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Review

CDK4/6 Inhibition in Cancer: Beyond Cell Cycle Arrest

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Pharmacologic inhibitors of cyclin-dependent kinases 4 and 6 (CDK4/6) have recently entered the therapeutic armamentarium of clinical oncologists, and show promising activity in patients with breast and other cancers. Although their chief mechanism of action is inhibition of retinoblastoma (RB) protein phosphorylation and thus the induction of cell cycle arrest, CDK4/6 inhibitors alter cancer cell biology in other ways that can also be leveraged for therapeutic benefit. These include modulation of mitogenic kinase signaling, induction of a senescence-like phenotype, and enhancement of cancer cell immunogenicity. We describe here the less-appreciated effects of CDK4/6 inhibitors on cancer cells, and suggest ways by which they might be exploited to enhance the benefits of these agents for cancer patients.

Development of Pharmacologic CDK Inhibitors

For a cell to divide, it must complete the cell cycle – a tightly regulated series of events governed in large part by the activity of cyclin proteins and their partner kinases, the CDKs. The CDKs that drive cell division are often hyperactive in cancers, and the sustained proliferative signaling that results is a well-recognized hallmark of malignancy [1]. Given the notion that inhibition of CDKs should halt otherwise uncontrolled cellular proliferation, scientists have long attempted to develop pharmacologic inhibitors of these CDKs. Nevertheless, many of the initial compounds lacked potency or selectivity. As a result, therapeutic targeting of the cell cycle machinery had remained an elusive goal.

The challenge stemming from a lack of specific CDK inhibitors has recently been met by the development of selective and potent inhibitors of CDKs 4 and 6 [2–4]. CDKs 4 and 6 specifically regulate cellular transition from the G1 phase of the cell cycle to the S phase, and CDK4/6 inhibitors effectively block the proliferation of sensitive cancer cells by inducing G1 cell cycle arrest [5]. Consequently, these agents have moved rapidly from research laboratories into clinical trials, and three have now received FDA approval for the treatment of metastatic breast cancer [6]. These early clinical successes have been celebrated, but it is clear that only a fraction of the clinical potential of CDK4/6 inhibitors has been realized. In this article we briefly introduce the CDK4/6 pathway, and then focus on novel therapeutic approaches that have the potential to maximize the utility of CDK4/6 inhibitors in the clinic.

Control of the G1–S Transition

To enter the cell cycle and commence DNA replication, a cell must pass from the G1 phase into the S phase through a tightly regulated restriction point [7]. According to the 'classical' cell cycle model, the G1–S transition begins when the balance between mitogenic stimulation (via growth factor receptor activation) and inhibition tips in favor of the former, triggering an increase in the levels of D-type cyclins (D1, D2, and D3) (Figure 1). D-type cyclins bind to CDK4 or CDK6, and the cyclin–CDK complexes then enter the nucleus where they are phosphorylated by the CDK-activating kinase (CAK) complex.

Highlights

Selective pharmacologic inhibitors of cyclin-dependent kinases 4 and 6 (CDK4/6) show immense promise as a treatment for a wide variety of cancers.

The primary mechanism of CDK4/6 inhibitor activity is suppression of RB phosphorylation, enforcing G1 cell cycle arrest, and thus inhibiting proliferation.

CDK4/6 inhibitors can also impact on other aspects of cancer cell behavior by inducing a senescence-like state, enhancing immunogenicity, and modulating kinase signaling.

CDK4/6 inhibitors may also exert their activity through a direct effect on other cell types within tumors, including immune cells.

Understanding the complex biological phenotypes induced by CDK4/6 inhibitors will ultimately allow the development of new therapeutic combinations to further benefit patients.

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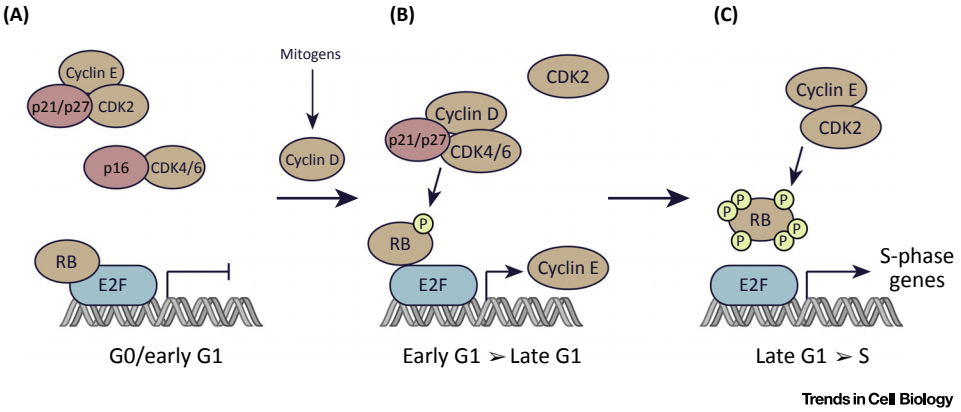

Figure 1. The Classical Model for Regulation of the G1–S Transition by Cyclins and CDKs. (A) In resting cells, CDK4/6 and CDK2 are inactive. D-type cyclin levels are low owing to the lack of a mitogenic stimulus, limiting CDK4/6 activity. Moreover, CDKs 4 and 6 are bound by INK4 family members (e.g., p16), establishing binary complexes that lack kinase activity. CDK2 complexes are inhibited by the CIP/KIP proteins p21 and p27. Collectively, the suppression of CDK4/6 and CDK2 leads to RB hypophosphorylation, and hence repressed expression of E2F target genes. This repression is mediated by direct blockade of the E2F transactivation domain by RB, as well as by the recruitment of histone modifiers to RB that further silence E2F target gene expression. (B) Levels of D-type cyclins increase in response to mitogenic stimuli owing to both enhancement of cyclin D gene expression and increased cyclin D protein stability. D-type cyclins bind to CDK4/6, forming complexes that are stabilized by p21 or p27. Cyclin D–CDK4/6 complexes then enter the nucleus and phosphorylate (P) RB. This partially derepresses expression of E2F target genes, including those for the E-type cyclins. The partial phosphorylation of RB facilitates progression through G1. (C) As the levels of E-type cyclins rise in late G1, CDK2 is activated, resulting in RB hyperphosphorylation and inactivation. Hyperphosphorylated RB is released from E2F, enabling the increased transcription of E2F target genes that is necessary for the cell to proceed into S phase.

In turn, activated CDK4/6 complexes phosphorylate the RB tumor-suppressor protein, as well as the related pocket proteins p107 and p130 [8–10]. RB phosphorylation by CDK4/6 partially derepresses the activity of the E2F family of transcription factors [11], thus facilitating the expression of E2F target genes including those for the E-type cyclins (cyclins E1 and E2). Cyclin E then binds to and activates CDK2, which hyperphosphorylates RB, further increasing the expression of E2F target genes that are crucial for initiation of DNA synthesis and entry into S phase [7,11–13]. Notably, RB not only inhibits the expression of S-phase genes by directly inhibiting E2F transactivation, but also by recruiting chromatin modifiers to DNA that can indirectly silence E2F target gene expression [14,15].

In reality, progression from G1 to S is not always regulated in such an orderly, stepwise manner. Indeed, RB contains several phosphorylation sites – amenable to phosphorylation by CDK4/6, CDK2, or both – and the consequences of phosphorylation at differing sites are distinct [16]. The capacity of these enzymes to facilitate the G1–S transition is probably a reflection of their net effects on RB phosphorylation at any given point in time. Indeed, the 'alternative' cell cycle model proposes that the G1–S transition can be facilitated by CDK4/6 or CDK2 acting alone or in concert. As such, CDK4/6-mediated phosphorylation of RB is likely not an absolute prerequisite for CDK2 activation [6].

Regulation of CDK4/6 and CDK2 activity is achieved in part by two families of endogenous inhibitory proteins. The first is the INK4 family, comprising $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$. These proteins bind to CDKs 4 and 6, forming binary complexes that lack kinase activity. The second is the CIP/KIP family, which includes $p27^{KIP1}$, $p21^{CIP1}$, and $p57^{KIP2}$. These proteins bind to CDKs more promiscuously and have more diverse functions, potently inhibiting several CDKs (including CDK4/6, CDK2, and CDK1), but also in some circumstances binding

to and stabilizing the cyclin D–CDK4/6 holoenzyme. These divergent functions may be regulated by both the amount and the phosphorylation status of the CIP/KIP proteins [17–20].

The description above does not reflect the full biologic complexity of CDK4/6 activity. For example, although preclinical data suggest that different tissues harbor dependencies on particular D-type cyclins or on CDK4 or 6, these proteins can compensate for each other owing to functional redundancy [21,22]. More strikingly, cells within some organ compartments can proliferate in the absence of all D-type cyclins [23], or without both CDK4 and CDK6 [22]. In these instances the G1 transition may be led by complementary cyclin–CDK complexes, including those containing CDK2 or CDK1 [22,24]. Although the notion that various cancer cells are particularly CDK4/6-dependent still renders the development of CDK4/6 inhibitors an attractive strategy, these observations immediately suggest pathways by which cancer cells could escape the effects of CDK4/6 inhibition.

Dysregulation of CDK4/6 Activity in Cancer

Many human cancers harbor genomic or transcriptional aberrations that activate CDK4/6 [25] through a wide variety of mechanisms described below. In theory, these cancers are expected to show greater CDK4/6 dependency, and have traditionally been considered ideal candidates for treatment with CDK4/6 inhibitors. However, as discussed later, not all these alterations have been shown to confer heightened sensitivity to CDK4/6 inhibitors [26].

Alterations in Cell Cycle Machinery Genes

Amplification of genes encoding D-type cyclins is commonly observed in human cancer, and correlates with increased levels of cyclin D protein. Based on data from large next-generation sequencing studies, cyclin D1 (*CCND1*) amplification is most commonly observed in cancers of the upper gastrointestinal tract, head and neck, breast, lung, and bladder [27,28], and amplification in breast cancer has been correlated with worse clinical outcomes [29]. *CCND2* and *CCND3* are also amplified in a variety of cancers, albeit less frequently [27]. In addition, numerous cancers demonstrate amplifications of *CDK4* (most notably liposarcoma and glioblastoma) and *CDK6* (upper gastrointestinal cancers and neuroendocrine carcinoma of the prostate) [27]. Specific genomic translocations and gene mutations can also markedly increase cyclin D levels in tumor cells. The resulting increase in CDK4/6 activity might be expected to confer sensitivity to CDK4/6 inhibitors, and this has indeed been shown to be the case [26]. For example, in mantle cell lymphoma the unique t11;14 translocation juxtaposes the *CCND1* and *IGH* immunoglobulin heavy-chain genes, leading to constitutive overexpression of cyclin D1 in a mitogen-independent fashion [30]. In addition, some tumors carry mutations in the 3′-untranslated regions (3′-UTRs) of *CCND1*, *CCND2*, and *CCND3*. These mutations can increase cyclin D levels by preventing degradation of cyclin D mRNA [26].

Loss of $p16^{INK4A}$ Function

Loss of $p16^{INK4A}$ function is a relatively common event in cancer, and is usually driven by homozygous deletion of the *CDKN2A* locus which encodes p16. Loss of p16 has been predicted to confer heightened CDK4/6 dependence in cancer cells [5]. Somatic *CDKN2A* deletion is most common in malignant peripheral nerve sheath tumors, glioblastoma, and carcinomas of the pancreas, head and neck, bladder, esophagus, and lung [27,28]. Importantly, however, a study examining a large panel of human cancer cell lines has recently called into question whether loss of $p16^{INK4A}$ function is actually associated with heightened sensitivity to CDK4/6 inhibition [26]. The reasons for this discrepancy are unclear, but may relate to the fact that *CDKN2A* mutant tumors show a propensity to upregulate CDK2 activity rapidly after exposure to CDK4/6 inhibitors, thus restoring RB phosphorylation and G1 progression [26].

Upstream Genomic Events that Increase Cyclin D Levels

In cancer cells that lack the genomic alterations described above, cyclin D levels and CDK4/6 activity are primarily regulated by mitogenic signaling. Notably, the mitogenic RAS–RAF–MEK–ERK pathway is a potent driver of *CCND1* transcription [31]. A large number of cancers demonstrate elevated cyclin D1 levels as a result of a variety of aberrations that activate the RAS–RAF–MEK–ERK pathway – including activating mutations in signaling effector proteins and upstream growth factor receptors [32]. Increased mitogenic signaling through a hyperactive phosphoinositide-3-kinase (PI3K)–AKT pathway also increases cyclin D1 levels by preventing its nuclear export and increasing its translation [33–35]. In some instances the importance of mitogen-induced cyclin D1 upregulation for tumor growth has been unequivocally demonstrated – for example, *Ccnd1* knockout mice are unable to form mammary tumors driven by *Erbb2* or *Ras* [36]. However, it has not been definitively demonstrated that higher levels of cyclin D1 protein in cancer cells are associated with CDK4/6 inhibitor sensitivity more generally.

Clinical Successes with CDK4/6 Inhibitors to Date

Three CDK4/6 inhibitors are currently approved for clinical use in the USA – palbociclib, ribociclib, and abemaciclib. All three agents are used as treatment for estrogen receptor (ER)-positive breast cancer based on the results of clinical trials demonstrating improvements in progression-free survival when CDK4/6 inhibitors are added to anti-estrogen therapy, the previous gold standard for treatment of ER-positive disease [37–39]. Abemaciclib is also approved for use as monotherapy in a similar patient population [40]. The rationale for focusing on breast cancer during the early development of CDK4/6 inhibitors was strong. Studies in transgenic animals had shown that cyclin D1 deficiency strongly and specifically impairs mammary epithelial proliferation, suggesting a key role for the CDK4/6 pathway in this tissue [21]. Moreover, cyclin D1 and CDK4 are essential for the formation and growth of several murine mammary tumors [36,41,42]. The first preclinical study of CDK4/6 inhibitors in breast cancer showed that a large proportion of human breast cancer cell lines are sensitive to palbociclib, as evidenced by reduced RB phosphorylation and cell cycle arrest [5]. Interestingly, cells demonstrating a luminal pattern of gene expression (as defined in [43]) were particularly sensitive to palbociclib, and luminal breast cancers are much more likely to express ER, providing reason to develop CDK4/6 inhibitors in ER-positive cancers. Moreover, *CCND1* is a known ER target gene, and combined inhibition of CDK4/6 and ER (with endocrine therapy) reduces tumor cell proliferation synergistically [5].

The clinical data beyond ER-positive breast cancer are sparser. Single-arm studies have been performed in a variety of cancer types bearing genomic abnormalities predicted to increase CDK4/6 inhibitor sensitivity, including HER2-positive breast cancer, mantle cell lymphoma, liposarcoma, melanoma, non-small cell lung cancer, glioblastoma, neuroblastoma, and malignant rhabdoid tumors [44–47]. Each study has shown evidence of clinical activity (partial response or prolonged stabilization of disease) in a subset of patients, but, in the absence of randomized data, it is difficult to know how CDK4/6 inhibitor activity compares to existing standards of care.

Improving the Efficacy of CDK4/6 Inhibitors as Cancer Therapy

A wealth of preclinical data generated over decades describe the regulation of CDK4/6 activity in proliferating cells, and one key to broadening the clinical impact of CDK4/6 inhibitors lies in reviewing these old data for clues. In addition, the promise of clinical efficacy has rekindled interest in the CDK4/6 pathway and has provided new research tools to understand it better.

Based on both old and new data, several lines of investigation have begun to emerge in an effort to develop CDK4/6 inhibitors rationally and swiftly (Figure 2, Key Figure).

Identifying Sensitive and Resistant Tumor Types

Clinically, there is significant heterogeneity in the response of individual tumors to CDK4/6 inhibitors [48,49] and it has therefore become imperative that we better understand biomarkers that predict sensitivity to these agents. Equally important is the problem of resistance to CDK4/6 inhibitors – for example, in breast cancer nearly all tumors acquire resistance to these agents after prolonged therapy, and the underlying mechanisms are also poorly understood [50]. As described above, CDK4/6 activity is upregulated in cancers through a variety of genomic alterations including cyclin D gene amplification and loss of *CDKN2A*, and an assumption has been made that cancers harboring these alterations should be the most susceptible to pharmacologic CDK4/6 inhibition. However, recent analyses of clinical specimens have called this assumption into question – for example, in randomized clinical trials of palbociclib for breast cancer, *CCND1* amplification and loss of p16 expression were not associated with enhanced CDK4/6 inhibitor response [51–53]. It is clear that more work will be necessary to identify predictors of sensitivity and resistance to these agents.

One approach is to select tumors originating from tissues wherein normal cells show a particular CDK4/6 dependence. A good example is the selection of breast cancers based on a mammary epithelial specific phenotype identified in *Ccnd1* knockout mice. Similarly, preclinical studies have shown responses to CDK4/6 inhibitors among hematopoietic cancers [54,55], in keeping with observations that *CDK6* is expressed at high levels in lymphoid organs, and that *Cdk6* knockout mice show specific defects in hematopoiesis and thymic development [22]. Other approaches are also being used to determine correlations between tumor sensitivities to CDK4/6 inhibition and their molecular profiles. In a recently published study, the sensitivity of 560 human cancer cell lines to the CDK4/6 inhibitor abemaciclib was assessed [26]. First, the authors confirmed the previously described and somewhat intuitive notion that lack of RB expression is associated with relative resistance to CDK4/6 inhibitors, and that the response to these agents is almost always RB-dependent [26,56]. This finding has also been strengthened through clinical reports describing the emergence of *RB1* mutations as tumors acquire resistance to CDK4/6 inhibitors [57]. Second, and in keeping with breast cancer clinical data, they did not find that *CCND1* amplification was associated with greater drug sensitivity. Instead, they identified a specific group of other genomic features predicted to increase cyclin D levels that are strongly associated with CDK4/6 inhibitor sensitivity. These features, collectively termed 'D-cyclin activating features' (DCAFs), include *CCND1* translocations (as seen in mantle cell lymphoma), amplification of *CCND2* or *CCND3*, mutations in the 3′-UTRs of *CCND1–3*, genomic loss of *FBXO31* (encoding a D-type cyclin ubiquitin ligase), and expression of K-cyclin, a cyclin D1 variant from the Kaposi's sarcoma virus [26]. Although particular DCAFs are more common in particular tumors types (e.g., *CCND1* 3′-UTR loss is more common in uterine and stomach cancers), many are distributed across a range of cancers. As a result of these findings, a clinical 'basket trial' of abemaciclib therapy is currently underway, recruiting patients with any cancer type whose tumors demonstrate a DCAF upon genomic profiling (NCT03310879).

Several intriguing questions arise from this work that are relevant to understanding the molecular predictors of CDK4/6 inhibitor response [26]. First, it is not clear why amplification of *CCND2* or *CCND3*, but not of *CCND1*, was associated with sensitivity to CDK4/6 inhibitors. Second, and counter to expectations, cell lines with *CDKN2A* alterations were found to be relatively resistant to abemaciclib as a result of their capacity to rapidly upregulate CDK2 activity after treatment. Third, *TP53* mutations were strongly associated with CDK4/6 inhibitor

Key Figure

Strategies for Deriving Maximal Clinical Benefit from CDK4/6 Inhibition

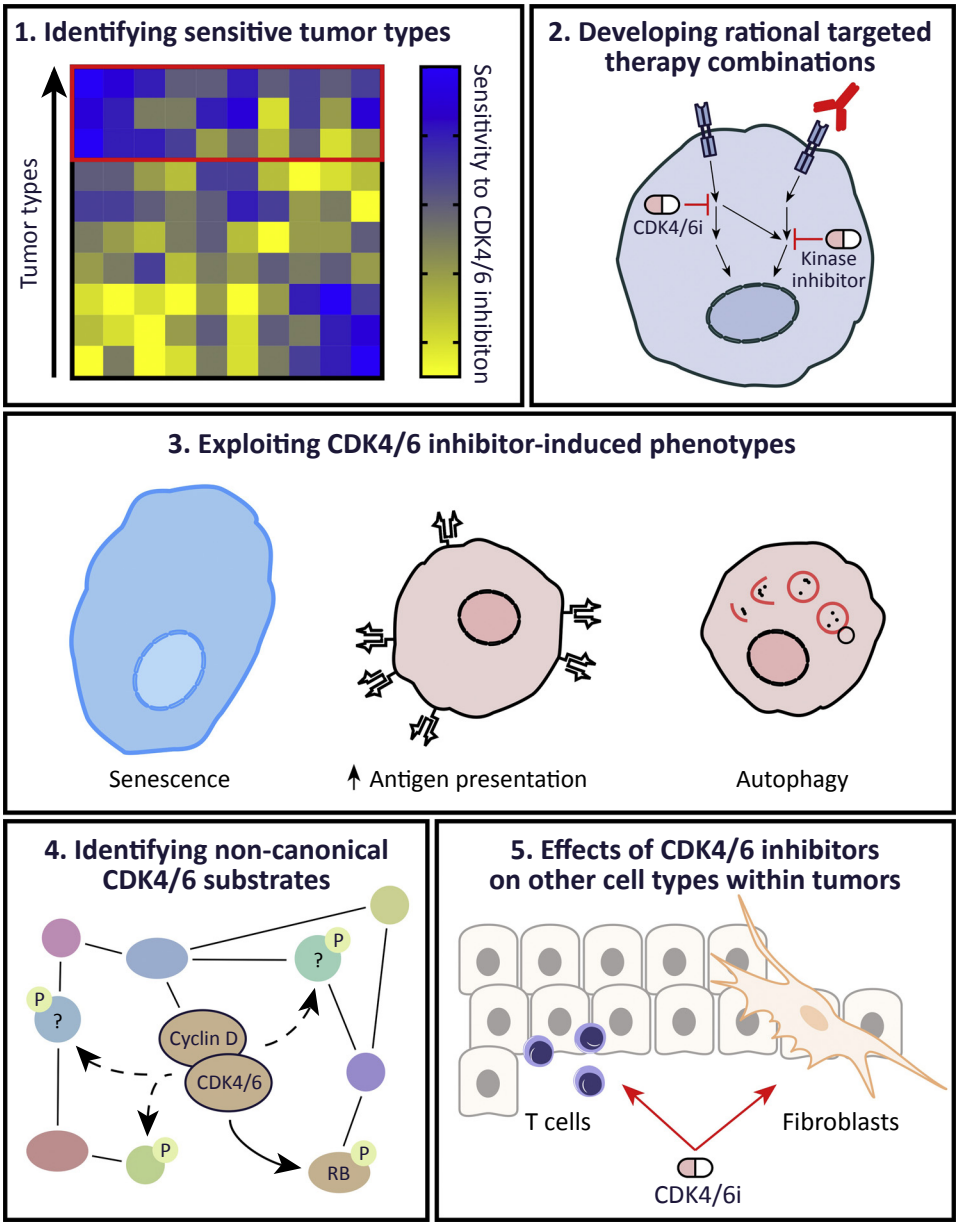

Figure 2. Although CDK4/6 inhibitors have shown impressive activity in the treatment of estrogen receptor-positive breast cancer, their full clinical potential has not been realized. This figure summarizes key areas that should be explored to expand the benefits of these agents in clinical practice. (1) Identification of sensitive tumor types using high-throughput multi-omic analyses. *In vitro* studies to determine molecular predictors of cancer cell sensitivity and resistance to CDK4/6 inhibitors are crucial for guiding patient selection in future clinical trials. Such studies should be performed using high-

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resistance, and the mechanisms for this are unclear. Further mechanistic studies will be necessary to strengthen the potential clinical implications of these findings.

One key challenge has been the development of accurate methods to identify tumors that have lost RB function, and which would therefore be expected to be CDK4/6 inhibitor-resistant. It is clear that tumors harboring genomic loss of *RB1* alleles and/or loss-of-function *RB1* mutations fall into this category, but these might only reflect a fraction of tumors lacking RB functionality [57,58]. One proposed surrogate marker of RB dysfunction is a high level of p16 as detected by immunohistochemistry. Indeed, RB and p16 levels are often inversely correlated in tumor cells as a result of a previously described feedback loop in which p16 is expressed in response to dysregulated E2F target gene expression [58–60], and *CDKN2A* and *RB1* lesions are mutually exclusive in most cancers. Despite this, clinical studies to date have failed to demonstrate that either the mRNA or protein levels of *CDKN2A*/p16 or *RB1*/RB are predictive of benefit from CDK4/6 inhibitors [51,52]. More broadly, analysis of breast cancer specimens from randomized clinical trials have failed to consistently identify any biomarker of CDK4/6 inhibitor resistance at either the mRNA of protein levels [50–52,61,62].

Developing Rational Targeted Therapy Combinations

The CDK4/6 pathway intersects with several key mitogenic signaling pathways in cancer cells, providing a strong rationale for combining CDK4/6 inhibitors with other targeted therapies. A prototypic case is the combination of CDK4/6 inhibitors and endocrine therapies (e.g., tamoxifen, aromatase inhibitors, fulvestrant) in ER-positive breast cancers [5,63,64]. Synergy between these agents can in part be explained by a 'two-hit' mechanism, whereby endocrine therapy limits *CCND1* transcription and CDK4/6 inhibitors block kinase activity directly (Figure 3) [65]. However, there are several additional interactions between these pathways – for example, in

throughput techniques to maximize efficiency. Examples include CRISPR or RNAi screens (ideally performed both with and without concomitant CDK4/6 inhibitor treatment), pharmacologic compound screens that identify synergistic CDK4/6 inhibitor-containing combinations, and bioinformatic analyses that determine associations between cancer cell CDK4/6 inhibitor sensitivity and their genomic and transcriptomic profiles. (2) Understanding crosstalk and cooperativity between parallel pro-survival signaling pathways. The CDK4/6 signaling pathway intersects with other key mitogenic pathways (e.g., PI3K–AKT; RAS–RAS–MEK–ERK; steroid hormone signaling) in tumor cells. Synergistic antitumor effects have been observed through coinhibition of CDK4/6 and these other pathways using combination regimens incorporating either small-molecule kinase inhibitors (depicted as pills) or inhibitory monoclonal antibodies (shown in red). Relatively few studies have systematically explored the impact of CDK4/6 inhibition on the phospho-proteome and signal transduction networks in cancer cells, and more are urgently required. (3) Exploiting biologic phenotypes induced by CDK4/6 inhibitors in cancer cells. Short-term treatment of cancer cells with CDK4/6 inhibitors induces RB-dependent G1 arrest. However, more prolonged exposure can induce profoundly different biological phenotypes in tumor cells, in a manner also reflective of sustained RB activation. Capitalizing upon these phenotypes might improve the efficacy of CDK4/6 inhibitors. For example, CDK4/6 inhibition invokes many hallmarks of cellular senescence (cellular enlargement and increased β-galactosidase activity), raising the question of whether they should be combined with 'senolytic' compounds (e.g., Bcl-2/Bcl-xl inhibitors). CDK4/6 inhibitors also increase tumor cell neoantigen presentation via cell-surface MHC class I, providing a rationale for immunotherapy combinations. In addition, CDK4/6 inhibitors can induce tumor cell autophagy, and cotreatment with autophagy inhibitors (e.g., bafilomycin, hydroxychloroquine) can increase their efficacy. (4) Identification of non-canonical CDK4/6 substrates. Aside from their canonical substrate RB, CDKs 4 and 6 also bind to and phosphorylate (P) a range of other protein substrates that are involved in diverse biologic processes. Key examples are the transcription factor FOXM1, some glycolytic enzymes, and nuclear factor of activated T cell (NFAT) family members. Inhibiting the phosphorylation of these substrates with CDK4/6 inhibitors can have wide-ranging effects on tumor cell biology, even in cells that lack RB function. (5) Understanding effects in cells of the tumor stroma. CDK4/6 inhibition can impact on stromal cell biology within solid tumors. For example, fibroblasts (shown in yellow) can senesce in response to CDK4/6 inhibitor treatment, releasing cytokines that impair antitumor immunity and thus limit drug efficacy. Conversely, CDK4/6 inhibition can directly enhance effector functions in T lymphocytes (blue) to strengthen antitumor immune responses. Better understanding of the effects of CDK4/6 inhibitors on these and other non-tumor cells could result in novel strategies that enhance drug activity and/or mitigate therapeutic resistance. Abbreviation: CDK4/6i, CDK4/6 inhibitor.

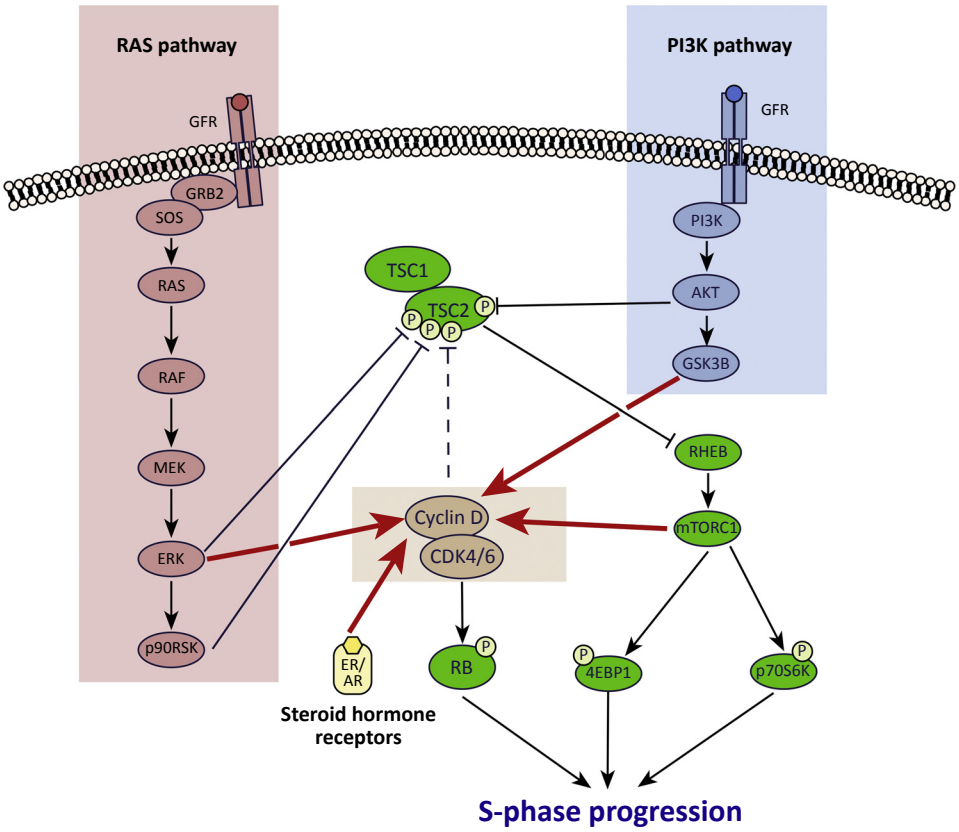

Figure 3. Crosstalk between the CDK4/6 and Mitogenic Signaling Pathways in Cancer. There is extensive crosstalk between mitogenic signaling pathways and the CDK4/6 pathway in cancer cells. First, mitogenic signaling increases cyclin D1 levels leading to increased CDK4/6 activity via several mechanisms (red arrows): PI3K pathway signaling reduces cyclin D1 turnover via GSK3β, RAS pathway signaling promotes ERK-dependent upregulation of transcription factors that drive cyclin D gene expression, mTORC1 increases cyclin D protein translation, and *CCND1* transcription is increased directly by activated ER (breast cancer) and AR (prostate cancer). In addition, these pathways also interact via their convergence on TSC2 and thus mTORC1: AKT, ERK, and p90RSK each directly phosphorylate (P) TSC2 to activate mTORC1, and cyclin D–CDK4/6 also bind to and probably phosphorylate TSC2 to increase mTORC1 activity (broken line). Rationale for synergistic combination therapy regimens: CDK4/6 inhibitors limit cell proliferation by reducing RB phosphorylation, but can also partially suppress TSC2 phosphorylation. Coinhibiting the PI3K and/or RAS pathway not only reduces cyclin D1 levels (further enforcing RB activation) but also increases the suppression of TSC2 phosphorylation, maximizing mTORC1 inhibition. Collectively, combined activation of RB and inhibition of mTORC1 potently blocks progression of cells into S phase. Abbreviations: AR, androgen receptor; ER, estrogen receptor; GFR, growth factor receptor; GSK3B, glycogen synthase kinase-3β; mTORC, mammalian target of rapamycin complex; PI3K, phosphoinositide-3-kinase; TSC, tuberous sclerosis complex.

breast cancers that have acquired endocrine therapy resistance, ER can directly activate E2F target gene transcription, and this can be reversed by CDK4/6 inhibitors [66]. Notably, androgen receptor (AR) signaling in prostate cancer shows several parallels to ER signaling in breast cancer. For example, both ER and AR drive *CCND1* transcription, and CDK4/6 inhibitors show *in vivo* activity in both hormone-sensitive and castrate-resistant prostate cancer [26,67]. Clinical development of CDK4/6 inhibitors in prostate cancer is now emerging as an important priority, and Phase II studies are currently underway (NCT02905318, NCT02494921).

The CDK4/6 and PI3K–AKT–mTOR pathways also interact extensively (Figure 3). On the one hand, PI3K pathway activation (triggered by signaling through upstream receptor tyrosine kinases as well as by mutations in *PIK3CA* or *AKT* [33,34]) increases cyclin D1 levels. On the other, cyclin D–CDK4/6 can reciprocally stimulate mTORC1 signaling, which lies downstream of PI3K [68]. Combination regimens containing inhibitors of CDK4/6 and receptor tyrosine kinase–PI3K–AKT signaling have proved to be effective in a variety of preclinical tumor models, often exhibiting synergism. Examples include combinations of CDK4/6 inhibitors with HER2 inhibitors in breast cancer [5,69,70], PI3K inhibitors in breast cancer, mesothelioma, and mantle cell lymphoma [71–73], mTOR inhibitors in cancers of the breast or head and neck [74,75], IGF-1R inhibitors in pancreatic cancer [76], and ALK inhibitors in neuroblastoma [77]. This synergy has complex mechanistic underpinnings: in brief, it is likely that the CDK4/6 and PI3K pathways both converge on the tumor suppressor, TSC2, a negative regulator of mTORC1 [68,69,78]. When TSC2 is phosphorylated, it degrades, which in turn increases mTORC1 activity. Activation of either the CDK4/6 or PI3K pathway can achieve this [68,79,80]. We have previously demonstrated an example wherein this interaction is responsible for therapeutic synergy: in HER2-positive breast cancer cells that are resistant to anti-HER2 agents, neither HER2 inhibitors nor CDK4/6 inhibitors can fully suppress TSC2 phosphorylation. When administered in combination, however, TSC2 phosphorylation is maximally suppressed, resulting in shutdown of mTORC1 activity, thus synergistically enhancing cell cycle arrest induced by CDK4/6 inhibitors [69,81]. Based on this mechanism, two randomized clinical trials are currently underway to determine whether CDK4/6 inhibitors can overcome therapeutic resistance in HER2-positive breast cancers (NCT02947685, NCT02675231).

The RAS–RAF–MEK–ERK signaling pathway also enhances CDK4/6 activity by driving *CCND1* transcription (Figure 3). This pathway is commonly activated by mutations in the *KRAS*, *NRAS*, and *BRAF* oncogenes [82]. Importantly, synergy between inhibitors of RAS pathway members and CDK4/6 is seen chiefly in cancer cells harboring activating mutations in *KRAS*, *NRAS*, or *BRAF* [83]. One important mechanism for this synergy is a double-hit on the CDK4/6 pathway, with coordinate lowering of cyclin D1 levels and inhibition of CDK4/6 resulting in maximal suppression of RB phosphorylation and hence G1 arrest [83,84]. Preclinically, inhibiting both the CDK4/6 and RAS pathways has demonstrated success using a pan-RAF inhibitor (inhibiting ARAF, BRAF, and CRAF protein kinases) in multiple tumor types, as well as inhibitors of BRAF (in *BRAF* mutant melanoma) and MEK (in *KRAS* mutant lung or colorectal cancer and *BRAF* mutant melanoma) [83–91]. Interestingly, the RAS pathway, like the PI3K pathway, can also activate mTOR through downstream phosphorylation of TSC2 [92,93], and combined CDK4/6 and MEK inhibition also leads to a synergistic suppression of mTORC1 signaling [94]. These results suggest that, in addition to activating their own canonical substrates, the CDK4/6, PI3K, and RAS pathways all converge upon TSC2 such that the benefits of combination regimens extend to include mTORC1 inhibition. This notion has been strengthened by recent studies showing that acquired resistance to combined CDK4/6 and MEK inhibition in melanoma can be mediated by activating mutations in *PIK3CA* and heightened mTORC1 activity [95,96].

Exploiting CDK4/6 Inhibitor-Induced Cellular Phenotypes

The initial effect of CDK4/6 inhibitors on responsive cancer cells is G1 cell cycle arrest. With more prolonged treatment, however, a range of other biological phenotypes have been observed. Each of these has its roots in the biology of the CDK4/6 pathway, and offers specific potential for the development of new CDK4/6 inhibitor-containing therapeutic combinations (Figure 2).

First, several investigators have reported that prolonged (several days or more) exposure of cancer cells to CDK4/6 inhibitors can induce a phenotype resembling cellular senescence. This is not altogether surprising, given the important role that RB activation plays in mediating senescence [97,98]. CDK4/6 inhibitor treatment can reproduce many of the morphological hallmarks of senescence including cellular enlargement, increased β-galactosidase activity, and development of senescence-associated heterochromatin foci (SAHF) both *in vitro* and *in vivo* [54,99,100]. That said, it is not clear whether CDK4/6 inhibitor-induced senescence is an exact phenocopy of classical senescence induced by DNA damage. For example, CDK4/6 inhibitor-induced senescence is reversible – as evidenced by resumption of tumor cell proliferation after prolonged drug treatment is withdrawn [99]. In addition, reports on whether CDK4/6 inhibition upregulates a transcriptional program for cytokines and chemokines known as the senescence-associated secretory phenotype (SASP) have been mixed, and the implications of this *in vivo* have not been explored [99,100]. Classical cellular senescence is a unique state associated with dramatic changes in several fundamental aspects of cell biology [101]. It is important that we now determine to what extent these features are recapitulated with CDK4/6 inhibition so as to understand how they might be exploited for therapeutic gain. For example, recent reports have described a series of candidate 'senolytic' compounds which show a specific capacity to induce apoptosis in cells that have undergone replicative senescence [101,102]. The efficacy of these compounds in CDK4/6 inhibitor pretreated cancer cells has not been assessed.

In some models, CDK4/6 inhibitor treatment can also induce cancer cell autophagy, evidenced by the presence of autophagolysosomes and the upregulation of various autophagic markers [103–105]. This is consistent with the fact that cyclin D–CDK4/6 activity restrains autophagy in normal epithelial compartments such as that of the mammary gland [106]. The mechanisms underlying CDK4/6 inhibitor-mediated autophagy induction are unclear, but autophagy may reflect a stress response triggered by a CDK4/6 inhibitor-mediated increase in reactive oxygen species (ROS) levels [103]. Importantly, inhibitors of autophagy (e.g., hydroxychloroquine, bafilomycin, and others) show synergy with CDK4/6 inhibitors in cancer cell lines, with the combination enhancing G1 arrest and the senescent phenotype [103,105]. Evidence of CDK4/6 inhibitor-induced autophagy in *in vivo* systems is currently lacking, but such studies would help determine whether combination CDK4/6 inhibition and autophagy inhibition might have utility in clinical trials.

Another intriguing phenomenon is that CDK4/6 inhibitors can increase cancer cell presentation of tumor neoantigens on major histocompatibility complex (MHC) class I molecules, thus enhancing antitumor immune responses [72,99,107]. In cells expressing functional RB, CDK4/6 inhibitors reduce expression of the E2F target, *DNMT1* (encoding a DNA methyltransferase). This results in DNA hypomethylation at some transposable genomic elements, including those containing endogenous retroviral (ERV) sequences. The resultant ERV transcription increases tumor cell double-stranded RNA (dsRNA) content, provoking a dsRNA response that triggers tumor cell production of type III interferons and ultimately the expression of interferon-stimulated genes, including those encoding the antigen-presentation machinery [99]. Interestingly, upregulation of antigen presentation-related genes has also been observed in cells rendered senescent through other means, such as replicative senescence [108], and it is possible that CDK4/6 inhibitor-induced enhancement of tumor cell antigen presentation is but one component of the senescent phenotype. Moreover, previous studies have suggested that functional RB might also be required for tumor cell expression of MHC class II molecules, although the mechanisms behind this are not known [109,110]. *In vivo*, the increased tumor immunogenicity observed after CDK4/6 inhibition potentiates the activity of immune checkpoint

blockade, a finding that has paved the way for clinical trials of combined CDK4/6 inhibition and immunotherapy in solid tumors [99,107,111].

Notably, although several groups have demonstrated enhanced antitumor immune responses after CDK4/6 inhibition in solid tumors [72,99,107,112], one report has described the opposite. In that study, CDK4/6 inhibition reduced phosphorylation of the ubiquitin ligase adaptor SPOP, thereby reducing ubiquitination of the programmed cell death protein ligand-1 (PD-L1). The resulting increase in tumor cell PD-L1 expression was associated with a reduction in effector T cell activity within murine mammary carcinomas [113]. Intriguingly, this suppression of antitumor immunity was associated with enhanced benefit of PD-1 blockade, thus also highlighting the promise of CDK4/6 inhibitor–immunotherapy combinations.

Identifying Non-Canonical CDK4/6 Substrates

Given that RB is the canonical CDK4/6 substrate, the overwhelming majority of CDK4/6 inhibitor research in cancer has focused on RB-proficient cells. Moreover, tumor cells with low *RB1* expression are generally insensitive to these agents *in vitro*, and loss of *RB1* function is an emerging mechanism of CDK4/6 inhibitor resistance [26,56,57]. However, it is clear that CDKs 4 and 6 directly phosphorylate dozens if not hundreds of other proteins [114,115], and that CDK4/6 inhibition can thus impact on tumor cell biology in an RB-independent manner. Discussing the range of CDK4/6 substrates and their implications for cancer treatment is beyond the scope of this article, but some notable examples include the Forkhead box 1 (FOXM1) transcription factor [115], key metabolic enzymes including 6-phosphofructokinase and pyruvate kinase M2 [116], the signal transduction protein IRS2 [117], the deubiquitinase DUB3 [118], and the ubiquitin ligase adaptor SPOP [113]. Given their protean cellular functions, the consequences of inhibiting the phosphorylation of these substrates are far-reaching, and have been shown to include modulation of tumor cell senescence, invasion and metastasis, metabolism, and immunogenicity [113,115,116,118].

Effects of CDK4/6 Inhibitors on Other Cell Types

Finally, we must recognize that CDK4/6 inhibitor treatment in living organisms not only impacts on tumor cells but also on other cell types within the tumor microenvironment and host, potentially impairing or enhancing responses to therapy. For example, the importance of the CDK4/6 pathway in regulating both proliferation and senescence in fibroblasts has been understood for many years [98,119], and a recent study demonstrated that CDK4/6 inhibitor-treated fibroblasts develop a senescent phenotype characterized by secretion of a large number of proinflammatory cytokines [120]. Hence, cancer-associated senescent fibroblasts may have the potential to enhance tumor growth by both directly stimulating tumor cells and also suppressing antitumor responses. Conversely, CDK4/6 inhibitors may have direct effects on specific lymphocyte populations that can potentiate antitumor immunity [99,121]. First, CDK4/6 inhibitor treatment of human $CD4^+$ T lymphocytes enhances their activity *in vitro*, as evidenced by upregulation of interleukin-2 secretion [112]. This occurs because CDK6 inhibition in particular enhances the nuclear translocation and activity of nuclear factor of activated T cell (NFAT) family members [107,112]. Second, CDK4/6 inhibitors suppress the proliferation of immunosuppressive regulatory T cells more than of other T cell subsets, potentially shifting the immune balance in favor of an antitumor immune response [99]. Also noteworthy is the fact that endothelial cell proliferation and angiogenesis are CDK4/6-dependent phenomena [122], and the effects of CDK4/6 inhibition on tumor angiogenesis are not known. Finally, CDK4/6 inhibitors have been shown to inhibit the proliferation of bone marrow hematopoietic stem and progenitor cells, mitigating the damage these cells can incur from exposure to cytotoxic chemotherapy, and providing a potential rationale for CDK4/6 inhibitors in the supportive care

of patients receiving conventional chemotherapy [123]. Continued exploration of the effects of CDK4/6 inhibitors on other cell types, including adaptive and innate immune cells, endothelial cells, and other mesenchymal cells, represents an important area of investigation.

Concluding Remarks

Pharmacologic inhibitors of CDKs 4 and 6 have changed the treatment landscape for breast cancer, and it is likely that their clinical indications will expand in the years to come. Although they were developed to inhibit tumor cell proliferation, their effects on cancer cells and other cells in the tumor microenvironment are clearly more extensive. Of particular importance, recent reports have highlighted that these agents can modulate intracellular kinase signaling, induce a senescence-like state, and enhance tumor cell immunogenicity. There are still many gaps in our understanding of how CDK4/6 inhibitors affect tumor cells, and why their impact differs in different cancer types (see Outstanding Questions). The key challenge now is to build upon this new information, much of it unexpected, and to then successfully integrate it into the design of new clinical trials. Only then will our patients stand the greatest chance of reaping the benefits of many decades of cell cycle research.

Outstanding Questions

Which cancer types should be prioritized in future CDK4/6 inhibitor trials? Answering this question will require a deeper understanding of the molecular determinants of cancer cell sensitivity to these agents.

To what extent are the phenotypes induced by CDK4/6 inhibitors *in vitro* (e.g., senescence, immunogenicity, autophagy) observed in human tumors? This can only be addressed by analyzing tumor biopsies obtained from patients that are receiving these agents.

How do CDK4/6 inhibitors modify kinase signaling in cancer cells that are controlled by different driving oncogenes (e.g., the PI3K pathway vs the RAS pathway)?

By what mechanisms do CDK4/6 inhibitors impact on the functions of T cells and other components of the adaptive immune system, and can they be used to enhance the effects of cancer immunotherapy?

What are the mechanisms by which tumor cells acquire resistance to CDK4/6 inhibitors?

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