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Cyclin D1, Cancer Progression and Opportunities in Cancer Treatment

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Abstract

Mammalian cells encode three D cyclins (D1, D2 and D3) that coordinately function as allosteric regulators of cyclin dependent kinase 4 and 6 (CDK4/CDK6) to regulate cell cycle transition from G1 to S phase. Cyclin expression, accumulation and degradation, as well as assembly and activation of CDK4/CDK6 are governed by growth factor stimulation. Cyclin D1 is more frequently dysregulated than cyclins D2 or D3 in human cancers and as such it has been more extensively characterized. Overexpression of cyclin D1 results in dysregulated CDK activity, rapid cell growth under conditions of restricted mitogenic signaling, bypass of key cellular checkpoints and ultimately neoplastic growth. This review discusses cyclin D1 transcriptional, translational, posttranslational regulation, and its biological function with a particular focus on the mechanisms that result in its dysregulation in human cancers.

Keywords

Cyclin D1; CDK4/CDK6; Proteasome; Posttranslational Regulation; Cancer

Introduction

The cell cycle refers to the experimentally determined intervals during which cells prepare for and subsequently duplicate their genome equally between two daughter cells. It is divided into four consecutive phases: G1 phase, during which cells accumulate mass and metabolites necessary for DNA replication; S phase, when DNA is replicated; G2, a gap phase that is essential to ensure accurate DNA replication; and M phase, DNA segregation and cell division. While the primary phases of cell division define states of proliferation and division, the majority of adult cells are maintained in a quiescent state (known as G0 phase), a resting state cells often enter post-mitotically or prior to terminal differentiation [1]. Unlike cells many terminally differentiate cells however, quiescent cells can reenter the cell cycle in G1 phase when exposed to appropriate mitogenic stimuli [2].

Transitions through the cell cycle are driven by cyclins and cyclin-dependent kinases (CDKs) [1]. Cyclins are the allosteric activators of cognate CDKs; their levels typically oscillate across the cell cycle, hence gaining the name cyclins. The cyclin family shares a homologous N-terminal 100-amino acid motif referred as the cyclin box that has a highly conserved three-dimensional structure and provides the binding interface for the appropriate CDKs [3]. CDKs define the partner kinases that can be activated only when they bind to

their cognate cyclins. Due to their biological significance, CDK activity is stringently regulated by the following mechanisms: the levels of cyclin partners; phosphorylation status; and the abundance of CDK inhibitory proteins, such as, the INK4 family: p16^{INK4A}, p15^{INK4B}, p18^{INK4C} and p19^{INK4D} and the CIP and KIP family: p21^{CDKN1A}, p27^{CDKN1B} and p57^{CDKN1C} [4].

D cyclins, including cyclins D1, D2 and D3, form active complexes with either CDK4 or CDK6, which in turn phosphorylate the retinoblastoma protein (Rb) and drive G1 to S phase progression [5]. D cyclins coordinate cell cycle progression with the extracellular stimulation (e.g. growth factor availability, nutrient availability and integrin-derived adhesion signaling) [6]. Given the role of D cyclins in mediating extracellular cues with cell proliferation, it is not surprising that overexpression of D cyclins or hyperactivation of their cognate CDK directly contributes to neoplastic growth. More specifically, cyclin D1 has attracted widespread attention due to the prevalence of its dysregulation in human cancers [7]. This review focuses on and discusses cyclin D1 structure, transcriptional, translational and posttranslational regulation, and its biological function. It also addresses the dysregulation of cyclin D1 in human cancers and the advancement and impact of new therapeutic inhibitors targeting CDK4/CDK6.

Transcriptional, Post-transcriptional and Translational Regulation of cyclin D1

1. β -catenin-dependent regulation of cyclin D1 transcription

Physiologically, Wnt/ β -catenin pathway regulates the development of various tissues and organs, including heart, liver, lung, brain, kidney and so forth [8]. Moreover, it also plays important roles in pathological conditions including gastric cancer, colorectal carcinoma, liver cancer and melanoma [9]. β -catenin mediates the canonical Wnt signaling pathway: the binding of Wnt to its receptor suppresses the degradation of β -catenin, which is mediated by the cytoplasmic β -catenin destruction complex. Reduced degradation and cytoplasmic accumulation of β -catenin results in increased nuclear translocation, where it associates with lymphoid enhancer factor/T-cell factor (LEF/TCF) and drives expression of key downstream target genes. The CCND1 gene, which encodes cyclin D1, represents a key target. β -catenin/LEF-1 complexes target motifs at -75 and -15 within the CCND1 promoter [10]. Importantly, cyclin D1 is necessary for β -catenin to drive colon carcinoma development [11]. It is also noteworthy that Wnt regulates cyclin D1 protein stability independent of β -catenin much as Ras-signaling regulates cyclin D1 accumulation and activation through multiple mechanisms [12, 13].

2. Epidermal Growth Factor Receptor (EGFR) and cyclin D1 expression

Cyclin D1 expression is responsive to a variety of growth factors [14], among which EGF is a classic mediator [15]. EGFR overexpression and/or hyperactivation correlates with poor prognosis in human cancers, including breast cancer, non-small cell lung carcinoma, and colon carcinoma [16]. As a mitogenic growth factor, EGF regulates prostate cancer cell proliferation at least partially through regulating cyclin D1 expression [17], and it regulates cyclin D1 accumulation at both mRNA and protein levels. ErbB2, also known as Neu or

Her2, is implicated in 20-30% of human breast cancers [18]. Here again, cyclin D1 expression is induced by Her2/Neu, Ras, Rac, Rho, c-Jun N-terminal kinase and p38 [19]; it is of equal importance that cyclin D1-CDK4 function is required for Her2-driven mammary carcinoma [19-21]. This work has contributing directly to the use and thus the success of CDK4/6 inhibitors in patients with HER2 positive breast cancers [22].

3. Phosphatidylinositol 3-kinase (PI3K) regulates cyclin D1

PI3K catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP₂) to form phosphatidylinositol 3,4,5-trisphosphate (PIP₃); PIP₃ in turn recruits Akt/PKB to the cell membrane, where it is phosphorylated and activated [23]. Activated Akt/PKB controls cell growth, differentiation, proliferation, motility and metabolism. Previous work revealed a role of PI3K in promoting G1/S cell cycle progression [24], suggesting a potential connection with D-type cyclins. Indeed, PI3K/Akt regulate nuclear accumulation of cyclin D1 through regulation of GSK3 β [12]. Consistently, dominant-negative (DN) alleles of either subunit of PI3K strongly suppress EGF-induced cyclin D1 accumulation [24]. Likewise, chemical inhibition of PI3K also reduces cyclin D1 at both mRNA and protein levels upon EGF stimulation, while rapamycin, a well-known mTORC1 inhibitor, exhibits no effect on EGF-induced cyclin D1 regulation [24]. Cumulatively, this supports a model where PI3K is indispensable for EGF-induced cyclin D1 upregulation. In glioma cells, cyclic-AMP response element binding (CREB) protein acts as a critical hub that mediates PI3K-Akt-induced cyclin D1 upregulation upon mitogenic stimulation [25]. Modulation of cyclin D1 by the PI3K-Akt signaling pathway represents one mechanism of growth factor-dependent sensing by cyclin D1.

4. NF- κ B-dependent control of cyclin D1

The NF- κ B transcription factor family, including p65 (RelA), RelB, c-Rel, p50/p105 (NF- κ B1), and p52/p100 (NF- κ B2), participates in various physiological and pathological processes including inflammation, tumorigenesis and tumor progression [26]. Members of the NF- κ B family contain conserved Rel homology domain that mediates dimerization, nuclear localization, DNA binding and their interaction with inhibitory I κ B proteins (I κ B). NF- κ B directly binds to cyclin D1 promoter and controls cyclin D1 transcription [27]. Other related studies implicated c-Rel, RelB and p52 in the regulation of cyclin D1 transcription in mammary tumors of transgenic mice [28], suggesting a key role of NF- κ B-dependent regulation of cyclin D1 during mammary gland tumorigenesis.

Post-transcriptional Control (Alternative Splicing) of cyclin D1

The gene encoding cyclin D1, CCND1, contains five coding exons, from which two transcripts are derived (cyclins D1a and D1b) (Figure 1) [29, 30]. Cyclin D1a is transcribed from an mRNA transcript derived from all five exons. The N-terminal region of cyclin D1a has a conserved Rb binding LXCXE motif; the middle contains cyclin box with the greatest homology between D Cyclins (Cyclin box is the domain that interacts with CDKs and CDK inhibitors: p21, p27 and p57); the C-terminal domain regulates protein stability. As discussed subsequently, this domain contains a threonine residue (Thr-286) that is phosphorylated by glycogen synthase kinase-3 β (GSK-3 β) [12]; phosphorylation of this

residue is both necessary and sufficient for ubiquitylation-dependent degradation. In contrast to D1a, cyclin D1b is encoded by an mRNA where intron 4 is not spliced, resulting in a unique C-terminus. Alternative splicing of CCND1 occurs primarily in the context of cancer and splicing factors implicated in its generation include ASF/SF2 and Sam68 [31, 32]. As a result of this alternative splicing, cyclin D1b loses its key regulatory motif encoded by exon 5 that directs its ubiquitylation-dependent degradation; the consequence is cyclin D1b accumulation in the nucleus and ultimately tumorigenesis [30, 33].

Posttranslational Regulation of cyclin D1

Cyclin D1 is highly labile, with a half-life of 10-30 minutes, and its degradation depends on cell cycle phases [12, 34]. Protein degradation is directed by polyubiquitylation, and thereafter, destruction via the 26S proteasome. Cyclin D1 degradation requires site-specific phosphorylation by GSK-3 β at a conserved threonine residue, Thr-286. Mutation of this threonine to a non-phosphorylatable residue dramatically stabilizes cyclin D1, inhibits its nuclear export and triggers the constitutive activation of CDK4/CDK6 within the nuclear compartment [12, 35, 36]. This nuclear dysregulation ultimately drives p53 inactivation, rampant genomic instability and neoplastic transformation in vitro and tumorigenesis in vivo [35, 37-40]. Although transcriptional regulation of cyclin D1 is complicated and is likely responsive to an underappreciated number of transcriptional regulators, post-transcriptional control ultimately dictates the overall accumulation of cyclin D1 in both normal and tumor cells due to its relative instability.

Protein ubiquitylation requires the concerted and coordinated function of three enzymes: E1 ubiquitin activating enzyme, E2 conjugating enzyme, and E3 ubiquitin ligase. The E3 ligase directs substrate specificity; it contains the largest family members and is generally the key regulatory component in this pathway. E3 ligases are classified into three categories: Homologous to E6-Associated Protein C-Terminus (HECT), Really Interesting New Gene (RING) and U-box [41]. Among these, cyclin D1 ubiquitylation is directed by the RING family E3 ligases. As discussed below, the SKP1-Cullin 1-F-box (SCF) is the primary subclass that directs cyclin D1 ubiquitylation [42]. Within this subclass, SKP1 and Cullin 1 are core components; while the F-box proteins, composed of ~ 80 family members, determine the substrate specificity. F-box proteins are defined by an F-box motif that is so coined for its homology with cyclin F [43]. F-box proteins are divided into three classes: Fbxw (with WD40 repeats as substrate binding domain), Fbxl (with Leucine-rich repeats as substrate binding domain) and Fbxo (with “other” substrate binding domains) [44]. The following section discusses the E3 ligases that have been implicated in regulating cyclin D1 ubiquitylation and degradation.

1. Fbxo4

Fbxo4 and alpha α B-crystallin, identified through the purification of cyclin D1 under conditions that favor stabilization of substrate-E3 ligase binding, were subsequently implicated as the major F-box protein binding to Thr-286 phosphorylated cyclin D1 [34, 45]. It was also noted that α B-crystallin is indispensable for Fbxo4-dependent binding to phosphorylated cyclin D1. Fbxo4-mediated cyclin D1 degradation involves the following

steps: i) cyclin D1 phosphorylation; 2) CRM1-dependent nuclear export, and 3) cytoplasmic polyubiquitylation and degradation (Figure 2) [46]. Phosphorylation of cyclin D1 at Thr-286 by GSK-3 β is required for both binding to CRM1, which in turn directs nuclear export and recognition by Fbxo4 [47]. GSK-3 β also phosphorylates Fbxo4; this phosphorylation generates a 14-3-3 ϵ binding site and it is necessary for Fbxo4 homodimerization [48], a regulatory event required for efficient cyclin D1 ubiquitylation. The importance of phosphorylation and dimerization is emphasized by the identification of mutations in human cancers that directly abrogate phosphorylation/dimerization, which in turn lead to cyclin D1 accumulation in human esophageal squamous cell carcinoma and melanoma [34, 47]. In tumor cells, the overexpression and/or hyperactivation of mitogenic signaling pathways activate PI3K-Akt signaling, which phosphorylates and inactivates GSK3 β . This “hypersignaling” directly impacts the Fbxo4-cyclin D1 axis, resulting in dysregulation of nuclear cyclin D1-CDK4, and finally tumorigenesis [46, 48, 49].

While Fbxo4 is subject to point mutations in certain cancers, findings in hepatocellular carcinoma (HCC) reflect a different mechanism. In HCC, sequencing analysis revealed four Fbxo4 isoforms: Fbxo4 α (full length), Fbxo4 β (with 7 amino acids encoded by a read through from intron 5, thus causing a sequence replacement for exon6), Fbxo4 γ (missing 168-245 nt of exon1) and Fbxo4 δ (missing exon6) [50]. Only Fbxo4 α regulates cyclin D1 ubiquitylation-dependent degradation. The mechanisms that regulate the alternative splicing and generation of different isoforms, and their impacts on cancers remain to be clearly established.

2. Fbxo31

Cellular senescence can be triggered by the attrition of chromosomal telomeric ends or via stress conditions that include low nutrient levels, oncogene activation, reactive oxygen species, and radiation treatment. Among these, oncogene-induced senescence is considered as an important mechanism for tumor suppression. Fbxo31 was identified in screening for factors that regulate senescence. Fbxo31 levels can be induced by DNA-damage, and interestingly, elevated Fbxo31 levels reversely correlate with cyclin D1 levels. Follow-up investigation suggested that Fbxo31 is a checkpoint protein that arrests cells upon genotoxic stress treatment [51]. Another work has revealed that Fbxo4 is also a major regulator of cyclin D1 stability following DNA damage [52]. In fact, Fbxo4 is subject to hemizygous mutations in human melanoma; moreover, Fbxo4 knockout mice overexpress cyclin D1 in all tissues, including melanocytes. Of equal importance, Fbxo4 loss cooperates with BRAF^{V600E} to promote the development of metastatic melanoma in a cyclin D1-dependent manner [47].

3. SKP2

The Cullin 1-SKP2-SKP1 E3 ligases make significant contribution to the regulation of the G1/S transition. Key substrates include the CDK inhibitors p21 and p27 [53-57], which have been validated biochemically and in cells through loss of function experiments. Cyclin D1 has also been suggested to be a substrate [58]. This conclusion was based on SKP2 loss of function analysis and its binding to cyclin D1 in co-immunoprecipitation experiments. However, SKP2 E3 ligase has not been shown to ubiquitylate cyclin D1, suggesting this

regulation may be an indirect effect. In addition, previous reports have already demonstrated that both p21 and p27 could stabilize cyclin D1 through inhibition of its nuclear export [53]. Taken together, SKP2 loss-mediated cyclin D1 upregulation likely reflects decreased ubiquitylation and degradation of p21 and p27; increased levels of p21 or p27 in turn contribute to cyclin D1 stabilization.

4. β -TrCP

β -TrCP, a WD40-repeat containing F-box and β -transducin-repeat containing protein, regulates cell division and signaling pathways that contribute to tumorigenesis [59]. β -TrCP recognizes substrate with a specific phosphorylated motif: DSG(X)₂S [42]. β -TrCP-mediated cyclin D1 ubiquitylation and degradation is found in a condition treated with a compound, named STG28, a derivative of troglitazone [60]. It has been shown to suppress cyclin D1 as well as cell cycle regulatory proteins, such as β -catenin and androgen receptor [61, 62]. In a work investigating the mechanism how STG28 regulates cyclin D1 expression, the E3 ligase β -TrCP was implicated as an active partner that interacts with cyclin D1 instead of the reported ligases, such as, SKP2, Fbxo4 and Fbxw8. Ubiquitylation assay suggests β -TrCP is a E3 ligase that controls cyclin D1 stability upon STG28 treatment [60]. The interaction between cyclin D1 and β -TrCP depends on Thr-286 phosphorylation. Given that cyclin D1 lacks a β -TrCP binding motif, the precise mechanism of regulation is likely indirect and remains to be elucidated.

5. Cdc27/APC3 and the Anaphase Promoting Complex/Cyclosome (APC/C)

As a conserved E3 ubiquitin ligase, APC/C is critically important for the fidelity of mitosis and directly regulates anaphase progression [63]. APC/C promotes the degradation of securin that facilitates the division of two daughter genomes. In addition to a variety of mitotic substrates, APC/C has been implicated in the regulation of cyclin D1 degradation via a direct binding [64]. Additional work provided direct support that suggests Cdc27/APC3 not only associates with cyclin D1, but also promotes cyclin D1 ubiquitylation [64]. Cdc27-mediated cyclin D1 degradation depends on a D-box for interaction and RK residues at position 179/180 for ubiquitylation [64]. How APC/C and under what physiological conditions contributes to cyclin D1 regulation remains unclear. Given that D1 is destroyed in G1 phase following DNA damage in an SCF-dependent manner, it seems unlikely that APC/C-dependent degradation would play a significant contribution at least in normal cells. However, in cells where Fbxo4 for example has been deleted, APC/C-dependent control may be important for maintaining mitotic viability. If this is the case, it might also represent a therapeutically tractable event.

Discrepancies in the E3 Ligases that Regulate cyclin D1 Ubiquitylation

It is apparent from the above discussion that cyclin D1 ubiquitylation is likely to reflect the activity of more than a single E3 ligase. It is not uncommon to have redundancy in the regulation of key growth regulatory proteins. For example, c-Myc polyubiquitylation can be catalyzed by at least three distinct E3 ligases [65-68]. With regard to cyclin D1 and each distinct E3 ligase, it remains important to evaluate the regulation in model organisms and multiple cell lines. For example, although transient knockdown of SKP2 results in its

accumulation, cyclin D1 does not accumulate in SKP2 knockout mouse embryonic fibroblasts (MEFs). In addition, SKP2 regulates p21 and p27, two factors that control cyclin D1 nuclear export [53], and thus upon acute SKP2 loss, any cyclin D1 accumulation observed would likely reflect an indirect regulation. Fbxo4 has been knocked-out in mice by two independent groups. Here again, discrepancy was noted with one group observing significant cyclin D1 overexpression and tumor susceptibility in tissues sensitive to cyclin D1 overexpression [47, 49], furthermore, tumor and biochemical data support the validity of Fbxo4 as one definitive regulator of cyclin D1 ubiquitylation and abundance; the second group failed to observe significant cyclin D1 overexpression when altering any ligase tested [69]. The reasons for the discrepancy remain unclear; it could reflect context-dependency or tissue specificity.

Stress-dependent Regulation of cyclin D1

1. Cyclin D1 and the DNA Damage Response (DDR)

Nuclear cyclin D1 accumulation leads to uncontrolled cell cycle progression [35]. Given the capacity of cyclin D1 to drive inappropriate cell division, it is not surprising that dysregulation of cyclin D1 might generate genome instability. Investigation of the mechanisms that underscore nuclear cyclin D1-dependent neoplastic growth revealed overexpression of the constitutively nuclear and stable cyclin D1 (T286A), but not wild type cyclin D1, promotes stabilization and mis-expression of the DNA replication licensing factor Cdt1, which then triggers DNA re-replication and DNA damage [37]. As a result, cyclin D1 T286A increases the incidence of DNA damage-induced chromatid breaks that favor the occurrence of “second hit”, and contribute to overt malignancy. Is this activity of non-phosphorylatable cyclin D1 relevant in the context of tumors that harbor wild type cyclin D1 but have mutations in an upstream E3 ligases? Indeed, loss of Fbxo4, for example, results in nuclear accumulation of cyclin D1 and mis-regulation of Cdt1 [52]. This likely reflects the fact that nuclear export of cyclin D1 is constitutive, and ubiquitylation-dependent destruction is the key event in preventing cyclin D1 nuclear accumulation.

In the context of DNA damage, cyclin D1 proteolysis depends on the activation of ATM and GSK-3 β , which in turn trigger Fbxo4-dependent cyclin D1 ubiquitylation [52]. As anticipated, nuclear cyclin D1-CDK4 drives genomic instability and facilitates neoplastic transformation and tumorigenesis in the absence of ATM [70]. Cyclin D1 also intersects with genome integrity through additional mechanisms. Genome-wide screening showed direct interaction between cyclin D1 and DDR proteins, such as Rad51 [71]. RAD51, as a recombinase, plays a critical role in homologous recombination, which keeps the genomic stability and normal cell cycle. Radiation enhances the interaction between Rad51 and cyclin D1; therefore, cyclin D1 is recruited to DNA damage sites in a BRCA2-dependent manner [71]. Loss of cyclin D1 inhibits Rad51-mediated DDR and increases cellular sensitivity to radiation. Cyclin D1-dependent effects on transcription have also been implicated in DDR [40, 72]. The effects of nuclear cyclin D1 in mediating DDR highlight a novel mechanism of genomic instability and pave the molecular basis for utilizing CDK inhibitors to treat tumors with cyclin D1 dysregulation.

2. The Unfolded Protein Response (UPR) suppresses cyclin D1 protein synthesis

The UPR defines the cellular response to unfolded and/or misfolded proteins in the endoplasmic reticulum (ER). This stress results in the activation of three signal transducers: PKR-like ER kinase (PERK) and Inositol-requiring enzyme 1 (IRE1), both of which harbor intrinsic protein kinase activity and Activating Transcription Factor 6 (ATF6), a transmembrane transcription factor that is activated by proteolytic cleavage [73]. UPR activation triggers a rapid G1 arrest, providing cells an opportunity to abrogate stresses and damages prior to cell apoptosis. The characterization of this response revealed that the arrest is a direct consequence of the inhibition of cyclin D1 protein synthesis with no alterations in gene transcription or protein degradation [74]. The major regulator of protein synthesis following UPR engagement is PERK. Under stress conditions, PERK phosphorylates eIF2 α , which inhibits the global gene translation [75-77]. Additional analysis demonstrated that PERK activation is both necessary and sufficient for cyclin D1 downregulation during the UPR [78, 79]. Therefore, cyclin D1 suppression mediated by UPR is regarded as a conserved response, which coordinates cell proliferation with the homeostasis of both extracellular and intracellular environment, and keeps cell survival under stress conditions.

Substrates of cyclin D1-CDK4 Complex

The cyclin D-CDK4/6 kinase is unusual among the larger family of proline directed kinases in that it is highly specific and few bona fide substrates have been identified and validated. The best characterized substrate for the cyclin D-CDK4/6 kinases is Rb (and related p107, p130) [80-82]. Hyperphosphorylation of Rb leads to de-repression of E2F family transcription factors and transcriptional activation of genes that control cell cycle progression, development and metabolism [5]. Analysis of primary tumors and tumor-derived cell lines has established that Rb and its related family proteins are the key substrates of cyclin D1-CDK4/6. First, loss of Rb is mutually exclusive with cyclin D1 mutation or amplification. Second and of equal importance, the fact that cyclin D1 activity is superfluous in cells lacking Rb is another genetic evidence for the kinase substrate relationship. Importantly, this key observation establishes the patient population that will be benefited from anti-CDK4/6 therapy. In an attempt to broaden our understanding of substrates, an unbiased systematic substrate screen was utilized and 68 potential candidates were implicated [83]. However, the majority of these candidates remain to be validated. Substrates that have been biochemically and functionally validated include Smad3, Forkhead Box M1 (Foxm1), Nrf1 and the protein arginine methyltransferase 5 (PRMT5) co-factor, MEP50 [84].

1. Smad3

Smad3 is a critical downstream mediator of transforming growth factor beta (TGF- β) [85]. Smad3, as a transcription factor, regulates the transcription of cell cycle regulators including p15, p21 and c-Myc [86]. In the presence of TGF- β , Smad3 associates with E2F4, E2F5, DP1, p107, as well as Smad4 to form transcriptional inhibitory complexes [87]. The oscillation of Smad3 in a cell cycle-dependent manner suggested its potential as a cyclin-CDK substrate. Biochemical analysis confirmed phosphorylation by both CDK4 and CDK2 at Thr8, Thr178 and Ser212 [88]. Phosphorylation of Smad3 inhibits its transcriptional

function and thus its anti-proliferative activity. As such, hyperactivation of CDK, as is frequently observed in cancers, promotes tumorigenesis and resistance to TGF- β through Smad3 phosphorylation. In addition, suppression of CDK-mediated Smad3 phosphorylation leads to decreased cell migration and invasion, finally inhibition of xenograft growth of triple negative breast cancer cells [89].

2. Foxm1

Foxm1, a member of Forkhead superfamily of transcription factors, contributes to embryonic development and tissue homeostasis, as well as pathological conditions, such as, tumorigenesis and tumor progression [90]. It regulates the expression of a large spectrum of genes that control cell cycle progression, cell proliferation, differentiation, migration and survival, DNA damage response, and blood vessel formation [91]. Unbiased screening identified Fxom1 is a direct substrate of cyclin D1-CDK4 complex. Phosphorylation of Fxom1 enhances its stability and transcriptional activity, resulting in cell cycle re-entry and suppression of senescence in melanoma cells but not in melanocytes [83]. This study provided a previously unanticipated molecular basis for the utilization of CDK4/CDK6 inhibitors to treat melanoma.

3. Nuclear respiratory factor 1 (Nrf1)

Nrf1, a nuclear encoded, mitochondrial transcription factor, increases mitochondrial respiratory function through direct transcription of genes that mediate respiratory activities and cell size [92]. Cyclin D1 has been linked with mitochondrial function through a work demonstrating the capacity of the cyclin D1/CDK4 kinase to directly phosphorylate Nrf1 on Serine 47 [93]. Phosphorylation of Nrf1 in turn reduces the expression of Nrf1-dependent genes. Based upon these results, a model is suggested wherein the overexpression of cyclin D1, and by extension, overactivation of the cyclin D1/CDK4 kinase, in tumor cells will reduce mitochondrial respiration with a consequent shift towards cytosolic glycolysis. The Warburg effect, as it is termed, is critical for increasing biosynthetic precursors that are needed to support a high rate of cell proliferation and growth [94].

The Biological Functions of cyclin D1

1. Cyclin D1 and cell cycle regulation

Cyclin D1, with its partner CDKs, regulates G1/S transition through Rb phosphorylation [81, 95]. Small polypeptide inhibitors of CDK4/CDK6 efficiently block Rb phosphorylation in vivo. Moreover, Rb is also phosphorylated by cyclin E-CDK2 in the late G1 phase. The hyperphosphorylation of Rb triggers reduced affinity for E2F, thereby permitting E2F activation and transcription of client genes required for cell division [1]. In human tumors, the cyclin D1-CDK4 axis shows a high frequency of alterations, highlighting the importance of this pathway for tumor progression. With the recent advent of small molecule inhibitors of CDK4/CDK6, it is critical to discern key contributions of cyclin D1 with CDK - dependent and -independent effects in order to develop rational and successful therapeutic regimes. The additional discussion below will introduce our current understanding of major activities of cyclin D1 with regard to these functions.

2. Cyclin D1-dependent transcriptional regulation

Gene transcription is a multi-step process that includes the recruitment of transcription factors and co-activator complexes to modify the chromatin at or near the transcription start site. In addition to CDK regulation, cyclin D1 has also been implicated in the regulation of gene transcription. Its function as a transcriptional regulator invokes both CDK -independent and - dependent mechanisms.

With regard to the former mechanism, cyclin D1 can associate with a variety of transcriptional regulators including chromatin modifying enzymes such as histone acetyl transferases P/CAF, NcoA/SRC1a, AIB-1, GRIP-1, TFIID, and TAF_{II}250 [96]. Cyclin D1 can also function as a co-repressor through recruitment of Histone Deacetylases (HDAC) 3; this repression can be alleviated by trichostatin A treatment [97]. Cyclin D1 also interacts with sequence specific DNA binding proteins such as the estrogen receptor, the androgen receptor and the myb like protein, DMP1 [98-100]. It is interesting to note that such association is generally correlated with transcriptional repression.

The binding and regulation of the above transcription factors is a CDK-independent activity of cyclin D1, raising the question of whether any transcriptional activities of cyclin D1 are CDK-dependent and thus could be modulated by small molecule CDK4 inhibitors. Indeed purification of nuclear, oncogenic cyclin D1 alleles from tumor tissues led to the identification of PRMT5-MEP50 as a target of the cyclin D1-CDK4 complex [38]. PRMT5 symmetrically dimethylates proteins; for example, the methylation of histones 3 and 4, resulting in heterochromatinization and transcriptional silencing [101]. In the context of cyclin D1-driven malignancies, cyclin D1-CDK4 directs PRMT5-MEP50 to the Cul4A/B promoters, thereby repressing transcription of these genes. It is the phosphorylation of MEP50 by cyclin D1-CDK4 that is integral to this regulation; phosphorylation of MEP50 also increases the catalytic activity of PRMT5 [38]. Histones are not the only target of cyclin D1-CDK4 activated PRMT5. More recent work revealed that cyclin D1-CDK4 can inactivate p53 through PRMT5-dependent p53 methylation, thereby permitting tumor progression while maintaining wild type p53 [40, 102]. Collectively, cyclin D1 has both direct and indirect impacts on transcriptional regulation. Additional work is required to determine how these activities contribute to normal versus neoplastic growth and whether such activities represent drugable targets.

3. Cyclin D1 and metabolism

Dysregulated metabolism is implicated as a major contributor to a number of human diseases, including cancer, obesity and diabetes. Notwithstanding, the cyclin D1-CDK4 axis impacts specific aspects of metabolic regulation. Peroxisome-proliferator-activated receptor (PPAR)- γ coactivator-1 α (PGC-1 α) regulates mitochondrial biogenesis, and acts as a transcriptional regulator that controls the expression of antioxidant genes, energy fuel selection, muscle fiber differentiation and transformation [103]. A recent study revealed that cyclin D1-CDK4 can modulate PGC-1 α acetylation, putatively through GCN5 phosphorylation; moreover, the cyclin D1 T286A mutant can constitutively acetylate PGC-1 α [104]. Acetylation of PGC-1 α inhibits its activity on gluconeogenic genes, such as, PCK1 and G6PC [104]. In addition, insulin deactivates GSK-3 β through PI3K-Akt signaling

pathway, resulting in cyclin D1 nuclear accumulation; therefore, insulin utilizes cyclin D1-CDK4 machinery to regulate glucose homeostasis independent of their function in cell cycle regulation. Further support stems from work demonstrating that CDK4/CDK6 inhibition increases mitochondrial number, leading to increased reactive oxygen species (ROS) [105]. In addition to regulation of gluconeogenesis, cyclin D1 can suppress glucose-induced key lipogenic genes, such as, carbohydrate response element-binding protein (ChREBP) and hepatocyte nuclear factor 4 α (HNF4 α); this regulation occurs via both CDK4 -dependent and -independent mechanisms, highlighting the direct relationship between the components of cell cycle and the transcriptional reprogramming of lipid metabolism [106]. Cyclin D1-CDK4 can also regulate mitochondrial function through direct phosphorylation of the mitochondrial transcription factor, Nrf1 [93]. How these findings will influence the development of future therapies, particularly with regard to combining CDK4 inhibitors with small molecules that target metabolic pathways, will be of interest. For example, a recent study reported the combined therapy using CDK4/CDK6, mTOR and MEK inhibitors can synergistically suppress pancreatic adenocarcinoma development [105].

4. Cyclin D1 and cell migration

Cell migration directly contributes to embryogenesis, immune response, wound repair, and tumor metastasis [107]. Surprisingly, cyclin D1 deletion was noted to increase the migratory behavior of MEFs [108]. To ascertain functional intersections, a genome-wide screen was undertaken. The screen revealed that cyclin D1 suppresses the expression of Rho-activated kinase II (ROCKII) and thrombospondin 1 (TSP-1), both of which are important regulators for cell migration [108]. Mechanistically, loss of cyclin D1 correlates with increased phosphorylation of the ROCK II substrates: LIM kinase, cofilin and myosin light chain, in cyclin D1 knockout MEFs. How cyclin D1 regulates ROCKII remains to be firmly established. Interestingly, as a physiological inhibitor of CDK4, p27 can increase migration through suppression of RhoA activity [109]; this regulatory effect appears to be cyclin D1-independent. As the master regulator of miRNA maturation, Dicer also contributes to cyclin D1-dependent cell migration. Cyclin D1 knockdown reduces cell migration in Dicer^{+/+} but not in Dicer^{-/-} HCT116 cells [110]. Tumor metastasis correlates with poor prognosis and very few interventions effectively targeting metastatic diseases make treatment much more difficult. If D cyclins directly contribute to metastatic diseases, the advent of small molecule regulators of the cyclin D/CDK kinases could have tremendous clinical impacts. However, this concept is yet to be interrogated.

Cyclin D1 Dysregulation in Human Cancers

Cyclin D1 is overexpressed and/or amplified in a large fraction of human cancers [111]. Cancers that frequently harbor cyclin D1 genomic alterations include pancreatic cancer (~ 25% - 82%) [112], non-small cell lung carcinoma (~ 5% - 76%) [111, 113, 114], breast cancer (~ 15% - 70%) [115], head and neck squamous cell carcinoma (HNSCC) (~ 20% - 68%) [116, 117], melanoma (~ 0% - 65%) [118], endometrial cancer (~ 26% - 56%) [119, 120], and colorectal carcinoma (~ 2.5% - 55%) [7]. In mantle cell lymphoma (MCL), cyclin D1 overexpression is the result of t(11;14)(q13;q32) rearrangement, and this rearrangement accounts for more than 90% MCL patients, making this translocation a hallmark of MCL

[121]. Multiple myeloma has IgH translocation with cyclin D1: 11q13 (CCND1) (~ 16%), which accounts for cyclin D1 overexpression in ~ 30% - 50% cases [7].

While the t(11;14)(q13;q32) translocation is a hallmark of MCL, it is unlikely the only factor that contributes to cyclin D1 overexpression and dysregulation. Screening of primary MCL revealed that cyclin D1 has mutations in the 3' untranslated region (3'UTR) either from 3'UTR deletion or point mutations that create a premature polyadenylation signal [122]. These mutations result in cyclin D1 mRNA stabilization and cyclin D1 upregulation (~ 4-10%) [16]. As discussed above, alternative splicing of CCND1, resulting in the expression of cyclin D1b, has been observed in a variety of cancers, including carcinomas of the breast, esophagus and prostate [7, 30].

In addition, cyclin D1 mutations can directly perturb its degradation. In esophageal and uterine cancers, mutations that directly target the GSK-3 β phosphorylation site, or disruption of the adjacent nuclear export signal are frequently observed [123, 124]. In addition to mutations in cyclin D1, Fbxo4 also undergoes hemizygous missense mutations (S8R, S12L, P13S, L23Q, G30N and P76T), accounting for 14% of the primary esophageal tumors [34]. Such mutations result in cyclin D1 overexpression, being consistent with Fbxo4 mediated cyclin D1 degradation. Mutations in cyclin D1 (P287S, P287T and delta289-292) have also been reported in endometrial cancers (~ 4%) [125]. Other dimensions of cyclin D1 upregulation depend on the activation of mitogenic signaling pathways, such as, Ras-MEK-Erk, PI3K-Akt, ErbB2 oncogenic pathways [14, 126-132], and loss of microRNAs (miRNAs) that control cyclin D1 mRNA stability, for example, miR-15a and miR-16 in prostate cancers [133].

Clinicopathological studies showed cyclin D1 overexpression correlates with tumor metastasis and poor prognosis in a series of human cancers [7, 123, 134]. The following are some representative examples: cyclin D1 levels directly correlate with tumor size, lymph node metastasis and advanced clinical stages of HNSCC [116]; other work supports the use of cyclin D1 expression as a prognostic indicator to evaluate the survival of patients with lung cancer and breast cancer [7]; finally, in tumors such as pancreatic adenocarcinoma, cutaneous melanoma, endometrial cancer, colorectal carcinoma and MCL, cyclin D1 influences local invasion, metastasis, and patients' prognosis [135]. The importance of cyclin D1 in the above tumors emphasizes the potential of utilizing CDK inhibitors for treatment.

Therapeutic Inhibition of the cyclin D1-dependent Kinases

The critical role of cyclin D1-CDK4 in regulating cell cycle progression and the hyperactivation of cyclin D1-CDK4 in human tumors makes this complex an attractive target for cancer treatment. Cyclin D1 does not possess enzymatic activity, making it a challenging therapeutic target. However, its catalytic partners CDK4/CDK6 can be targeted, therefore, highly specific inhibitors have been developed [136]. Among a variety of inhibitors, those with the highest degree of specificity for CDK4/CDK6 kinases include PD0332991 (Palbociclib) [22], LY2835219 (Abemaciclib) [137] and LEE011 (Ribociclib) [138]. The above three inhibitors exhibit strong efficacy in regard to suppressing Rb phosphorylation

with IC₅₀ at the nanomolar range. Palbociclib was the first CDK4/CDK6 inhibitor approved by FDA to treat ER (+), Her2 (-) locally advanced or metastatic breast cancers [139]. Abemaciclib was also recently received FDA approval for treating patients with refractory hormone-receptor-positive (HR+) advanced or metastatic breast cancers. Besides breast cancers, these inhibitors are undergoing extensive investigations in various clinical trials; the activities and efforts to evaluate CDK4/CDK6 inhibitors in a variety of indications have recently been reviewed in depth [1]. In addition, several other compounds are also undergoing clinical trials; for more detailed information, refer to Table 1, which lists the information on these inhibitors tested in various tumors in different clinical trials.

Future Prospects

Extensive studies illustrated the critical roles of cyclin D1-CDK4 in normal cell cycle regulation and their dysregulation in human cancers [140]. Because cyclin D1 responds to a variety of cell signaling pathways, oncogenic signaling pathways commonly regulate cell proliferation and tumor growth through enhancement of cyclin D1 expression and/or inhibition of cyclin D1 degradation. During the past decade, details regarding the mechanism of cyclin D1 post-translation have been revealed providing key insights that have clinical importance with the advent of highly specific CDK4/6 small molecule inhibitors.

As requisite functional partner kinases of cyclin D1, suppression of CDK4/CDK6 activity successfully blocks cyclin D1-mediated cell cycle progression, making these protein kinases attractive therapeutic targets. The currently available small molecule inhibitors exhibit strong efficacy in the nanomolar range. The evaluation of the activities in various clinical trials (from Phase I to Phase IV) in various solid tumors and leukemia/lymphoma is providing hope for clinical efficacy. The high efficacy of these inhibitors opens a new era for targeted cancer therapy. However, like all other chemotherapeutic chemicals, CDK inhibitors exhibit some degree of side effects, the most common of which is neutropenia. Generally speaking, these side effects can be tolerated by the majority of the patients. Another emerging question is the development of resistance to CDK4/CDK6 inhibitors in preclinical studies; therefore, it is urgent to dissect the detailed mechanisms how tumor cells develop resistance to these inhibitors. To eliminate the possibility of developing resistance, combined therapy may help to some extent. By solving the above questions, in the near future, cancer patients will be benefited from CDK inhibitors as officially approved medicine.

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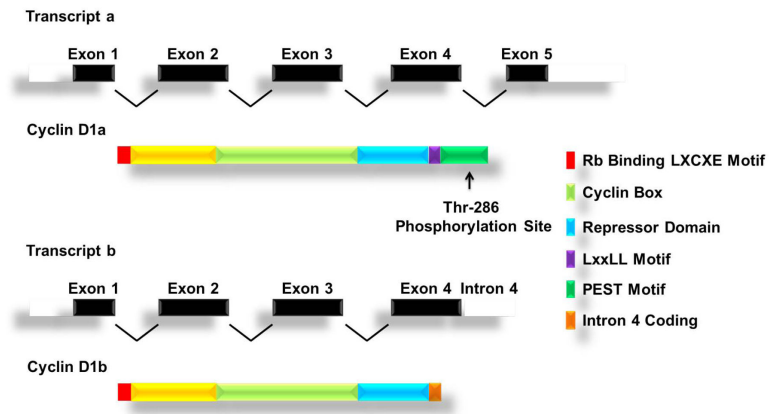


Figure 1. The Structures of Two Transcripts of cyclin D1. Schematic illustration of cyclin D1a (top) and cyclin D1b (bottom).

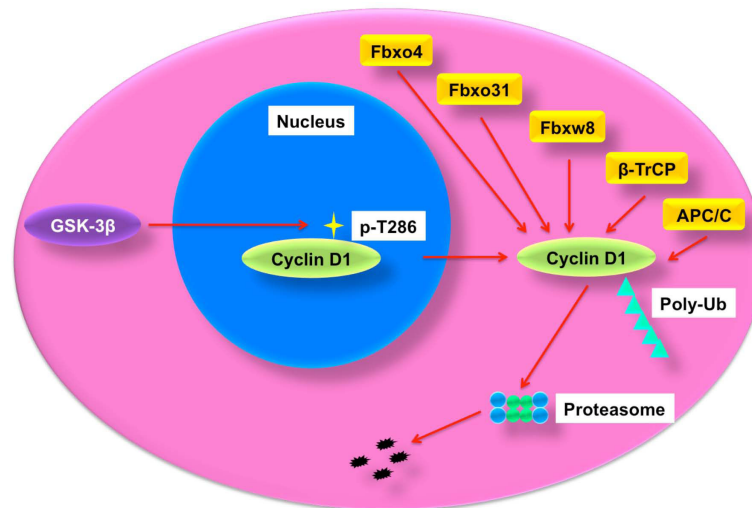


Figure 2. Ubiquitin Proteasome-mediated cyclin D1 Degradation. Phosphorylation is the first step for cyclin D1 degradation. GSK-3 β phosphorylates cyclin D1 at Thr-286. After phosphorylation, cyclin D1 is transported from nucleus to cytoplasm, where it is recognized by different E3 ligases, including Fbxo4, Fbxo31, Fbxw8, β -TrCP and APC/C. After polyubiquitylation, cyclin **D1** is targeted to proteasome for degradation.

Table 1

Clinical Trials of Inhibitors Targeting CDK4/CDK6

Chemical	Clinical Trial Stages (Access: September 5 th , 2016)	ClinicalTrials.gov Identifier No.
BAY1000394, Roniciclib (Bayer)	Phase I: Advanced Malignancies	NCT01188252, NCT01335256, NCT02047890, NCT02390154, NCT02457351
CYC065-01 (Cyclacel Pharmaceuticals, Inc.)	Phase I: Advanced Cancers	NCT02552953
	Phase I: Healthy volunteers	NCT01913314, NCT02059148, NCT02256267, NCT02327143, NCT02387814, NCT02482935, NCT02672423, NCT02677844, NCT02884089
	Phase I: Neoplasm Metastasis, Lymphoma	NCT01394016, NCT02014129, NCT02117648, NCT02688088, NCT02745769
	Phase I: Recurrent Solid Tumors, Breast Neoplasms	NCT02057133, NCT02644460, NCT02857270, NCT02784795
	Phase I: Non-small Cell Lung Carcinoma	NCT02079636, NCT02411591
LY2835219, Abemaciclib (Eli Lilly and Company)	Phase II: Stage IV Squamous Non-small Cell Lung Cancer	NCT02450539, NCT02779751
	Phase II: Mantle Cell Lymphoma	NCT01739309
	Phase II: Melanoma, Sarcoma, Metastatic Breast Cancer	NCT02102490, NCT02308020, NCT02441946, NCT02675231, NCT02747004, NCT02846987, NCT02831530
	Phase III: Breast Cancer, Non-small Cell Lung Cancer	NCT02107703, NCT02152631, NCT02246621, NCT02763566
	Phase I: Healthy volunteers	NCT02388620, NCT02431481
	Phase I: Squamous Cell Carcinoma of the Head and Neck, Myelofibrosis, Neuroblastoma	NCT01747876, NCT02370706, NCT02429089, NCT02780128
	Phase I: Hormone Receptor Positive, HER2-negative, Advanced Breast Cancer, Glioblastoma, Glioma, Advanced Solid Tumors or Lymphoma	NCT01237236, NCT01857193, NCT01898845, NCT02154776, NCT02333370, NCT02345824, NCT02414724, NCT02586675, NCT02599363, NCT02608216, NCT02734615, NCT02754011
LEE011 (Novartis Pharmaceuticals)	Phase I/II: Non-small Cell Lung Cancer, Liposarcoma, Prostate Cancer, Glioma	NCT02292550, NCT02343172, NCT02494921, NCT02555189, NCT02607124
	Phase I/II: Breast Cancer, Melanoma, Metastatic or Advanced Solid Tumors	NCT01543698, NCT01777776, NCT01781572, NCT01872260, NCT02088684, NCT02657343, NCT02703571, NCT02732119, NCT01820364
	Phase II: Gastrointestinal Cancer, CDK4/6 Pathway Activated Tumors, Soft Tissue Sarcoma, Melanoma, Hepatocellular Carcinoma	NCT01820364, NCT02159066, NCT02187783, NCT02420691, NCT02524119, NCT02571829
	Phase II: Breast Cancer, Teratoma, Relapsed ER Positive Ovarian, Fallopian Tube, Primary Peritoneal or	NCT02300987, NCT02632045, NCT02657928, NCT02712723, NCT02774473

Chemical	Clinical Trial Stages (Access: September 5 th , 2016)	ClinicalTrials.gov Identifier No.
P276-00 (Piramal Enterprises Limited)	Endometrial Cancer	NCT01958021, NCT02278120, NCT02422615
	Phase III: Advanced, Metastatic Breast Cancer	NCT00407498
	Phase I: Advanced Refractory Neoplasms	NCT00882063, NCT00898287
	Phase I/II: Pancreatic Cancer, Refractory Multiple Myeloma	NCT00824343, NCT00899054, NCT01903018,
	Phase I/II: Head and Neck Squamous Cell Carcinoma	NCT00835419
	Phase II: Melanoma	NCT01602887, NCT01756768, NCT01756781, NCT01802476, NCT01821066, NCT01844323, NCT01904747, NCT01906125, NCT01918176, NCT01953731, NCT02041273, NCT02059330, NCT02083640, NCT02085538, NCT02097329, NCT02131298, NCT02222441, NCT02311946, NCT02334800
	Phase I: Healthy volunteer	NCT01320592, NCT01976169, NCT02255461, NCT02499146, NCT02626507
	Phase I: Relapsed and Refractory Acute Leukemia and High-Risk Myelodysplasia, Breast Cancer, Central Nervous System Tumors	NCT00420056, NCT01111188, NCT00141297, NCT01522989, NCT02030483, NCT02159755 NCT02501902,
	Phase I: Non-Hodgkin Lymphoma, Mantle Cell Lymphoma, Advanced Solid Tumor Malignancies, Multiple Myeloma, Metastatic Pancreatic Ductal Adenocarcinoma	NCT00721409, NCT01684215, NCT02101034, NCT02202200, NCT02310243, NCT02448771, NCT02599714
	Phase I/II: Breast Neoplasms, Melanoma, Carcinoma, Squamous Cell of Head and Neck, Solid Tumors, MLL-rearranged Acute Leukemia	NCT01709370, NCT01723774, NCT02008734, NCT02022982, NCT02040857, NCT02384239, NCT02448420, NCT02536742, NCT02549430, NCT02592083, NCT02592746, NCT02603679, NCT02668666, NCT02690480, NCT02738866, NCT02760030, NCT02764541, NCT02774681, NCT02778685
PD0332991, Palbociclib (Pfizer)	Phase II: ER (+), HER2 (-) Advanced Breast Cancer	NCT02059213, NCT02465060, NCT02499120, NCT02693535
	Phase II: Advanced Refractory Solid Tumors or Lymphomas, Prostate Cancer, Head and Neck Squamous Cell Carcinoma	NCT01291017, NCT01356628, NCT01907607
	Phase II: Non-small Cell Lung Cancer, Advanced Gastrointestinal Stromal Tumors, Liver Cancer	NCT00555906, NCT01536743
	Phase II: Ovarian Epithelial Carcinoma, Multiple Myeloma	NCT01037790, NCT01209598, NCT02530320, NCT023334527, NCT028066648
	Phase II: Oligodendroglioma, Oligoastrocytoma, Liposarcoma, Cancer, Metastatic Urothelial Carcinoma, Metastatic Melanoma, Pancreatic Neuroendocrine Cancer	NCT02154490, NCT02785939
	Phase II/III: Squamous Cell Lung Carcinoma	

Chemical	Clinical Trial Stages (Access: September 5 th , 2016)	ClinicalTrials.gov Identifier No.
	Phase III: Breast Neoplasms	NCT01740427, NCT01864746, NCT01942135, NCT02028507, NCT02142868, NCT02297438, NCT02600923
	Phase IV: Breast Neoplasms	NCT02679755
R547 (Hoffman-Roche)	Phase I: advanced solid tumors	NCT00400296
	Phase I: Advanced Solid Tumors	NCT00292864
SNS-032, BMS-387032 (Sunesis Pharmaceuticals)	Phase I: B-lymphoid Malignancies, Chronic Lymphocytic Leukemia, Mantle Cell Lymphoma, Multiple Myeloma	NCT00446342

Note: 1. Clinical trials are accessed till September 5th 2016.

2. Clinical trials listed above don't include trials with "Terminated" and "Withdrawn".