Targeting Cell-cycle Machinery in Cancer

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SUMMARY

Abnormal activity of the core cell-cycle machinery is seen in essentially all tumor types and represents a driving force of tumorigenesis. Recent studies revealed that cell-cycle proteins regulate a wide range of cellular functions, in addition to promoting cell division. With the clinical success of CDK4/6 inhibitors, it is becoming increasingly clear that targeting individual cell-cycle components may represent an effective anti-cancer strategy. Here, we discuss the potential of inhibiting different cell-cycle proteins for cancer therapy.

Introduction

The cell-cycle is a highly regulated process enabling cell growth, duplication of genetic material and cell division. Progression from one cell-cycle phase to another is driven by the core cell-cycle machinery operating in cell nucleus. This machinery is composed of proteins called cyclins and their catalytic partners, the cyclin-dependent kinases (CDKs) (Malumbres and Barbacid, 2009). Different cyclin-CDK complexes become activated in specific phases of the cell-cycle and phosphorylate their target proteins (Box 1, Figure 1).

In normal cells, the activity of cell-cycle proteins is tightly controlled by their cell-cycle-specific transcription, protein degradation (Box 2, Figure 2), as well as by several CDK-inhibitor proteins (Sherr and Roberts, 2004). All these mechanisms are frequently dysregulated in human cancers, resulting in aberrant activation of cell-cycle proteins. Indeed, genetic lesions within the core cell-cycle machinery, resulting in its hyperactivation, play a causative role in development of most tumor types (Malumbres and Barbacid, 2009). For these reasons, targeting cell-cycle proteins seems to represent an effective way of halting tumor growth.

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AUTHOR CONTRIBUTIONS

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The enthusiasm for inhibiting cyclin-CDK kinases was initially mitigated by the widely held belief that these proteins are essential for proliferation of normal non-transformed cells. However, genetic experiments revealed that individual cyclins and CDKs are largely dispensable for proliferation of normal tissues (Sherr and Roberts, 2004). In contrast, these proteins are essential for proliferation of specific cancer types, depending on the genetic lesions they carry (Malumbres and Barbacid, 2009). The most clear-cut success of targeting cell-cycle machinery are inhibitors of CDK4 and CDK6. Introduction of these compounds to the clinics represented a breakthrough in the treatment of breast cancers, and will likely have a profound effect on treatment of many other tumor types (Alvarez-Fernandez and Malumbres, 2020). Quite unexpectedly, recent work revealed that cell-cycle proteins play additional roles in tumor development by affecting not only tumor cells, but also its microenvironment, for example by modulating the anti-tumor immune response (Deng et al., 2018; Goel et al., 2017; Zhang et al., 2018). Hence, inhibition of these proteins is likely to affect several aspects of the tumorigenic process.

Despite the current success with CDK4/6 inhibitors (CDK4/6i), targeting cell-cycle proteins for cancer treatment is still at its infancy. In this review, we discuss the current state of affairs and provide our view about the most promising future directions.

D-type Cyclins and CDK4/6 in Cancer

Amplification or rearrangements of genes encoding D-cyclins (*CCND1, CCND2, CCND3*), CDK4 or CDK6, and overexpression of these proteins was documented in a very large number of tumor types (Cancer Genome Atlas Research, 2013). Also, inactivation of CDK4/6-inhibitor proteins, in particular p16^{INK4A}, represents a frequent feature of many tumor types (Figure 3, (Cancer Genome Atlas Research, 2013)). All these molecular lesions result in hyperactivation of cyclin D-CDK4/6 kinase in tumor cells and abrogation of RB1-mediated suppression of the cell-cycle. This, in turn, drives uncontrolled cell proliferation that becomes uncoupled from the external mitogenic and anti-mitogenic signals (Alvarez-Fernandez and Malumbres, 2020).

Targeted overexpression of D-cyclins or CDK4 in transgenic mice resulted in development of tumors, confirming the role of deregulated cyclin D-CDK4/6 kinases in tumorigenesis (Otto and Sicinski, 2017). Conversely, genetic ablation of D-cyclins, CDK4 or CDK6 reduced or prevented formation of tumors. For example, mice lacking cyclin D1, CDK4, or expressing kinase-inactive cyclin D1-CDK4/6 complexes are resistant to development of breast cancers (Alvarez-Fernandez and Malumbres, 2020). Importantly, an acute and global shutdown of cyclin D1 in mice bearing Her2-driven mammary carcinomas halted tumor growth and triggered senescence exclusively in tumor cells, while having no obvious impact on animals' physiology (Choi et al., 2012). Cell-cycle arrest and senescence of tumor cells was also seen upon ablation of Cdk4 in mice with Kras-driven lung cancers (Puyol et al., 2010). Collectively, these observations documented the requirement for cyclin D-CDK4/6 activity in tumor maintenance. They also revealed a differential reliance of tumor cells - but not of most normal cell types - on individual D-cyclins or CDKs for proliferation.

Intriguingly, analyses of a mouse glioma model revealed that cyclin D1-Cdk4 contributes to progression of glial tumors also by acting in non-tumor cells. Specifically, ablation of cyclin D1 or Cdk4 impeded activation of tumor-associated microglia, thereby inhibiting tumorigenesis (Ciznadija et al., 2011). Others reported increased expression of cyclin D1 in breast cancer-associated stromal cells. Importantly, overexpression of cyclin D1 in stromal fibroblasts promoted breast tumorigenesis, via secretion of pro-inflammatory cytokines and osteopontin (Pestell et al., 2017). These observations, together with a recent demonstration of a role for CDK4/6 in anti-tumor immunity (see below) reveal that cyclin D-CDK4/6 affects tumorigenesis both by tumor-intrinsic mechanisms as well as by affecting tumor microenvironment.

CDK4/6 Inhibitors

The first CDK4/6-specific inhibitor – palbociclib – was synthesized in 2001 and showed efficacy against a wide range of human cancer cell lines and xenografts, including breast cancers (Fry et al., 2004). Finn et al. (2009) demonstrated that breast cancer cell lines representing hormone receptor-positive (HR^+), luminal-type mammary carcinomas were most sensitive to CDK4/6 inhibition This evidence, together with mouse genetic experiments described above, led to the use of CDK4/6i (palbociclib, ribociclib and abemaciclib) in clinical trials for breast cancer patients (Table 1, Figure 4).

The very first clinical trial began in 2007 and demonstrated palbociclib's efficacy against mantle cell lymphoma (Leonard et al., 2012). Starting in 2015, phase II and III clinical trials have been carried out with palbociclib (PALOMA series), ribociclib (MONALEESA) and abemaciclib (MONARCH) (Alvarez-Fernandez and Malumbres, 2020). These trials investigated the utility of combining CDK4/6i with standard endocrine-therapy (an estrogen receptor-antagonist fulvestrant or an aromatase-inhibitor, letrozole) in patients with advanced HR^+/$HER2^-$ breast cancers. Addition of either of these CDK4/6i dramatically extended progression-free survival and prolonged the overall survival of patients. Moreover, abemaciclib extended progression-free survival when used as monotherapy in women with HR^+/$HER2^-$ metastatic breast cancer (Finn et al., 2015; Gao et al., 2020). Consequently, all three CDK4/6i were approved by the US Food and Drug Administration (FDA) for treatment of patients with HR^+/$HER2^-$ metastatic or advanced breast cancer.

Palbociclib and ribociclib represent highly specific inhibitors of CDK4 and CDK6, while abemaciclib also inhibits several other kinases (Klein et al., 2018). For palbociclib and ribociclib, neutropenia, thrombocytopenia and anemia represent the rate-limiting toxicities (Finn et al., 2016; Hortobagyi et al., 2016). This likely represents an on-target effect due to cyclin D3-CDK6 inhibition in hematopoietic cells (Sicinska et al., 2003). In contrast, these side-effects are less pronounced in patients treated with abemaciclib, which may be caused by the higher potency of this compound in inhibiting CDK4 rather than CDK6 (Sledge et al., 2017). For this reason, abemaciclib can be administered continuously, while palbociclib and ribociclib require an intermittent dosing schedule (Klein et al., 2018). The most frequent side-effects in abemaciclib-treated patients are gastrointestinal symptoms (Sledge et al., 2017). While the molecular mechanism is unknown, it was postulated that inhibition of CDK9 by abemaciclib may be responsible for this phenomenon (Chen et al., 2016).

It remains to be seen whether the ability of abemaciclib to inhibit other kinases represents an advantage or disadvantage over the more selective CDK4/6i. A recent PALLAS study revealed that addition of palbociclib to adjuvant endocrine therapy in patients with early-stage HR$^+$/HER2$^-$ breast cancer did not improve invasive disease-free survival, compared with adjuvant endocrine therapy alone (Mayer et al., 2021). In contrast, a similar study with abemaciclib reported a significant prolongation of invasive disease-free survival (Johnston et al., 2020). It is possible that the ability of abemaciclib to inhibit other kinases may be responsible for the therapeutic effect. It is also likely that the efficacy of abemaciclib as a single-agent is in part due to its off-target effects.

An important issue for treatment with CDK4/6i it to develop biomarkers of sensitivity and resistance. It has been widely documented that tumor cells which lost RB1 expression do not arrest their proliferation upon CDK4/6 inhibition (O'Leary et al., 2016). Indeed, intact RB1 status represents the best predictive feature for CDK4/6i efficacy. Other predictors are not clear. Cancers with genomic activation of *CCND1–3* genes were reported to be particularly susceptible to CDK4/6i (Gong et al., 2017). However, in *post hoc* analyses from the PALOMA trials, neither *CCND1* amplification nor the levels of cyclin D1 mRNA correlated with responsiveness to CDK4/6i (Finn et al., 2020). The predictive value of CDK4 gene amplification and protein overexpression is unclear, with different studies linking these lesions to increased sensitivity or resistance (Alvarez-Fernandez and Malumbres, 2020). Immunohistochemical detection of Thr172-phosphorylated CDK4 (an activating phosphorylation carried out by the CDK-activating kinase, CAK) in breast cancer was shown to predict susceptibility to palbociclib treatment (Raspé et al., 2017). This observation may provide a predictive marker, which can be assessed in tumor samples. On the other hand, high levels of cyclin E1 mRNA in metastatic lesions were shown to correlate with resistance to palbociclib (Turner et al., 2019). Despite the progress, more markers are needed to reliably predict patients' response to CDK4/6 inhibition.

Consistent with the growth-promoting function of cyclin D-CDK4/6, treatment with CDK4/6i causes cell-cycle arrest of tumor cells. In addition, some tumors respond to CDK4/6 inhibition by undergoing senescence (Alvarez-Fernandez and Malumbres, 2020). Several different mechanisms involving RB1 as well as FOXM1 (which also represents CDK4/6 phosphorylation substrate), were proposed to explain this phenomenon (Alvarez-Fernandez and Malumbres, 2020). Kovatcheva et al., (2017) postulated that expression of chromatin-remodeling enzyme ATRX determines the choice between quiescence and senescence in response to CDK4/6 inhibition.

Inhibition of CDK4/6 was also shown to affect tumor metabolism. Wang et al. (2017) observed that in tumor types expressing high levels of cyclin D3-CDK6 (such as T-ALL), inhibition of CDK4/6 reduces the flow of glucose-derived carbon into the pentose phosphate and serine pathways, thereby depleting antioxidants NADPH and glutathione. This, in turn, increases the levels of reactive oxygen species and causes tumor cell apoptosis. Franco et al. (2016) reported that treatment with CDK4/6i induces tumor cell metabolic reprogramming and increases the rate of oxidative phosphorylation.

Several recent studies revealed that treatment with CDK4/6i modulates the anti-tumor immune response of the host, via multiple mechanisms. First, inhibition of CDK4/6 preferentially impedes proliferation of immuno-inhibitory CD4$^+$FOXP3$^+$ Treg cells, with smaller effect on other T cell subtypes, such as cytotoxic CD8$^+$ T cells (Deng et al., 2018; Goel et al., 2017; Ruscetti et al., 2018; Schaer et al., 2018). This decreases the Treg to CD8$^+$ ratio of intratumoral T cells and facilitates tumor cell killing by the CD8$^+$ cells. Second, inhibition of CDK4/6 awakens expression of endogenous retroviral elements in tumor cells, resulting in increased production of type-III interferons (Goel et al., 2017; Hurvitz et al., 2020; Schaer et al., 2018). This, in turn, increases presentation of tumor antigens. Third, inhibition of CDK4/6 triggers dephosphorylation and nuclear translocation of NFAT transcription factors, resulting in T cell activation, which enhances the anti-tumor immune response (Deng et al., 2018; Schaer et al., 2018). Lastly, inhibition of CDK4/6 causes upregulation of a checkpoint protein PD-L1 in tumor cells (Jerby-Arnon et al., 2018; Jin et al., 2019; Zhang et al., 2018). Importantly, studies using mouse cancer allografts revealed that treatment with CDK4/6i strongly synergizes with anti-PD-1/PD-L1 immune checkpoint blockade (Deng et al., 2018; Goel et al., 2017; Jerby-Arnon et al., 2018; Zhang et al., 2018). Since the action of CDK4/6i on host immune cells does not dependent of tumor's RB1 status, these findings raise a possibility that CDK4/6i could be used to stimulate host antitumor immunity and to boost the effectiveness of immune checkpoint blockade in case RB1-negative tumors.

Inhibition of CDK4/6 was also shown to impair DNA damage repair through homologous recombination, following cytotoxic chemotherapy. Salvador-Barbero et al. (2020) demonstrated that sequential treatment of pancreatic cancers first with taxanes (or other chemotherapeutic compounds) followed by CDK4/6i strongly potentiates the anti-tumor effect of the former due to repression of DNA repair machinery.

The intrinsic (pre-existing) or acquired resistance to CDK4/6i represents the major obstacle in their clinical effectiveness. Since this issue was the subject of a recent review (Alvarez-Fernandez and Malumbres, 2020), we will only briefly mention the major mechanisms. These include: (i) inactivation of RB1; (ii) overexpression of CDK6 through amplification of the *CDK6* gene or via other mechanisms; (iii) activation of cyclin E-CDK2 that by-passes cyclin D-CDK4/6; (iv) activation of FGFR1/2 or EGFR/ERBB2 → RAS-RAF-MEK-ERK or PI3K-AKT signaling (Alvarez-Fernandez and Malumbres, 2020); (v) lysosomal sequestration of CDK4/6i (Fassl et al., 2020; Llanos et al., 2019); (vi) activation of AURKA identified by a recent genome-wide study (Wander et al., 2020).

While CDK4/6i represent a breakthrough in breast cancer treatment, they are now being tested in clinical trials for many different cancer types. These trials are exploring various combination treatments involving CDK4/6i; several additional synergies with CDK4/6 inhibitors have been demonstrated in pre-clinical studies and will likely enter the clinics (Malumbres, 2019). One of the main challenges will be to develop better biomarkers for predicting sensitivity and resistance of tumors to CDK4/6i and the efficacy of combinatorial treatments against tumors carrying defined sets of genomic lesions. Efforts should be made to develop more selective compounds that inhibit only CDK4 or CDK6, as agents that selectively inhibit CDK4 could be potentially used at higher doses without encountering

bone marrow toxicity. Another possibility is the use of degrader compounds that would degrade CDK4 and/or CDK6 (Box 3). Such compounds are expected not only to inhibit CDK4/6 kinase, but also to liberate cyclin D-CDK4/6-bound $p21^{CIP1}/p27^{KIP1}$ inhibitors, which would then inhibit cyclin E-CDK2.

Cyclin E

Overexpression of E-cyclins and hyperactivation of cyclin E-CDK2 kinase have been documented in many human tumors (Figure 3). Upregulation of cyclin E can be driven by *CCNE1* or *CCNE2* gene amplification, increased activity of c-MYC or E2F transcription factors (which transcriptionally induce cyclin E), or inactivation of the F-box protein, FBXW7 (CDC4), which mediates cyclin E degradation (Hwang and Clurman, 2005). Moreover, several tumor types express proteolytically cleaved, 'low-molecular weight' (LMW) cyclin E (Caruso et al., 2018). LMW-cyclin E binds CDK2 with an increased affinity and forms hyperactive cyclin E-CDK2 complexes that are resistant to inhibition by $p21^{CIP1}$ and $p27^{KIP1}$ (Porter et al., 2001; Wingate et al., 2005).

In most cases studied, overexpression of cyclin E conferred poor clinical outcome (Hwang and Clurman, 2005) and was linked to resistance to several therapeutic compounds. For instance, elevated cyclin E levels represent a mechanism of resistance against trastuzumab in $HER2^+$ breast cancers (Scaltriti et al., 2011). In HR^+ breast cancers, the presence of cytoplasmic cyclin E correlates with resistance to aromatase inhibitors as well as to treatment with anti-estrogens plus CDK4/6i (Alvarez-Fernandez and Malumbres, 2020). Amplification of *CCNE1* confers resistance to platinum-based chemotherapy in high-grade serous ovarian cancer (Etemadmoghadam et al., 2009). Also, overexpression of cyclin E2 was implicated as a mechanism of resistance to the endocrine therapy (Caldon et al., 2012).

Analyses of mouse cancer models confirmed the causative role of cyclin E-overexpression in tumorigenesis. Transgenic mice overexpressing cyclin E1 in T cells develop clonal lymphomas upon treatment with N-methylnitrosourea, or when crossed with $p27^{Kip1}$-null mice (Geisen et al., 2003; Karsunky et al., 1999). Mice engineered to overexpress cyclin E1 in mammary glands develop mammary gland hyperplasias that progress at a low rate to mammary carcinomas (Bortner and Rosenberg, 1997). Mammary-specific expression of LMW-cyclin E1 triggers breast cancer development in a much higher fraction of mice than that of full-length cyclin E1. Importantly, the tumorigenic function of LMW cyclin E critically depends on Cdk2, as genetic ablation of Cdk2 completely blocked breast cancer occurrence in LMW-cyclin E transgenic mice (Akli et al., 2011)

While overexpression of cyclin E causes tumorigenesis, genetic ablation of E-cyclins was shown to impede tumor formation. Constitutive knockout of cyclin E prevented development of hepatocellular carcinomas, while an acute shutdown of E-cyclins in tumor-bearing mice blocked liver cancer progression (Geng et al., 2018; Sonntag et al., 2018). Moreover, mice with reduced levels of E-cyclins (cyclin $E1^{+/-}E2^{-/-}$ and $E1^{-/-}E2^{+/-}$) show decreased susceptibility to Myc-driven breast cancers (Zhou et al., 2020).

It is not entirely clear how overexpressed cyclin E contributes to tumorigenesis. Given its well-established role in cell-cycle progression, a likely scenario is that overexpressed cyclin E drives uncontrolled cell proliferation. However, depletion of cyclin E in cyclin E1-amplified ovarian cancer cell lines triggered apoptosis, suggesting that cyclin E may play a pro-survival role in these cells (Etemadmoghadam et al., 2010). High levels of cyclin E in tumor cells were also shown to lead to genomic and chromosomal instability (Teixeira and Reed, 2017). Overexpressed cyclin E causes replication stress in S phase and chromosome segregation errors in M phase, resulting in genomic instability. Human mammary epithelial cells overexpressing cyclin E were shown to enter mitosis with short unreplicated genomic segments at a small number of specific loci, leading to anaphase anomalies and ultimately deletions. Interestingly, incompletely replicated regions were preferentially located at late-replicating domains and regions characterized by a paucity of replication origins (Teixeira and Reed, 2017). Other studies implicated overexpressed cyclin E in inhibiting APC[CDH1] activity, resulting in the abnormal accumulation of APC[CDH1] substrates as cells enter mitosis. This, in turn, impairs progression through mitosis, thereby contributing to polyploidy (Caldon et al., 2013). Overexpression of cyclin E was also implicated in erroneous MCM-loading, interference with nucleotide biosynthesis, induction of DNA-damage in stalled forks, uncontrolled DNA-replication firing and replication-transcription collisions, and all these mechanisms may contribute to genome instability (Mazumder et al., 2007; Teixeira and Reed, 2017). Another possible role of cyclin E in promoting tumorigenesis was provided by the observations that cyclin E-CDK2 phosphorylates c-MYC, and that this event prevents oncogene-induced senescence (Campaner et al., 2010; Hydbring et al., 2010).

All these functions of cyclin E are carried out in the cell nucleus, consistent with nuclear localization of full-length cyclin E. Intriguingly, proteolytically cleaved LMW-cyclin E is localized in the cytoplasm (Delk et al., 2009), suggesting that cyclin E may promote tumorigenesis by acting in this compartment. Lucenay et al. (2016) reported that cytoplasmic cyclin E physically interacts with ATP citrate lyase, an enzyme in *de novo* lipogenesis pathway, and enhances its enzymatic activity.

It is currently assumed that cyclin E exerts its oncogenic functions by activating its catalytic partner, CDK2. However, some groups proposed that cyclin E might play a kinase-independent role in driving proliferation of tumor cells. Sweeney et al. (1998) reported that in breast cancer cells, there is no evident relationship between CDK2 activity and cyclin E expression, with most of cyclin E residing in inactive complexes containing CDK-inhibitors p21[CIP1] and p27[KIP1]. In another study, cyclin E mutants which were unable to activate CDK2, transformed rat embryonic fibroblasts in collaboration with H-Ras (Geisen et al., 2003). Cyclin E was also shown to localize to centrosomes and to promote S phase entry in a CDK2-independent fashion (Matsumoto and Maller, 2004). Ectopic expression of cyclin E mutant deficient in activating CDK2 but retaining the centrosomal localization sequence accelerated S phase entry and promoted polyploidy (Matsumoto and Maller, 2004). Recent studies of mouse liver cancer models revealed that while cyclin E is essential for tumor progression, Cdk2 is not (Geng et al., 2018; Sonntag et al., 2018). Consistent with these findings, *in vitro* cultured human liver cancer cell lines require cyclin E protein, but not cyclin E-associated kinase, for tumor cell proliferation (Geng et al., 2018). These

observations raise a possibility that cyclin E might play a kinase-independent role in tumorigenesis. However, the molecular basis of such possible function remains unknown.

Targeting cyclin E may represent an attractive therapeutic strategy. Indeed, depletion of cyclin E arrested proliferation of cancer cell lines representing many tumor types (Hwang and Clurman, 2005). Importantly, mouse genetic experiments revealed that E-type cyclins are dispensable for proliferation of normal, non-transformed tissues of postnatal mice (Geng et al., 2007). Collectively, these observations indicate that targeting of cyclin E may selectively inhibit the proliferation of tumor cells, while sparing normal tissues. One possible approach to target cyclin E could employ 'degrader' compounds (Lai and Crews, 2017) (Box 3), which would selectively deplete E-cyclins. Such approach would extinguish all cyclin E functions, thereby assuring the therapeutic effect.

CDK2

While the role of cyclin E in tumorigenesis is well established, the requirement for CDK2 in tumor growth is still the subject of debate. Mouse knockout experiments revealed that CDK2 is not required for normal development and physiologic cell proliferation, except for meiotic divisions in male and female germ cells (Berthet et al., 2003; Ortega et al., 2003).

Analyses of several human cancer cell lines revealed that CDK2 is dispensable for their proliferation (Tetsu and McCormick, 2003). In contrast, CDK2 is required for proliferation of melanoma cells, where its expression is regulated by the melanocyte lineage transcription factor MITF (Desai et al., 2013). CDK2 also plays an essential function in ovarian cancer cells with *CCNE1* amplification. Depletion of CDK2 or inhibition of CDK2 kinase in these cells arrested tumor cell proliferation and in several instances triggered tumor cell apoptosis (Au-Yeung et al., 2017; Etemadmoghadam et al., 2013; Yang et al., 2015). Inhibition of CDK2 also impeded ovarian cancer tumorigenesis *in vivo* (Au-Yeung et al., 2017), which was attributed to suppression of metastatic colonization (Yang et al., 2015). In diffuse large B-cell lymphomas, CDK2 was shown to promote tumor cell survival by maintaining the expression of the anti-apoptotic factor MCL1. Depletion of CDK2 resulted in down-regulation of MCL1 and triggered apoptosis of tumor cells (Faber and Chiles, 2007). Silencing of CDK2 also caused p53-dependent apoptosis of neuroblastoma cells with MYCN-amplification and overexpression (Molenaar et al., 2009). High levels of CDK2 were observed in glioblastoma multiforme, where CDK2 was shown to be essential for cell proliferation and confer resistance to radiation, as inhibition of CDK2 increased tumor cell radiosensitivity (Wang et al., 2016).

Hyperactivation of CDK2 – through different molecular mechanisms – was shown to represent one of the mechanisms of breast cancer resistance to CDK4/6i (Alvarez-Fernandez and Malumbres, 2020). Hence, inhibition of CDK2 may represent a therapeutic strategy to block proliferation of CDK4/6i-resistant tumors. Indeed, depletion or inhibition of CDK2 restored the sensitivity of resistant breast cancer cells to CDK4/6i (Fassl et al., 2020; Herrera-Abreu et al., 2016).

Several studies suggested the therapeutic value of combining CDK2 inhibition with other treatments. For example, in RB1-negative cyclin E-overexpressing triple-negative breast cancers (TNBC), inhibition of CDK2 was shown to act synergistically with chemotherapy (Rao et al., 2017). In medulloblastoma, CDK2 inhibition combined with targeting of MYC using BET bromodomain inhibitors synergistically suppressed tumor progression by causing cell-cycle arrest and apoptosis (Bolin et al., 2018). Combined inhibition of phosphatidylinositol 3-kinase (PI3K) and CDK2 induced cell death and enhanced antitumor activity in colorectal cancer xenografts (Beale et al., 2016). Treatment with CDK2 inhibitors led to a dramatic decrease in proliferation and enhanced apoptosis of cyclin E-overexpressing trastuzumab-resistant breast cancer cell lines and significantly reduced tumor growth of trastuzumab-resistant xenografts (Scaltriti et al., 2011). Depletion of CDK2 blocked cell-cycle progression, increased cell death and reduced colony formation in tamoxifen-resistant breast cancer cell lines (Johnson et al., 2010). Azimi et al. (2018) reported that upregulation of CDK2 confers resistance to BRAF and Hsp90 inhibitors in melanoma cells, and that CDK2 depletion sensitizes cells to these inhibitors. Since no CDK2-specific inhibitors are available, several of the studies utilized non-specific compounds that target other kinases. Other studies depleted CDK2 (using siRNAs or sRNAs), which may result different outcomes than kinase inhibition (Horiuchi et al., 2012a).

In addition to driving cell-cycle progression, CDK2 was shown to play other functions, which may be relevant in the context of therapeutic CDK2 inhibition. A recent study demonstrated that CDK2 phosphorylates proteins that regulate histone modifications, chromatin, transcription, and RNA/DNA metabolism (Chi et al., 2020). It remains to be seen how inhibition of CDK2 would affect these processes in cancer cells. Moreover, CDK2 was shown to phosphorylate and negatively regulate the stability of FOXP3, the master regulator of Tregs (Morawski et al., 2013). Hence, inhibition of CDK2 may increase the number of immunosuppressive Treg cells, which would impede the anti-tumor immune response. Despite this concern, the demonstrated essential role of CDK2 in certain tumor types justifies the efforts to develop specific CDK2 inhibitors. Such inhibitors would need to selectively target CDK2, but not a related kinase CDK1, given the essential function of the latter in cell proliferation (see below).

Cyclin A

Two A-type cyclins operate in mammalian cells, the ubiquitously expressed cyclin A2 and testes-specific cyclin A1 (Martínez-Alonso and Malumbres, 2020). Cyclin A2 expression is upregulated in many tumor types (Figure 3). In contrast, high levels of cyclin A1 were documented in a much narrower set of tumors, mainly in myeloid leukemias and in testicular and endometrial cancers (Horie et al., 2019; Yasmeen et al., 2003). Increased levels of cyclin A2 were shown to correlate with early relapse and poor overall survival in hepatocellular carcinoma and endometrioid adenocarcinoma (Santala et al., 2014; Yam et al., 2002). In contrast, other authors reported that cancers with low levels of cyclin A2 were more aggressive than those with high cyclin A2 expression (Aaltomaa et al., 1999; Bendris et al., 2014; Mashal et al., 1996). Arsic et al. (2012) observed that depletion of cyclin A2 enhanced the invasiveness of transformed cells via down-regulation of RhoA activity. Consistent with

these observations, colorectal cancer metastases were found to express decreased levels of cyclin A2, as compared to primary tumors.

Bayard et al. (2018) reported the presence of a distinct subgroup of hepatocellular carcinomas containing genomic activation of the *CCNA2* gene driven by diverse mechanisms. These include structural rearrangements resulting in *CCNA2* fusion with several different genes, as well as viral (HBV or AAV2) insertions within the *CCNA2* gene. Interestingly, viral insertions induce the expression of abnormal *CCNA2* transcripts, predicted to generate a truncated cyclin A2 protein. Hepatocellular carcinomas displaying *CCNA2* activation share the molecular features with liver cancers overexpressing cyclin E1, and together they define a distinct subgroup of tumors characterized by a unique gene expression profile indicative of E2F and ATR pathway activation. Cyclin A2/E1-overexpressing tumors display a specific structural rearrangement signature. These rearrangements are enriched at active chromatin regions and were shown to activate *TERT* promoter, which likely contributes to tumorigenesis (Bayard et al., 2018). A recent analysis of gastric cancer cell lines revealed that cyclin A2 expression is elevated in *KRAS*-mutant gastric tumors, causing an increased sensitivity to PLK1-inhibitors due to mitotic catastrophe and apoptosis upon PLK1-inhibition. These results suggest that cyclin A2 levels may serve as biomarker of PLK1-inhibitor sensitivity (Lee et al., 2020).

Analyses of most mouse cancer models support a pro-tumorigenic role of overexpressed cyclin A2. Transgenic mice overexpressing cyclin A2 in mammary glands exhibited hyperplasia and nuclear abnormalities suggestive of pre-neoplastic alterations (Yam et al., 2002). Conversely, deletion of cyclin A2 in oncogene-transformed mouse embryonic fibroblasts suppressed their tumorigenic potential (Gopinathan et al., 2014). Moreover, hepatocyte-specific ablation of cyclin A2 significantly delayed incidence of liver cancers triggered by expression of oncogenic Ras plus p53-silencing (Gopinathan et al., 2014). Transgenic mice overexpressing cyclin A1 in the myeloid lineage displayed abnormal myelopoiesis; however cyclin A1 overexpression alone was not sufficient to induce myeloid leukemia (Yam et al., 2002). In contrast to these findings, mice with hypomorphic *CCNA2* alleles were more susceptible to spontaneous and induced tumor formation, particularly in the lung. Splenocytes and fibroblasts derived from these animals displayed increased chromosomal instability and chromosome segregation defects (Kanakkanthara et al., 2016). These results indicate that cyclin A2-insufficiency may promote neoplastic transformation.

The full range of molecular functions of cyclin A in tumorigenesis remains unclear. Elevated cyclin A levels may accelerate cell-cycle progression. Moreover, cyclin A2-CDK2 directly phosphorylates AKT, promoting its oncogenic function (Liu et al., 2014). Cyclin A2 and its associated CDK activity are required for the induction of progesterone receptor target genes in breast cancer cells (Moore et al., 2014). A tumor-suppressive role for cyclin A was suggested by the observations that cyclin A2-CDK2 phosphorylates p53 protein and stimulates its sequence-specific DNA-binding (Wang and Prives, 1995). Cyclin A2-CDK1 and A2-CDK2 can also phosphorylate MDM2, which weakens its inhibitory interaction with p53 (Zhang and Prives, 2001). These two mechanisms are expected to increase p53 activity in cyclin A2-overexpressing cells.

It is not clear whether targeting cyclin A2 would represent a therapeutic option, given the essential requirement for cyclin A in adult organisms. Conditional ablation of cyclin A in mouse hematopoietic lineage resulted in a rapid lethality, indicating that a global inhibition of cyclin A would likely have life-threatening consequences (Kalaszczynska et al., 2009). Given that cyclin A can activate both CDK2 and CDK1, it is possible that a specific inhibition of cyclin A2-CDK2 (or A2-CDK1) may have a more selective effect on cancer cells.

DBF4–CDC7

Overexpression of CDC7 was documented in a wide range of human malignancies (Figure 3). In most of the tumor types studied, CDC7 overexpression correlates with poor clinical outcome and aggressive tumor phenotype (Montagnoli et al., 2010). Intriguingly, overexpression of CDC7 was found predominantly in tumors or tumor cell lines with mutant p53 (Bonte et al., 2008). Importantly, human cancer cells with mutated p53 were reported to be particularly sensitive to CDC7-inhibition. Depletion/inhibition of CDC7 using si/shRNAs or chemical CDC7i triggered apoptosis of p53-mutant tumor cells. In contrast, cells expressing wild-type p53 responded to CDC7 depletion/inhibition by undergoing a transient cell-cycle arrest (Montagnoli et al., 2004; Rodriguez-Acebes et al., 2010). It has been argued that inhibition of CDC7 in p53 wild-type cells triggers p53-dependent cell-cycle arrest. In contrast, p53-mutant cancer cells attempt to continue DNA synthesis upon CDC7-inhibition, which results DNA-damage and ultimately cell death (Sawa and Masai, 2009). However, several other investigators concluded that inhibition of CDC7 is equally effective against tumors regardless of p53 status, with some studies reporting cell-cycle arrest, and other apoptosis as the main outcome (Iwai et al., 2019; Wang et al., 2019).

Wang et al., (2019) demonstrated that treatment of p53-mutant liver cancer cells with a CDC7i, XL-413, induces tumor cell senescence. Furthermore, the senescent cells can be killed by inhibition of the mTOR pathway. Others reported that simultaneous treatment with another CDC7i, PHA-767491 and 5-fluorouracil (5-FU) elicited a synergistic antitumor effect in hepatocellular carcinoma (Li et al., 2015). However, PHA-767491 is not CDC7-specific, as it also has a considerable activity against CDK9 (Natoni et al., 2011)(Figure 4) and other CDKs. Indeed, it was shown that PHA-767491 enhances the efficacy of 5-FU by inhibiting CHK1 phosphorylation (through CDC7-inhibition) (Yang et al., 2019) and by down-regulating MCL1 (a target of CDK9) (Cidado et al., 2020). In another study, administration of PHA-767491 synergized with multiple EGFR tyrosine kinase-inhibitors to overcome the resistance to EGFR-targeted therapy in various TNBC cell lines. Again, the synergistic effect was in large part dependent on CDK9 inhibition (McLaughlin et al., 2019). PHA-767491/NMS-1116354 and XL-413/BMS-863233 have been used in phase I clinical trials (Table 1). These compounds did not move into phase II, suggesting that they elicited significant toxicity. Phase I clinical trials involving two other compounds, LY-3143921 and TAK-931 are either in progress or have been completed but results have not been disclosed.

In summary, the utility of targeting CDC7 in cancer treatment remains inconclusive. It is likely that the toxic effects of anti-CDC7 compounds observed in the clinics represented an off-target effect of inhibiting other kinases, such as CDK9. It remains to be demonstrated

whether CDC7 inhibition would selectively kill p53-mutant tumor cells in an *in vivo* setting, while having a transient and reversible effect on normal tissues.

Cyclin B and CDK1

High levels of cyclins B1 and B2 were observed in a wide spectrum of tumors (Figure 3) and linked to tumor aggressiveness and poor prognosis (Martínez-Alonso and Malumbres, 2020). Suzuki et al. (2007) reported that in breast cancers, the presence of nuclear cyclin B1 is associated poor clinical prognosis, whereas cytoplasmic cyclin B1 has no predictive value. Hassan et al. (2002) demonstrated that overexpression of cyclin B1 in head and neck squamous cell carcinomas renders tumors resistant to radiation therapy, as evidenced by significantly increased recurrence of cyclin B1-overexpressing tumors following radiotherapy. It was proposed that overexpressed cyclin B1 expedites G2/M transition, a stage during which cancer cells are most sensitive to this treatment.

Cyclin B1 has been proposed to represent a tumor antigen. Yu et al. (2002) observed that in contrast to cell-cycle-regulated expression of cyclin B1 in normal cells, in tumor cells cyclin B1 is constitutively expressed at high levels in the cytoplasm. This leads to recognition of cyclin B by the host immune system (Vella et al., 2009; Yu et al., 2002). Indeed, it was reported that during lung tumorigenesis, aberrant expression of cyclin B1 in premalignant lesions correlates with T cell-dependent antibody responses against cyclin B1 (Egloff et al., 2006). Anti-cyclin B1 antibody responses were also reported in patients with prostate, breast, colorectal and hepatocellular cancers (Egloff et al., 2006). Intriguingly, anti-cyclin B1 antibodies and anti-cyclin B1 memory T-cells were also found in healthy individuals with no cancer history, suggesting that events other than cancer may lead to aberrant cyclin B1 expression and anti-cyclin B1 immunity. Importantly, vaccine-elicited anti-cyclin B1 immunity was shown to protect mice against cyclin B1-overexpressing tumors (Vella et al., 2009).

Studies on colorectal cancers and pituitary adenomas indicated that cyclin B may have tumor-suppressive properties. The authors postulated that low levels of cyclin B1 are associated with poor survival of patients, and that overexpression of this protein correlates with decreased invasion and metastasis of cancer cells. This phenomenon was attributed to ability of cyclin B1 to regulate EMT markers, such as E-cadherin (Fang et al., 2015; Li et al., 2019).

Transgenic mice engineered to overexpress cyclins B1 and B2 are prone to formation of tumors (e.g. colon, lung cancer), which has been attributed to defects in chromosome segregation (Nam and van Deursen, 2014). While both cyclins B1 and B2 can induce aneuploidy when overexpressed, they do it through distinct mechanisms. *In vitro* analysis of MEFs from cyclin B-overexpressing mice revealed that cyclin B1 inhibits separase activation, leading to anaphase bridges, whereas cyclin B2 triggers Aurora-A-mediated Plk1 hyperactivation, which results in accelerated centrosome separation and lagging chromosomes (Nam and van Deursen, 2014). Furthermore, cyclin B2–Cdk1 acts antagonistically to p53 to control Aurora-A-mediated centrosome disjunction, with p53 keeping Aurora A protein levels under control through transcriptional and post-translational

inhibitory mechanisms, and cyclin B2–Cdk1 activating the residual Aurora-A pool (Nam and van Deursen, 2014).

Deregulated CDK1 activity in tumorigenesis is typically associated with aberrant expression of B-type cyclins (Nam and van Deursen, 2014) rather than CDK1-overexpression. Liver-specific ablation of Cdk1 was shown to confer resistance against tumorigenesis induced by activated Ras and silencing of p53 (Diril et al., 2012). However, analyses of knockout mice indicated that Cdk1 is essential for proliferation of normal cells (Santamaría et al., 2007). Hence, inhibition of CDK1 is expected to block proliferation of non-transformed tissues. Intriguingly, it was reported that cells transformed with c-MYC oncogene as well as c-MYC-overexpressing TNBC cells undergo apoptosis when treated with CDK1-inhibitors (Goga et al., 2007; Kang et al., 2014). A similar effect was observed using mouse models of MYC-driven lymphomas or liver tumors, as well as TNBC xenografts (Goga et al., 2007; Horiuchi et al., 2012b; Kang et al., 2014). This pro-survival function of CDK1 was attributed to the ability of CDK1 to phosphorylate BIRC5 (survivin), an inhibitor of apoptosis. Indeed, inhibition of CDK1 downregulated survivin expression (Goga et al., 2007). Another proposed mechanism was p53-independent upregulation of pro-apoptotic BIM protein upon CDK1-inhibition (Horiuchi et al., 2012b; Kang et al., 2014). These observations raise a possibility that a transient inhibition of CDK1 may kill MYC-overexpressing tumor cells, while causing only reversible cell-cycle arrest in normal tissues. However, inhibition of Cdk1 was also shown to cause apoptosis of embryonic stem cells (Huskey et al., 2015). Hence, the impact of CDK1-inhibition on survival of stem cell compartments (such as hematopoietic stem cells) within the adult organism needs to be evaluated.

Cyclin H–CDK7

Cyclin H-CDK7 kinase was shown to play two distinct roles, by serving as the CDK-activating kinase (CAK), as well as a component of a general transcription factor TFIIH (Box 1).

Amplification of the *CDK7* gene was detected in several cancer types and shown to correlate with aggressive pathological features and poor prognosis (Figure 3) (Sava et al., 2020). The CDK7 protein is overexpressed in oral squamous cell carcinomas and in TNBC, and correlates with reduced overall and disease-free survival (Sava et al., 2020). On the other hand, increased CDK7 levels observed in estrogen receptor-positive breast cancers inversely correlate with tumor grade and size, and were hence described as a favorable prognostic marker (Sava et al., 2020).

The first 'CDK7-specific' inhibitor, BS-181 had a significantly higher potency towards CDK7 than to other CDKs; however, it demonstrated some off-target activity (Table 1). BS-181 was shown to arrest the growth of multiple breast and gastric cancer cell lines through cell-cycle inhibition and induction of apoptosis (Sava et al., 2020). In xenograft models, BS-181 significantly reduced the growth of MCF-7 breast and BGC823 gastric tumors, with no detected overall toxicity. Importantly, treatment with BS-181 led to a reduction of RNA-polymerase II CTD phosphorylation, suggestive of stalling of mRNA

transcription (Sava et al., 2020). While BS-181 demonstrated low bioavailability, administration of its improved analog, ICEC0942 (CT7001), resulted in a significant decrease in tumor growth concomitant with the reduction in RNA-polymerase II CTD phosphorylation *in vivo* (Patel et al., 2018). However, it was not until the development of the first covalent CDK7-inhibitor, THZ1 (Kwiatkowski et al., 2014), that the transcriptional function of CDK7 in cancer became intensely studied. While THZ1 showed a similar antiproliferative potency to that of BS-181, cancer cell line profiling indicated that some cell lines (e.g. T-ALL) were particularly sensitive to THZ1-treatment (Kwiatkowski et al., 2014). A genome-wide analysis in T-ALL cells demonstrated that treatment with THZ1 disproportionally affects transcription of the *RUNX1* gene. Importantly, RUNX1 - whose high expression in T-ALL cells is driven by a large super-enhancer domain - plays a key role in the core transcriptional regulatory circuitry and is required for growth and survival of leukemic cells (Kwiatkowski et al., 2014).

Several other studies reported high THZ1 efficacy in cancers addicted to expression of other super-enhancer-driven genes. Chipumuro et al. (2014) reported that THZ1 disrupted the transcription of amplified *MYCN* gene in neuroblastoma cells, which led to significant tumor regression in an *in vivo* model. Such effect was highly selective for MYCN-overexpressing cells and correlated with preferential downregulation of super-enhancer-associated genes. Christensen et al. (2014) found that in small-cell lung cancer, expression of super-enhancer-associated transcription factor genes, including *MYC*, is highly vulnerable to THZ1-treatment. Finally, Wang et al. (2015) identified an "Achilles cluster" of TNBC-specific genes that are especially sensitive to CDK7-inhibition and are frequently associated with super-enhancers. The authors proposed that CDK7 mediates the transcriptional addiction of TNBC cells to these genes, and hence its inhibition triggers apoptotic cell death of tumor cells.

Collectively, these observations indicated that inhibition of CDK7 specifically affects expression of super-enhancer-driven genes. However, the emergence of an even more selective CDK7-inhibitor, YKL-5–124, has challenged this model. Treatment of cancer cell lines with YKL-5–124 prevented CDK1 and CDK2 T-loop phosphorylation by CAK, and led to cell-cycle arrest at the G1/S boundary and inhibition of E2F-driven gene expression. Strikingly, YKL-5–124-treatment had no effect on RNA-polymerase II CTD phosphorylation (Olson et al., 2019). At this time, it became evident that the effects of the THZ1 compound were primarily due to its off-target inhibition of other transcriptional CDKs, namely CDK12 and CDK13, which contributed to the dramatic inhibition of super-enhancer-associated genes (Olson et al., 2019). In the revised, current view CDK7-inhibition is thought to mainly affect cell-cycle progression. Unexpectedly, a recent observation showed that YKL-5–124-treatment activates immune-response signaling in small-cell lung cancer cells, leading to secretion of essential pro-inflammatory cytokines/chemokines, which in turn can activate CD8^{+} T-cells (Zhang et al., 2020). It remains to be seen whether this phenomenon truly represents an on-target effect of CDK7-inhibition, or it is again due to inhibition of another kinase. Nevertheless, treatment with YKL-5–124 was shown to inhibit small-cell lung cancer growth *in vivo* and to enhance tumor response to anti-PD-1 immunotherapy (Zhang et al., 2020). These results indicate that more studies are needed to decipher the full range of normal, physiological functions of CDK7.

Currently, there are three active phase I clinical trials involving CDK7-inhibitors SY-5609, SY-1365 and CT7001 in patients with breast, ovarian and advanced solid malignancies (Table 1). The newest generation of most specific CDK7-inhibitors has not yet been included in clinical trials. It remains to be seen whether these selective CDK7-inhibitors perform better than the compounds which also inhibit CDK12 and CDK13. The clinical efficacy of CDK12/13-inhibitors (which do not target CDK7) also need to be explored.

Non-specific pan-CDK inhibitors

A large number of clinical trials conducted to date utilized non-specific pan-CDKi which target several CDKs (Table 1). The first-generation pan-CDKi included flavopiridol/ alvocidib and R-roscovitine/seliciclib (Table 1, Figure 4). Of these inhibitors, flavopiridol has been most extensively studied in clinics (Otto and Sicinski, 2017). Flavopiridol, which inhibits CDK1, CDK2, CDK4, CDK6, CDK7, and CDK9, was shown to cause cell-cycle arrest of tumor cells in G1 and G2 phases (Asghar et al., 2015).Administration of flavopiridol induced apoptosis in several mouse tissues, leading to organ atrophy, an effect attributed to inhibition of CDK9 (Otto and Sicinski, 2017). Despite these limitations, flavopiridol was tested in phase I clinical trials. While in these trials flavopiridol was active as a single agent against some solid malignancies, clinical phase II studies reported insufficient efficacy. However, some evidence of clinical activity was observed in hematological malignancies (Otto and Sicinski, 2017), and flavopiridol received orphan drug designation from the FDA. On the other hand, R-roscovitine (inhibitory activity against CDK1, CDK2, CDK5, and CDK7) failed to show antitumor activities in preclinical and clinical studies as a monotherapy (Asghar et al., 2015). However, it sensitized various cancers to DNA-damaging agents and radiation, which is consistent with its ability to block DNA double stranded break end-resection and to impair DNA repair via homologous recombination (Lin et al., 2018). Moreover, sequential treatment with R-roscovitine followed by doxorubicin was shown to kill TNBC cells. This effect was attributed to the ability of R-roscovitine to arrest tumor cells in the G2/M phase, thereby priming them for DNA-damage. Inactivation of the p53 pathway seems to be required for such combination-induced cytotoxicity, as this treatment was shown to specifically affect p53-mutant TNBC (Jabbour-Leung et al., 2016).

The second-generation of pan-CDKi with increased potency, such as dinaciclib, AT7519, milciclib, TG02, CYC065 and RGB-286638, has been developed (Otto and Sicinski, 2017). Dinaciclib (inhibitory activity against CDK1, CDK2, CDK5, and CDK9) (Figure 4) demonstrated superior (over 100-fold higher) potency in inhibiting RB1 phosphorylation and a more than 10-fold higher therapeutic index than flavopiridol (Otto and Sicinski, 2017). Additionally, dinaciclib inhibited cell-cycle progression in over 100 tumor cell lines of various tumor types and induced the regression of established solid tumors in a range of mouse xenograft models (Asghar et al., 2015). Phase I clinical trials reported partial responses and tolerable toxicity (Mitri et al., 2015; Nemunaitis et al., 2013). However, results from randomized phase II trials of dinaciclib in solid tumors have been mostly disappointing (Mita et al., 2014; Stephenson et al., 2014). On the other hand, promising clinical data obtained in patients with chronic lymphocytic leukemia (CLL) led to a randomized phase III study of dinaciclib in refractory CLL (Ghia et al., 2017).

Dinaciclib-treatment may provide an effective strategy in MYC-overexpressing TNBC and MYC-driven B cell lymphomas. Administration of dinaciclib caused tumor regression in mouse models of these tumor types (Otto and Sicinski, 2017). This effect may be due to the synthetic lethal interaction between CDK1-inhibition and MYC-overexpression (see above). Currently, a phase I study is investigating the use of dinaciclib in patients with MYC-overexpressing breast cancers. Dinaciclib also demonstrated synergistic activity when combined with the AKT-inhibitor MK-2206, and caused strong tumor growth inhibition in pancreatic cancer xenografts (Otto and Sicinski, 2017).

A recent pre-clinical study revealed that dinaciclib treatment induces type I interferon gene signature within the tumor and elicits immunogenic cell death. Moreover, administration of dinaciclib strongly augmented the efficacy of anti-PD-1 immune checkpoint blockade in syngeneic mouse tumor models. Animals treated with anti-PD1 antibody and dinaciclib showed increased T-cell infiltration and dendritic cell activation within the tumor, indicating that this combination improves the anti-tumor immune response (Hossain et al., 2018).

Since these pan-CDKi compounds inhibit multiple CDKs, they cause significant toxicities in normal tissues, thereby severely narrowing their therapeutic window. These shortcomings further highlight the urgent need to develop highly selective CDK-inhibitors, or inhibitors that target defined CDK subsets.

Future Outlook

The success of CDK4/6-inhibitors in the clinics represents only the beginning of targeting cell-cycle proteins in cancer treatment. In the next years, compounds which inhibit other cell-cycle proteins will be tested in pre-clinical studies and ultimately in cancer patients. One of the main challenges will be to identify tumor subsets that are dependent on particular cell-cycle proteins.

Another important challenge will be to delineate the full range of functions of cell-cycle proteins, which may be relevant in the context of cancer therapy. The role of cell-cycle proteins in anti-tumor immune response, acting both within tumor cells as well in the immune system is only beginning to be appreciated, and will be the subject of intense studies. Several other properties of tumor cells as well as of the surrounding stroma were postulated to be regulated by cell-cycle proteins, and further studies are needed to support these findings *in vivo* in the context of human cancers.

An open question is the advantage of highly specific inhibitors, versus compounds that inhibit several kinases. As we discussed, selective CDK4i may show less on-target toxicity than currently used CDK4/6i. However, since upregulation of CDK6 (and CDK2) represents mechanisms of CDK4/6i resistance (Alvarez-Fernandez and Malumbres, 2020), such narrowly acting compounds may enable acquisition of resistance. It is therefore plausible that less specific compounds, targeting for instance CDK4/6 and CDK2 may result in better clinical outcomes. The same principle applies to 'CDK7-specific' inhibitors, where at least some of the therapeutic effects were mediated by inhibition of CDK12 and CDK13 (Olson et

al., 2019). When CDK2i become available, it will be of interest to compare compounds that inhibit only cyclin E-CDK2, versus ones that target all CDK2-containing complexes.

It will also be important to design inhibitors with altered chemical properties. It was recently shown that all currently approved CDK4/6i become 'trapped' in the lysosomes of some TNBC due to their weakly basic structure (Fassl et al., 2020; Llanos et al., 2019), preventing them from reaching their targets in the nucleus. Importantly, less basic derivatives of a CDK4/6i ribociclib were shown to escape the lysosomal sequestration and to arrest proliferation of ribociclib-resistant TNBC (Fassl et al., 2020). Hence, a new generation of CDK4/6i may extend the use of these compounds to tumor types that are currently resistant to CDK4/6i therapy.

The emerging technologies will also allow to convert the existing inhibitors into degrader compounds (Box 3). Degradation of the kinase may result in a more sustained effect than its inhibition. On the other hand, removal of the kinase subunit, while leaving the cyclin protein intact, may trigger compensatory mechanisms, such as binding of a related CDK to the intact cyclin. Such an outcome may mitigate the effects of CDK degradation. For instance, it was shown that small-molecule inhibition of CDK2 kinase has more profound effects on cell proliferation than CDK2 depletion (Horiuchi et al., 2012a). These different scenarios need to be tested experimentally *in vivo* using mouse cancer models.

In summary, this exciting field will remain an area of intense investigation for several years to come. It will undoubtedly yield many new therapeutic modalities which will have a major impact on the treatment of cancer patients.

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Box 1.

The cell-cycle.

The cell-cycle is composed of four phases: Gap 1 (G1), DNA-synthesis (S), Gap 2 (G2) and mitosis (M) (Figure 1). Mitogenic signals received during G1 phase cause upregulation of D-type cyclins (D1, D2, D3), which bind and activate their catalytic partners, CDK4 or CDK6. The activity of cyclin D-CDK4/6 kinase is negatively regulated by the INK4 family of inhibitors (p16^{INK4A}, p15^{INKB}, p18^{INK4C}, p19^{INK4D}) (Figure 1). The formation of stable cyclin D-CDK4/6 complexes requires KIP/CIP proteins (p21^{CIP1}, p27^{KIP1}, p57^{KIP2}), which serve as 'assembly factors' for cyclin D-CDK4/6. In contrast, KIP/CIP proteins are inhibitors of CDK2- and CDK1-containing complexes (Sherr and Roberts, 2004). Cyclin D–CDK4/6 kinases phosphorylate various cellular targets, most importantly, the retinoblastoma protein (RB1), as well as related RBL1 (p107) and RBL2 (p130) (Malumbres and Barbacid, 2001). In its unphosphorylated form, RB1 binds and inactivates or represses E2F transcription factors. Phosphorylation of RB1 by cyclin D-CDK4/6 partially inactivates RB1, leading to release of E2Fs, which then upregulate their transcriptional targets. Among E2F targets are two E-type cyclins (E1 and E2, collectively referred to as 'cyclin E'), which bind and activate CDK2. Cyclin E-CDK2 is responsible for completing phosphorylation of RB1, thereby unleashing the E2F transcriptional program and resulting in synthesis of proteins required for entry of cells into the S-phase (Hwang and Clurman, 2005). During progression through the G1 phase, several proteins – some of them representing E2F transcriptional targets – assemble on origins of DNA replication and form pre-replication complexes, composed of ORC1–6, CDC6, CDT1 and MCM2–7 DNA helicase. At the onset of the S-phase, DNA replication is triggered by a series of sequential phosphorylations carried out by DBF4-CDC7 and cyclin E-CDK2 (Moiseeva and Bakkenist, 2018) (Figure 1). These phosphorylation events promote binding of CDC45 and GINS to MCM2–7, resulting in formation of the CDC45-MCM2–7-GINS (CMG) complex and activation of DNA helicase (Moiseeva and Bakkenist, 2018). Subsequently, the role of cyclin E-CDK2 in firing of DNA replication origins is taken over by cyclin A-CDK2. Cyclin A can activate CDK1 and CDK2; it plays important roles during S-phase as well as in G2-M. The crucial function of cyclin A during G2/M-transition is to activate cyclin B-CDK1 (Figure 1). Cyclin B accumulates in the nucleus at the onset of mitosis, upon being phosphorylated by PLK1 (Martínez-Alonso and Malumbres, 2020). Cyclin B-CDK1 phosphorylates several proteins, most of them associated with events related to mitotic progression and cell division, such as cytoskeleton reorganization, nuclear envelope breakdown, chromosome condensation, mitotic spindle assembly and function, chromosome segregation, and cytokinesis (Martínez-Alonso and Malumbres, 2020). The CDK-activating kinase (CAK) represents the major driver of cell-cycle progression (Sava et al., 2020). This complex is composed of cyclin H, the catalytic subunit (CDK7) as well as a RING protein called MAT1, which stabilizes the complex. CAK activates cell-cycle CDKs by phosphorylating the critical threonine residue within their T-loops. Moreover, CAK-dependent phosphorylation stabilizes some cyclin-CDK complexes, such as cyclin B-CDK1. In addition to its cell-cycle role, CAK functions as a member of TFIIH, a general transcription factor that acts to recruit RNA polymerase II to gene promoters.

Within this complex, CDK7 phosphorylates the C-terminal domain of RNA-polymerase II (Sava et al., 2020).

Box 2.

Regulation of cell-cycle machinery by proteasomal degradation.

An important aspect of cell-cycle regulation is governed by multi-protein E3 ubiquitin ligase complexes, such as the anaphase-promoting complex or cyclosome (APC/C), Skp-Cullin-F-box containing complex (SCF), and BTB-Cul3-Rbx1 complex (BCR), which sequentially promote degradation of key cell-cycle regulators (Figure 2) (Dang et al., 2020; Rape et al., 2006). Recognition of specific degron motifs by components of E3 ligase complexes, such as CDC20, CDH1, FBXW7, CUL3, Cyclin F, SKP2 and βTrCP, leads to ubiquitination and proteasomal degradation of target proteins. APC/C^{CDH1} stabilizes G1 phase by maintaining low levels of mitotic and S-phase cyclins, thereby preventing premature initiation of S-phase (Malumbres and Barbacid, 2009). At the end of G1 phase, the APC/C^{CDH1} becomes inactivated by cyclin E-CDK2; this inactivation is further maintained by early mitotic inhibitor 1 (EMI1). During S phase, cyclin E is degraded via FBXW7-mediated ubiquitination (Otto and Sicinski, 2017), and it can be also degraded by the BCR complex (Singer et al., 1999). SKP2 is thought to act as an oncogene, by contributing to the ubiquitination and degradation of CDK inhibitors from the KIP/CIP family (Dang et al., 2020). βTrCP plays an important role in regulating CDK1 activity. It prevents premature activation of CDK1 by inducing degradation of CDC25A and EMI1, and it can contribute to CDK1 activation by mediating WEE1 degradation in G2 phase. Also during G2 phase, Cyclin F shuts down the activity of E2F, prevents the synthesis of replicative histones, contributes to the regulation of centrosomal duplication and mediates the degradation of CDH1 (Dang et al., 2020). In mitosis, cyclin A is degraded by APC/C^{CDC20} following nuclear envelope breakdown and before metaphase (Martínez-Alonso and Malumbres, 2020). Subsequently, degradation of cyclin B and securin (responsible for the anaphase onset) is executed by APC/C^{CDC20} (Martínez-Alonso and Malumbres, 2020). Finally, APC/C^{CDH1} function starts in anaphase where it modulates the stability of mitotic players such as Aurora A and PLK1; the APC/C^{CDH1} activity then continues into the next G1-phase (Dang et al., 2020).

Box 3.

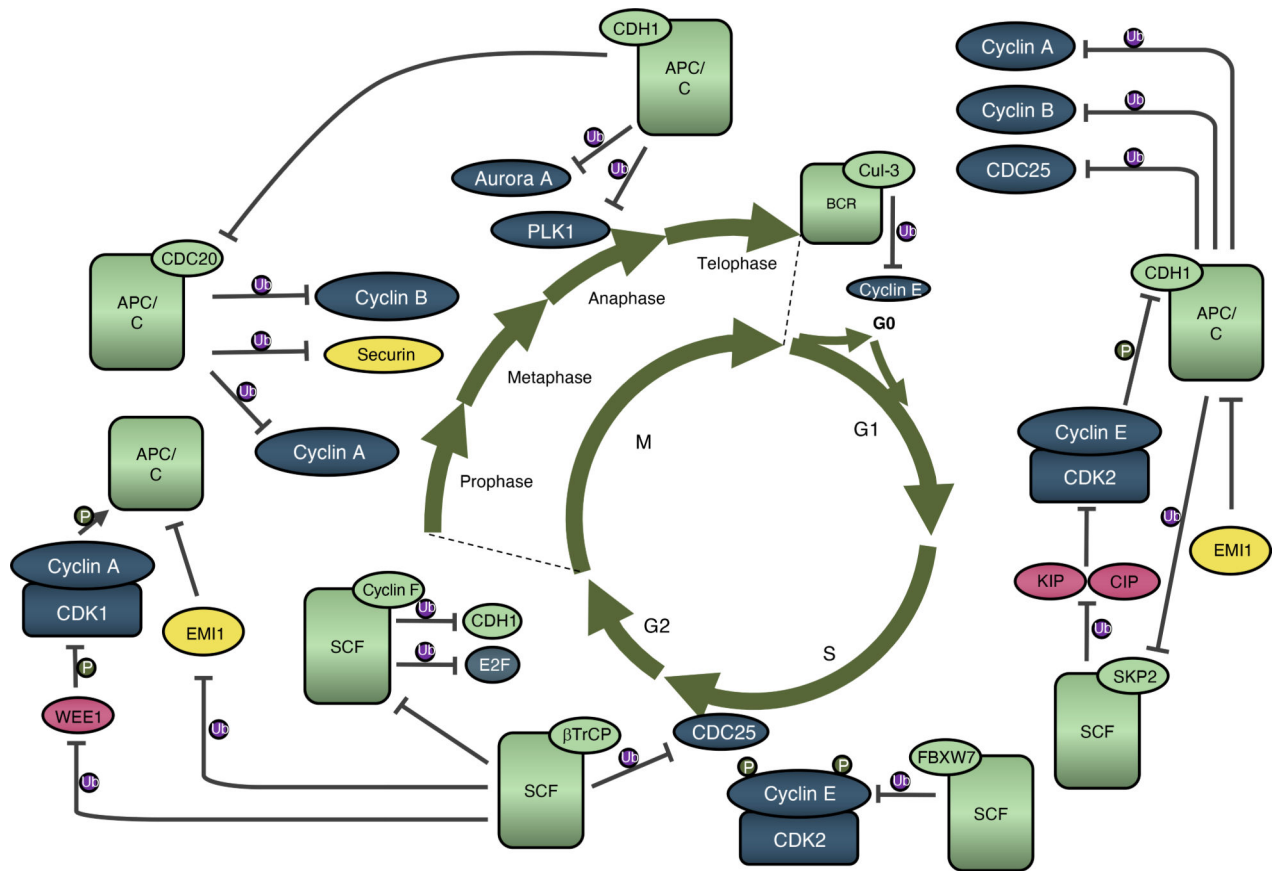### Targeted degradation of cell cycle proteins in cancer cells.

Traditionally, targeting the cell cycle machinery involves inhibition of the catalytic activity of the core enzymes. In recent years, an array of approaches involving targeted protein degradation (TPD) has been developed (reviewed in (Wu et al., 2020)). In principle, TPD can be achieved by endowing the target protein with a degradation domain or by adapting the existing chemical inhibitors. The most popular approach is called PROteolysis TArgeting Chimeras (PROTAC). This approach involves bi-functional molecules, or "molecular glues", in which one domain binds the target protein and the other interacts with a component of an E3 ubiquitin ligase, such as CRBN, pVHL or β-TCRP. This recruits the E3 ligase to the target protein, thereby triggering its degradation by the ubiquitin-proteasome system. TPD has proven to represent a powerful tool in basic research and it holds promise in targeting of currently "undruggable" proteins. Several TPD approaches are being developed and applied to target members of the cell cycle machinery in the context of cancer therapy. Selective CDK4/6 inhibitors have been used to synthesize PROTACs that target these kinases (Brand et al., 2019; Jiang et al., 2019; Rana et al., 2019). For example, Brand et al. (2019) developed a compound in which palbociclib has been conjugated to pomalidomide, a phthalimide-based moiety that is expected to recruit CRL4^{CRBN} E3 ligase complex. Surprisingly, this compound selectively depleted CDK6, but not CDK4 in acute myeloid leukemia cells, thereby allowing interrogation of CDK6-dependent pathways and functions. Jiang et al. (2019) described a panel of imide-based degrader molecules capable of targeting both CDK4 and CDK6, or selectively degrading either of these kinases. These molecules were further adapted to additionally degrade lymphoid transcription factors Ikaros (IKZF1) and Aiolos (IKZF3), known targets of imides and of some imide-based degraders. The authors reported that in mantle cell lymphoma cell lines, degradation of IKZF1/3 coupled with dual CDK4/6 degradation had a stronger anti-proliferative effect than degradation of CDK4/6 or IKZF1/3 alone, or a selective CDK4/6i. De Dominici et al. (2020) identified a CDK6-selective PROTAC that suppressed tumor burden of Philadelphia chromosome-positive acute lymphoblastic leukemia *in vivo* more effectively than CDK4/6i. These findings are consistent with the observations that CDK6 plays kinase-independent, transcriptional functions (Kollmann et al., 2013). Degradation of CDK6 is expected to remove both catalytic and transcriptional CDK6 functions, while CDK6 inhibitors block only its enzymatic activity. Hence, in addition to reduced toxicity stemming from their excellent sensitivity, degraders can help to exploit cancer cell dependencies on non-catalytic functions of target proteins. Teng et al. (2020) reported the development of a CDK2 and CDK5 dual degrader, which displayed a considerable selectivity over CDK1, CDK4/6 as well as transcriptional CDKs (CDK7 and CDK9). The authors demonstrated that this compound, through its ability to degrade CDK2, inhibited proliferation of cyclin E1-overexpressing human ovarian cancer cells. These observations point to CDK2-degradation as a potentially valuable therapeutic strategy in ovarian and other cancers that overexpress cyclin E1.
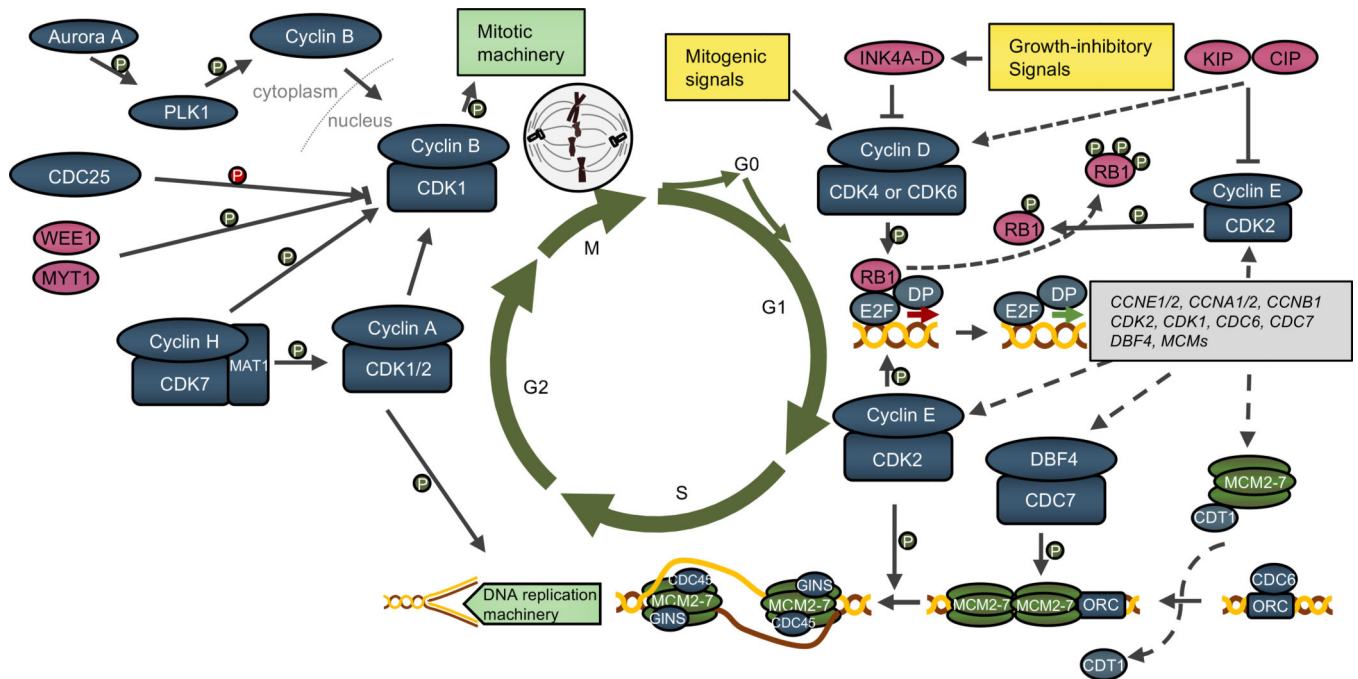
Figure 1. Overview of the cell-cycle.

Please see Box 1 for details. Activation of cyclin D-CDK4/6 kinases triggers a cascade of events resulting in activation of E2F-dependent transcriptional program. DP1, the dimerization partner of E2F transcription factors. Examples of E2F targets are provided in a grey rectangle. Multiple proteins (such as ORC, CDC6, MCMs) assemble on DNA replication origins during the G1 phase, resulting in DNA origin licensing. Subsequent phosphorylations (P) by DBF4-CDC7 and cyclin E-CDK2 activate the DNA helicase and trigger origin firing and entry of cells into the S-phase. During S-phase, cyclin A-CDK2 phosphorylates components of the DNA replication machinery. In G2-phase, cyclin A activates cyclin B-CDK1. Cyclin B is localized in the cytoplasm during G2-phase, but it translocates into the nucleus upon being phosphorylated by PLK1. Inhibitory phosphorylations of CDK1 provided by WEE1 and MYT1 kinases are removed by the CDC25 phosphatase family. Activation of cyclin-CDK complexes is also carried out by cyclin H-CDK7-MAT1 (CAK1) complex. Upon activation, cyclin B-CDK1 phosphorylates components of the mitotic machinery. Blue and pink ovals denote, respectively, positive and negative regulators of the cell-cycle.

Figure 2. Regulation of cell-cycle machinery by proteasomal degradation.
E3 ubiquitin ligases ascertain unperturbed cell cycle progression by governing timely
degradation of various cell-cycle proteins. Light green rounded rectangles and ovals
represent ubiquitin ligase complexes and their respective activators/f-box proteins. Ub in a
purple circle indicates ubiquitination. FBXW7 - F-Box and WD Repeat Domain Containing
7; Cul-3 – Cullin 3; SKP2 – S-Phase Kinase Associated Protein 2; βTrCP - Beta-Transducin
Repeat Containing E3 Ubiquitin Protein Ligase.
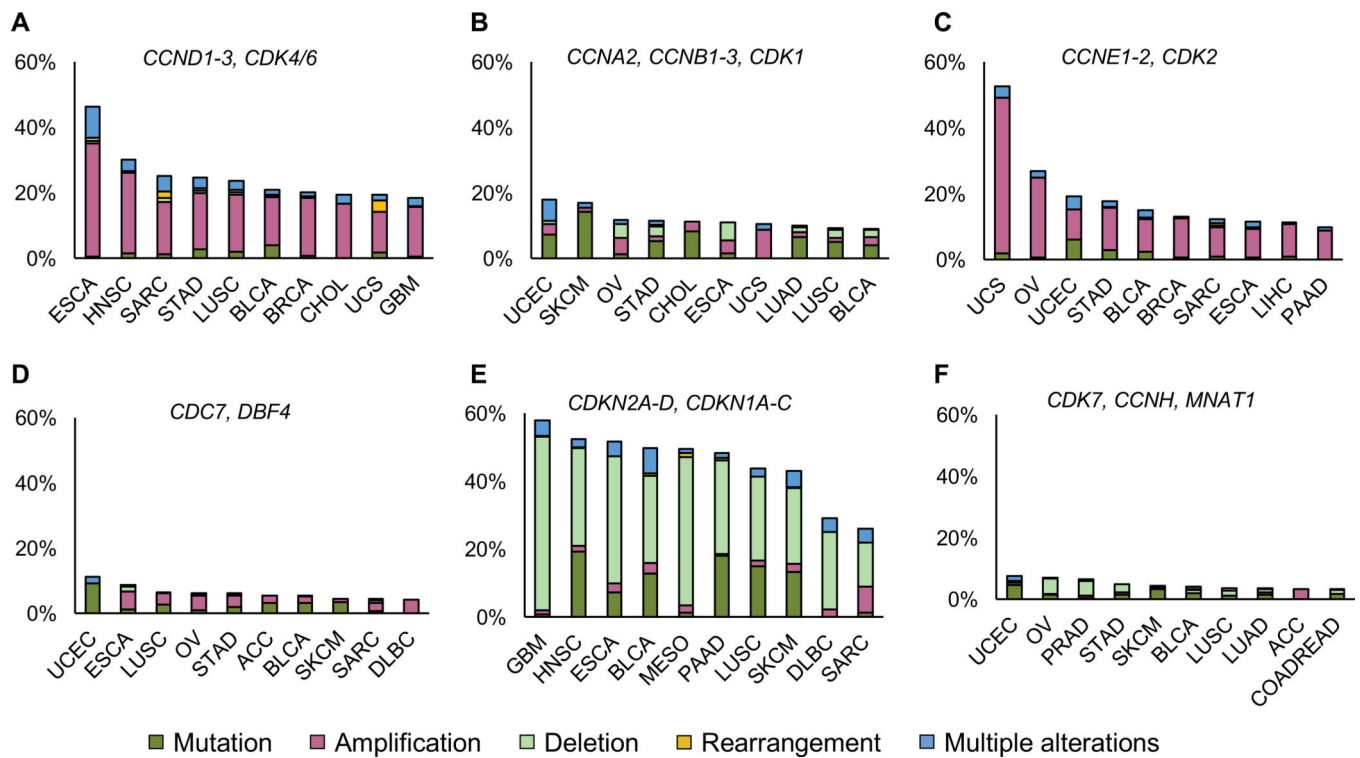
Figure 3. The landscape of genetic alterations within the core cell-cycle machinery genes.
Data according to The Cancer Genome Atlas PanCancer (n=10,967 samples). Shown is the frequency of alterations within groups of the indicated cell-cycle genes. Ten most affected cancer types are shown for each group, together with percentages of cases displaying genetic alterations. Tumor type designation: ACC - Adrenocortical Carcinoma; BLCA - Bladder Urothelial Carcinoma; BRCA - Breast Invasive Carcinoma; CESC - Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma; CHOL – Cholangiocarcinoma; COADREAD - Colorectal Adenocarcinoma; DLBC - Diffuse Large B-cell Lymphoma; ESCA - Esophageal Carcinoma, GBM - Glioblastoma Multiforme, HNSC - Head and Neck Squamous Cell Carcinoma, LIHC - Liver Hepatocellular Carcinoma, LUAD – Lung Adenocarcinoma, LUSC- Lung Squamous Cell Carcinoma, MESO – Mesothelioma, OV - Ovarian Serous Cystadenocarcinoma, PAAD - Pancreatic Adenocarcinoma, PRAD - Prostate Adenocarcinoma, SARC – Sarcoma, SKCM - Skin Cutaneous Melanoma, STAD - Stomach Adenocarcinoma, UCEC - Uterine Corpus Endometrial Carcinoma, UCS - Uterine Carcinosarcoma, UVM - Uveal Melanoma.
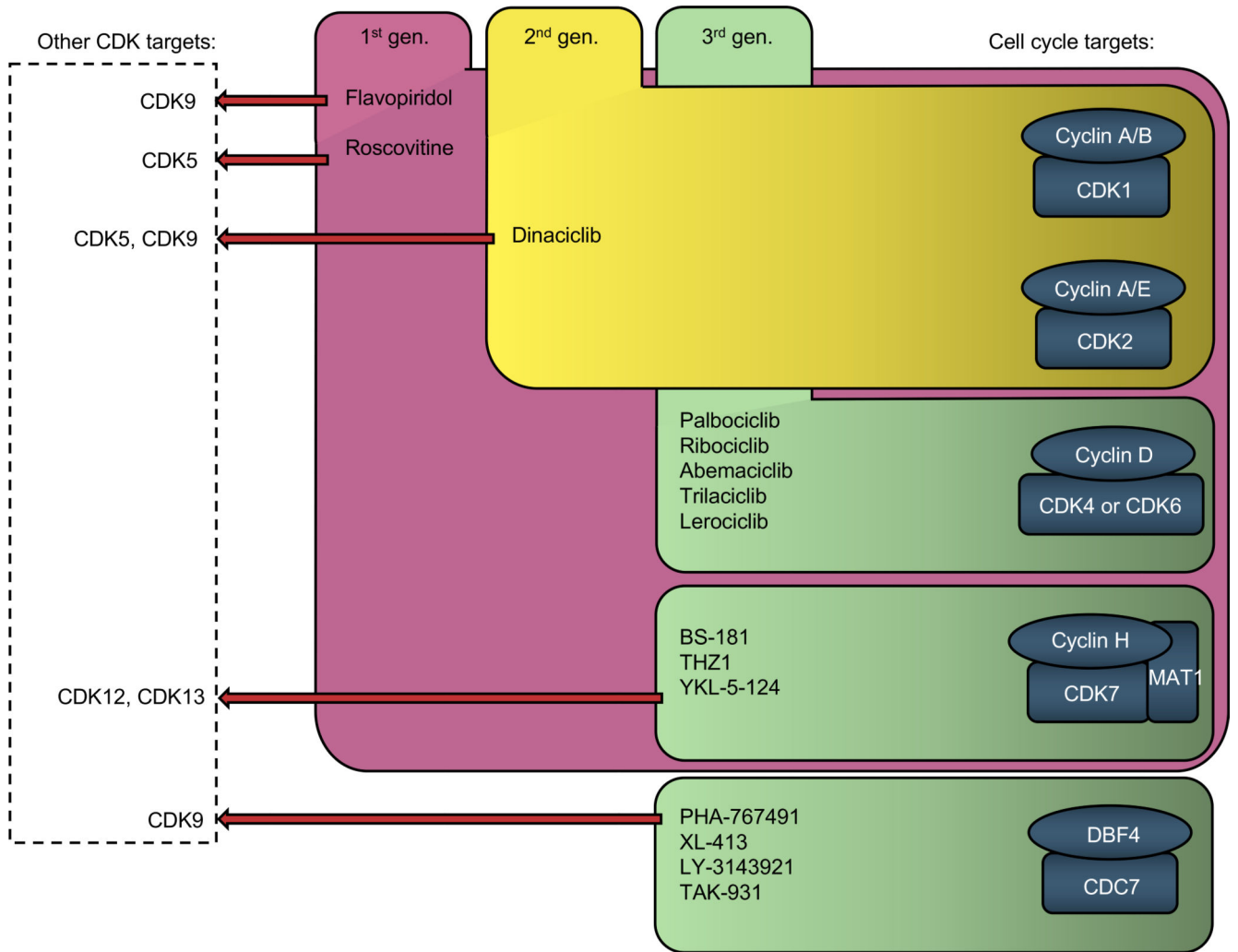
Figure 4.
Inhibitors of cell-cycle proteins and their major non-cell-cycle CDK targets.

Table 1.

Inhibitors of cell-cycle proteins in clinical development.

Drug name (alternative names)	Primary target (IC50)	Off target (IC50)	Clinical trials/clinical utilization	Reference
1st generation (Pan-kinase inhibitors)				
Flavopiridol (Alvocidib, L86-8275, HMR-1275)	CDK9 (20 nM), CDK1 (30 nM), CDK2 (40 nM), CDK4 (20–40 nM), CDK6 (60 nM)	CDK7 (875 nM), MAPK, PKC, EGFR	66 phase I or II clinical trials in different malignancies; 5 active trials (MDS/AML); not registered for clinical use in oncology	(Shapiro, 2006)
Roscovitine (Seliciclib, CYC202)	CDK5 (0.16 μM), CDK1 (0.65 μM), CDK2 (0.7 μM)	ERK	3 phase I or II clinical trials in different malignancies; no active trial and no registration for clinical use in oncology	(Ali et al., 2009; Jabbour-Leung et al., 2016)
2nd generation (Multiple-kinase inhibitors)				
Dinaciclib (SCH727965)	CDK2 (1 nM), CDK5 (1 nM), CDK1 (3 nM), CDK9 (4 nM)		18 phase I, II and III clinical trials in different malignancies; the only phase 3 trial in CLL (results to be posted); 4 active trials; no registration for clinical use in oncology	(Parry et al., 2010; Ghia et al. 2017)
AT7519	CDK9 (<10 nM), CDK5 (13 nM), CDK2 (47 nM)	CDK4 (100 nM), CDK6 (170 nM), CDK1 (210 Nm), CDK7 (2400 nM), GSK-3β	5 phase I or II clinical trials in heamotologic malignancies; 1 active trial; no registration for clinical use in oncology	(Otto and Sicinski, 2017)
Milciclib (PHA-848125)	CDK2 (45 nM)	CDK7 (150 nM), CDK4 (160 nM), CDK5 (265 nM), CDK2 (363 nM), CDK1 (398 nM), TrKa	4 phase I or II clinical trials in thymic and liver carcinomas; 1 active trial; no registration for clinical use in oncology	(Otto and Sicinski, 2017)
SB1317 (TG02)	CDK2 (13 nM)	JAK2, FLT3	7 phase I or II clinical trials in various solid tumors and hematological malignancies; 3 active trials; no registration for clinical use in oncology	(Otto and Sicinski, 2017)
CYC065	CDK2 (5 nM), CDK9 (26 nM)		3 phase I active clinical trials in hematological malignancies; no registration for clinical use in oncology	(Otto and Sicinski, 2017)
RGB-286638	CDK9 (1 nM), CDK1 (2 nM), CDK2 (3 nM), CDK4 (4 nM), CDK3 (5 nM), CDK5 (5 nM)	CDK7 (44 nM), CDK6 (55 nM), GSK-3β, TAK1, JAK2, MEK1	1 withdrawn phase I clinical trial	(Otto and Sicinski, 2017)
3rd generation (Specific kinase inhibitors)				
Palbociclib (PD-0332991, Ibrance)	CDK4 (9–11 nM), CDK6 (15 nM)		registered for clinical use in hormone receptor positive, HeR2-negative advanced breast cancer patients; 215 phase I/II/III/IV clinical trials, including PALOMA series; being tested in various malignancies (173 active)	(Fry et al., 2004)
Ribociclib (LEE011, Kisqali)	CDK4 (10 nM), CDK6 (39 nM)		registered for clinical use in hormone receptor positive, HeR2-negative advanced breast cancer patients; 103 phase I/II/III clinical trials, including MONALEESA series; being tested in various malignancies (72 active)	(Hortobagyi et al., 2016)
Abemaciclib (LY2835219, Verzenio)	CDK4 (2 nM), CDK6 (10 nM)		registered for clinical use in hormone receptor positive, HER2-negative advanced breast cancer patients; 100 phase I/II/III/IV clinical trials, including MONARCH series;	(Sledge et al., 2017)

Drug name (alternative names)	Primary target (IC50)	Off target (IC50)	Clinical trials/clinical utilization	Reference
			being tested in various malignancies (81 active)	
Trilaciclib (G1T28)	CDK4 (1 nM), CDK6 (4 nM)		4 phase I and II clinical trials in small cell lung cancer and breast cancer; no registration for clinical use in oncology	(Tan et al., 2019)
Lerociclib (G1T38)	CDK4 (1 nM), CDK6 (4 nM)	CDK9 (28 nM), CDK5 (0.8–1.2 μM), CDK2 (1.5–3.6 μM), CDK1 (2.4 μM), CDK7 (2.4 μM)	3 phase I and II clinical trials in lung and breast cancer; no registration for clinical use in oncology	(Bisi et al., 2017)
PHA-767491 (NMS-1116354)	CDC7 (10 nM), CDK9 (34 nM)	CDK2 (240 nM), CDK1 (250 nM), CDK5 (460 nM), GSK-3β, MK2, PLK1, CHK2	2 withdrawn phase I clinical trials in advanced solid tumors	(Montagnoli et al., 2008)
XL-413 (BMS-863233)	CDC7 (3.4 nM)	PIM1, CK2	2 withdrawn phase I and II clinical trials in advanced solid tumors and hematologic malignancies	(Koltun et al., 2012)
LY-3143921	CDC7 (3.3 nM)		1 active phase I clinical trial in advanced solid cancers	
TAK-931 (Simurosertib)	CDC7 (<0.3 nM)		3 phase I and II clinical trials in various advanced solid tumors; one on-going	(Iwai et al., 2019)
BS-181	CDK7 (210 nM)	CDK2 (0.9 μM), CDK5 (3 μM), CDK9 (4.2 μM), CDK1 (8.1 μM), CDK4 (33 μM), CDK6 (47 μM)	not entered clinical studies yet	(Ali et al., 2009)
ICEC0942 (CT7001, Samuraciclib)	CDK7 (40 nM)	CDK2 (620 nM), CDK9 (1.2 μM), CDK1 (1.8 μM)	1 phase I/II on-going clinical trial in advanced solid tumors	(Patel et al., 2018)
THZ1	CDK7 (3.2 nM)		not entered clinical studies yet	(Kwiatkowski et al., 2014)
YKL-5-124	CDK7 (9.7 nM)	CDK2 (1300 nM) CDK9 (3020 nM)	not entered clinical studies yet	(Olson et al., 2019)
SY-5609	CDK7 (0.6 nM)	CDK2 (2900 nM) CDK9 (970 nM) CDK12 (770 nM),	1 phase I dose-escalation in select advanced solid tumors	(Sava et al., 2020)
SY-1365	CDK7 (84 nM)	CDK2 (2117 nM) CDK9 (914 nM) CDK12 (204 nM),	1 phase I dose-escalation/safety advanced solid tumors	(Sava et al., 2020)

[1]Inhibitors of cell- cycle kinases which entered clinical trials of any phase; MAPK - Mitogen-Activated Protein Kinase; PKC – Protein Kinase C; EGFR - Epidermal Growth Factor Receptor; ERK - Extracellular Signal-Regulated Kinase; JAK2 – Janus Kinase 2; FLT3 - Receptor-type tyrosine-protein kinase FLT3; GSK-3β - Glycogen Synthase Kinase 3 beta; TAK1 - Transforming Growth Factor-β Activated Kinase 1; MEK1 - Dual Specificity Mitogen-Activated Protein Kinase 1; MK2 - MAP Kinase-Activated Protein Kinase 2; PLK1 – Polo-Like Kinase 1; CHK2 - Checkpoint Kinase 2; PIM1 - Proto-oncogene Serine/Threonine-Protein Kinase 1; CK2 – Casein Kinase 2; MDS/AML – Myelodysplastic Syndrome/ Acute Myeloid Leukemia, CLL – Chronic Lymphocytic Leukemia