothelial growth factor). In the context of transcriptional control, CDK6 activity is influenced by the expression level and availability of CDK4. Mechanistically, CDK6 cooperates with the transcription factor c-JUN at the VEGF-A promoter (Kollmann et al., 2019). Paradoxically, this CDK4/6-mediated regulation of VEGF-A is not dependent on D-type cyclins, suggesting a mechanism independent of kinase activity (Kollmann et al., 2019). Finally, from a pharmacological standpoint, Roxyl-zv-5 J, a multitarget inhibitor of CDK4 and VEGFR2, has shown antitumor and antiangiogenic activity in xenograft models [56], validating the use of biological multitarget drug design.

In summary, CDK4 regulates some aspects of angiogenesis, and thereby may support tumor growth and metastasis. Importantly, whether this effect is mediated by the canonical role of CDK4 in cell cycle progression in endothelial cells or by an independent role remains to be critically addressed in the future. Notably, further study of the role of CDK4 in promoting the neovascularization of tumors is an interesting new avenue for new therapies. Evaluating whether CDK4/6i efficiency in metastatic cancers, notably HR+ , HER2-negative advanced/metastatic breast cancer (HR+/HER2- a/mBC), is partly due to this anti-angiogenic effect will be of interest for the cancer community.

5.2. CDK4 and the immune system

Immune checkpoint blockade is an established therapy for several cancer types, including melanoma and lung cancer. In particular, inhibition of the PD-1/PD-L1 pathway has proven efficient for some patients [18,96].

From the clinical point of view, most investigations into the role of CDK4 in the immune response utilize data from CDK4/6i treatment and relate to antitumor immunity in prostate, breast and lung cancers [9104]. These effects were proposed to be mediated by complementary effects on tumor cells and immune cells. First, CDK4/6is first showed a dual effect, both increasing tumor antigen presentation by tumor cells and suppressing the proliferation of regulatory T cells through the canonical RB-E2F pathway, resulting in the overall clearance of tumor cells [104]. Second, CDK4 has been linked to the regulation of PD-L1 expression in tumor cells [65,143]. Notably, PD-L1 protein abundance is regulated by CDK4-cyclin D and the Cullin 3–SPOP E3 ligase through proteasome-mediated degradation [143]. Consequently, the inhibition of CDK4/6 increased PD-L1 protein levels, promoting SPOP degradation and inactivation of the proteasome in primary human prostate cancer samples. Remarkably, the knockdown of only CDK4, but not CDK6, was able to induce PD-L1 expression, highlighting the specific importance of CDK4 relative to CDK6 in PD-L1 expression [143]. Moreover, canonical RB-dependent phosphorylation could also mediate PD-L1 expression in tumors. Indeed, RB phosphorylated at specific sites by CDK4 and CDK6 (serine 249 and threonine 252) was inversely correlated with PD-L1 expression in samples from prostate cancer patients [65]. Interestingly, phosphorylated RB interacts with NF-κB, transcriptionally inhibiting the expression of PD-L1 in prostate cancer cells [65]. When CDK4/6 were inhibited, the RB-mediated repression of NF-κB activity was released, and therefore, the expression of PD-L1 increased [65]. These effects of CDK4/6is are apparently paradoxical since the increased expression of PD-L1 resulted in blockade of the immune response against tumor cells. However, the inhibition of CDK4/6 may mediate the conversion of immunologically cold to hot tumors, rendering them sensitive to immune checkpoint therapy [62,92]. Finally, and beyond PD-L1, CDK4 and CDK6 blockade can also induce an antitumor response through DNA damage and the cGAS-STING pathway [35]. Remarkably, Cdk4 or Cdk6 deficiency triggered an increased level of endogenous

DNA damage, resulting in activation of the cGAS-STING signaling pathway to activate the type I interferon response, potentially activating CD8 T cells [35].

CDK4/6i also synergize with other classic antitumor agents, such as chemotherapeutic agents, demonstrating enhanced immunogenic responses [39] [117,138,145]. Another therapeutic strategy consists of using CDK4/6is to increase the efficiency of oncolytic virus to increase immunogenicity against glioblastoma cancer cells [139].

Altogether, these studies highlight the importance of CDK4 and CDK6 in modulating cancer immune surveillance in both tumor and immune cells. On the one hand, CDK4/6 decrease tumor antigen presentation, the type I interferon response, and PD-L1 expression in tumor cells. On the other hand, CDK4/6 impact the proliferation and activity of T cells. Thus, the effects of CDK4/6 inhibition largely favor global antitumor immunity through various complementary CDK4- and CDK6-mediated effects. Importantly and because most studies have used CDK4/6is, the relative contributions of CDK4 and CDK6 in regulating tumor immunity are not yet clearly elucidated and would require additional specific genetic studies.

6. Concluding remarks

CDK4 is especially important for regulation of the G1/S transition of the cell cycle in response to growth factors and other proliferative stimuli [43]. According to CDK4 and CDK6 expression levels, CDK4 may be dispensable for cellular proliferation because of a compensatory effect of CDK6 or eventually CDK2 [81]. Nevertheless, the use of CDK4/6is in the treatment of some types of breast cancer has revealed that CDK4 and CDK6 display other functions in cancer cells. While some targets of CDK4 and CDK6 overlap, others seem to be CDK4 specific, such as p53-R249S or SPOP (Table 2), and still others are probably CDK6 specific. Importantly, the relative contributions of CDK4-mediated functions may also depend on cancer subtype and grade. We can speculate that other functions of CDK4 could be as essential as regulation of the cell cycle and contribute to some aspects of tumorigenesis. In this review, we have revised some of these additional functions of CDK4, including most notably the control of metabolism, cell fate (senescence, cell death, autophagy), cell migration and other cellular functions related to the cytoskeleton and immune functions.

The increasing clinical use of CDK4/6i treatment points out the need to more precisely investigate the different outcomes controlled by CDK4 and CDK6. A better understanding of the mechanisms of CDK4 and CDK6 would help to design cotargeted and synergetic therapies but may also extend CDK4/6i use to a broader spectrum of diseases. The finding that CDK4 is a major regulator of cellular metabolism provide new tools to design innovative therapies for the treatment of cancer and, perhaps, metabolic diseases. The same is true for the role of CDK4 in cellular senescence or in the immune system, which offers the opportunity to include CDK4/6is in senotherapy or immunotherapy. Finally, exploring these noncanonical functions may also elucidated the potential side effects of CDK4/6is.

In conclusion, gaining a comprehensive understanding of the diverse functions of CDK4 provides valuable insights for the development of targeted therapies for cancer and other diseases. Further exploration of the roles of CDKs in these non-cell cycle-associated functions is warranted to unlock the full therapeutic potential of CDK inhibitors in cancer and beyond. Fig. 4.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

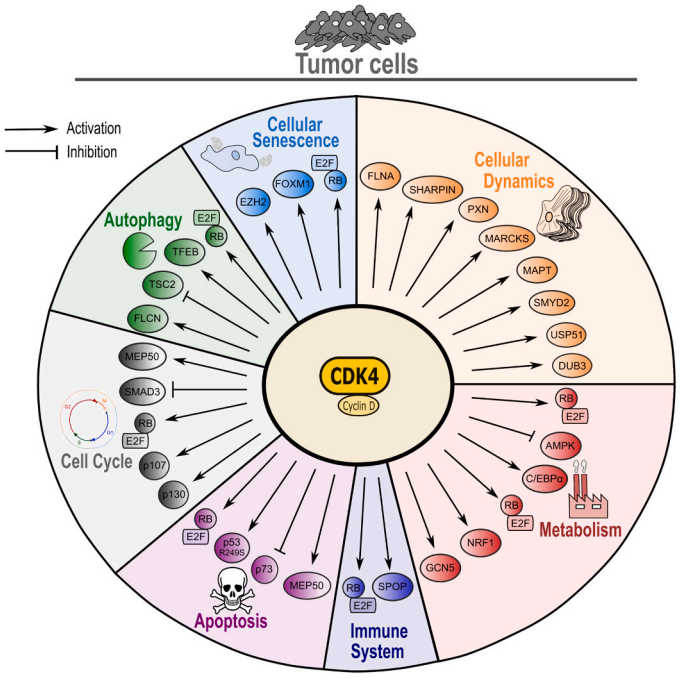

Fig. 4. CDK4 at the crossroad of multiple biological processes in tumor cells. CDK4 participates to tumorigenesis through multiple cell-cycle and non-cell cycle activities. CDK4 regulates through phosphorylation an important amount of proteins, leading to their subsequent activation, inhibition and/or stabilization. Altogether, these intermediate substrates mediate CDK4-dependent functions in Cell Cycle (Grey), Metabolism (Red), Cellular senescence (Light Blue), Autophagy (Green), Apoptosis (Purple), Cellular Dynamics (Orange) and Immune system modulation (Dark Blue).

Data Availability

No data was used for the research described in the article.

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