Molecular Targeting in Oncology

Cancer Drug Discovery and Development

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We dedicate this book to Scott Wadler, MD—our mentor, colleague and friend.

Dr. Scott Wadler was a gifted clinician, skilled educator and an expert thought leader in Oncology. His foresight and intuition suggested the need for a book on molecular targeting long before this became a buzz word in cancer research. His efforts forged the foundation of this book, and its structural organization is a reflection of his vision for how the field should think about the new era of molecularly targeted agents. His contributions were many, and he will be missed by patients, students and colleagues alike.

Introduction

In contrast to the premise that drug development in oncology has been empirically based, history shows that anticancer drugs have been targeted from the very beginning. The earliest anticancer drugs were targeted antimetabolites (purine and pyrimidine analogs and antifolates). Next came the alkylating agents. Certainly, l-phenylalanine mustard (melphalan) was targeted to melanin metabolism but was found to be more effective in myeloma than in melanoma. Cyclophosphamide was designed as a prodrug to be selectively metabolized in tumor cells. Clinical trials, however, demonstrated antitumor activity, but its metabolism was through the hepatic p450 system. The next class introduced, hormone antagonists, proved effective with less toxicity. Thus, the concepts were brilliant in the past, but our knowledge of the science was as yet inadequate. Our understanding of the biology of cancer only now has permitted much more elegant and effective therapeutic interventions such as Herceptin®, Gleevec®, and Avastin®.

In the 1980s and 1990s, starting with the development of rituximab (Rituxan) for B cell lymphomas, trastuzumab (Herceptin) for Her-2-positive breast cancer, and imatinib (Gleevec) for the treatment of chronic myelogenous leukemia (CML) and GI stromal tumors (GIST), the pharmaceutical industry has regrouped to rationally design drugs with novel mechanisms of action. Epidermal growth factor receptor (EGFR) inhibitors, Iressa® and Tarceva®, to inhibit signal transduction pathways, inhibitors of mammalian target of rapamycin (mTOR), inhibitors of histone deacetylases, and drugs that promote apoptosis are in clinical trials or have recently completed clinical trials.

In *Molecular Targeting in Oncology*, we have attempted to present an overview of the development of targeted therapies for the treatment of cancer with an emphasis on clinical application. Five sections cover the most important elements of drug development: General Strategies for Molecular Targeting in Oncology, Molecular Targeting for Specific Disease Sites, Classes of Drugs for Molecular Targeting in Oncology, Specific Drugs for Molecular Targeting in Oncology, and Challenges in Molecular Targeting in Oncology. These sections present different perspectives on how targeted therapeutics are being evaluated. The "Strategies" section focuses on approaches using targeted therapies to inhibit cell growth. The section on "Disease Sites" describes how clinicians are evaluating targeted therapies in specific organ systems. The third section on "Classes" of targeted therapies illustrates how various classes of pharmacologic and immunologic agents are developed for individual molecular targets. The "Drugs" section focuses on selected new drugs that have novel mechanisms of action. The final section deals with "Challenges" for the future of targeted therapeutics and includes chapters on appropriate patient selection, use of combination therapy, how to deal with tumor cell resistance, advances in targeted imaging, measurement of clinical effects, clinical trial design, and preclinical development of targeted agents. Although the structure of this book guarantees some overlap between chapters and sections, readers might start with the chapters that most interest them and use the supporting chapters to gain a better understanding of how targeted drug development is being viewed by basic science investigators, industry representatives, and government scientists.

The structure of *Molecular Targeting in Oncology* is designed to cover the flavor of the rapidly developing area of targeted therapies for the treatment of patients with cancer. Targeted therapies will likely continue as focus for future drug development as the molecular pathways mediating tumor initiation, progression, and metastasis are better defined and the ability to rationally design drugs using high-throughput technology becomes more firmly established. Therapeutic activity for some targeted agents, such as Gleevec, is remarkable, whereas others significantly reduce toxicity in cancer patients while providing comparable clinical response rates compared with conventional cytotoxic drugs. As further knowledge of the biology of the cancer cell expands over the next few years, the number of targeted agents will likely increase. Our challenge will be to determine how best to use these agents to improve the outcome for our patients with cancer.

Howard L. Kaufman, MD

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I General Strategies for Molecular Targeting in Oncology

1 The Cell Cycle

Therapeutic Targeting of Cell Cycle Regulatory Components and Effector Pathways in Cancer

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Summary

Dysregulation of cell cycle signaling is a pathognomonic feature of tumor initiation and progression. An understanding of the key cell cycle components dysregulated in cancer and the molecular mechanisms responsible has led to the generation of new targeted therapeutics. The development of therapies which selectively inactivate key genetic drivers in specific tumors and the use of molecular abnormalities within the cancer to selectively activate therapies exemplify mechanism-based therapies. The tyrosine kinase inhibitor signal transduction inhibitor-571 (STI-571) is a prototypic molecular-targeted therapy, which selectively targets aberrant Bcr-Abl kinase activity and produces a highly specific anti-cancer effect in chronic myelogenous leukemia (CML) patients. Novel therapies include inhibitors of tyrosine kinases, cyclin-dependent kinases or histone deacetylases, lytic viruses that kill cells with defective p53 function, and molecular mimics that induce or recapitulate endogenous tumor suppressors. These new approaches are derived from an understanding that dysregulated cell cycle control components drive tumorigenesis. Components of the cell cycle often play distinct roles in the biological processes of normal development, normal cell cycle progression in the adult animal, and during the process of tumorigenesis. The realization that cell cycle components play redundant roles in the cell cycle of embryogenesis, but are required for tumorigenesis, provides an additional, compelling rationale for targeting the aberrant cell cycle in cancer. Ultimately, the continued study of the mechanisms used by cancerous cells to evade cell cycle checkpoint control provides the groundwork for the development of rational cancer therapies aimed at improving both the efficacy of treatment and the quality of patient life.

Key Words: Cyclin-dependent kinase; CDK inhibitors; cell cycle; therapy; acetylation; p53; cyclin D1; EGFR; HDAC; STI-571; flavopiridol; CDK2 inhibitor.

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1. INTRODUCTION

A greater understanding of the molecular genetic changes within an individual patient's tumor has led to an alternative therapeutic approach, in which the key genetic drivers of tumorigenesis serve as targets for therapy. Aberrant function and expression of the cell cycle is a uniform feature of human tumors. Targeted therapies directed to abnormal cell cycle control protein function has led to the development of effective new therapies. Herein, we describe the components regulating the cell cycle and highlight recent progress in the field. We discuss preclinical and clinical therapeutic advances, targeting the cell cycle and new types of therapeutics under development.

2. THE CELL CYCLE IN NORMAL CELL DIVISION

Eukaryotic cells, upon stimulation by mitogenic signals, pass through a highly regulated sequence of events referred to as the cell cycle. The cell cycle is marked by four distinct phases: G_1 (Gap1) phase, S (DNA synthesis) phase, G_2 (Gap2) phase, and M (mitosis) phase. During the G_1 phase, the abundance of mitogenic signals determines DNA synthesis, apoptosis, or progression to a quiescent state (G_0 phase). After the commitment to cellular division, cells undergo DNA replication in S phase, passage through G_2 phase, cellular division in M phase, and ultimately return to G_1 phase.

The orderly progression of the cell cycle is controlled primarily by the cyclin-dependent kinases (CDKs). CDKs are serine/threonine-specific protein kinases whose catalytic activity is positively regulated by cyclins and negatively regulated by CDK inhibitors (CDKIs), the expression of which is tightly temporally regulated during cell division. The cyclin-CDK holoenzymes phosphorylate diverse substrates including the retinoblastoma tumor suppressor protein (pRb) and the related p130 and p107 proteins. Many of the cyclin-CDKs regulate cell cycle "checkpoints" that protect a cell from erroneous DNA replication and ensure the accuracy and precision of cell division. Since the discovery of the first CDK by Timothy Hunt in 1983 (CDC2 in yeast), at least 13 human CDKs have been identified which function in cell cycle regulation and other cellular processes (Table 1).

Passage through the G_1 restriction point is regulated by the expression of two G_1 cyclin families, the cyclin D family (D1, D2, and D3), and the cyclin E family (E1 and E2). The D cyclins interact with CDK4 and CDK6, and the E cyclins interact with CDK2, forming heterodimeric holoenzymes, which can phosphorylate pRb rendering it inactive and allowing passage from G_1 into S phase (Fig. 1). The D cyclins, in particular cyclin D1 that is the rate-limiting subunit in the formation of CDK4/6 holoenzymes, are sensitive to mitogenic stimuli and link extracellular proliferation cues to the underlying cell cycle program. Hyper-phosphorylation of pRb by cyclin D-CDK4/6 holoenzymes during mid-G_1 phase results in the release of E2F family members that direct the transcription of the E cyclins and components that are necessary for DNA replication in late G_1 phase. Cyclin E–CDK2 complexes lead to further pRb phosphorylation forming a positive feedback loop that precipitates entry into S phase. Some redundancy in cyclin D/E function may also exist, as transgenic expression of cyclin E in cyclin D1-deficient mice can rescue approximately one-third of the mice from cyclin D1-deficient phenotypes, suggesting complex partial redundancy in cyclin function *(1)*.

Table 1
The Cyclin–Dependent Kinases and their Heterodimeric Regulatory Cyclin Partners. The Proposed Functions are Shown on the Right.

CDK1	cyclin B ringo	Prophase to metaphase transition Regulation of topoisomerase 2 β-Amyloid-induced cytotoxicity
CDK2	cyclin A cyclin E	G_1/S transition, S phase and G_2 phase Centrosome duplication Regulation of Sp1-mediated transcription Apoptosis in thymocytes, mesangial cells DNA damage-induced apoptosis
CDK3	cyclin E	G_1/S transition
CDK4	cyclin D	G_1 phase
CDK5	p35, p25 p39, p29	Dopamine and glutamate signaling Neurite outgrowth, neurone migration Induction of acetylcholoine receptors Apoptosis Golgi membrane traffic Insulin exocytosis by β-cells Phototransduction Neurotransmitter release (VDCC[1])
CDK6	cyclin D	G_1 phase Neuronal cell death
CDK7	cyclin H	CDK1, 2 activation Basal transcription (TFIIH)
CDK8	cyclin C	Basal transcription Regulation of CDK7-cyclin H
CDK9	cyclin K cyclin T	Signal transduction RNA transcription (P-TEF-b) HIV-Tat-dependent transcription MyoD-mediated myocyte differentiation
CDK11	cyclin L	RNA processing or transcription Apoptosis Dopamine and glutamate signaling

[1]VDCC - voltage-dependent Ca2+ channel. Reproduced with permission from (74)

Cyclin D1 expression is critical in the proliferation of numerous cell types including hematopoietic, fibroblast, myocytes, and epithelial cells *(2,3)*. Cellular levels of cyclin D1 can be influenced by a number of mitogenic and oncogenic signals including mutations of Ras, Src, Rac, and ErbB2 (HER-2/neu) (*4–7*). The phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway both induces cyclin D1 expression and stabilizes the abundance of cyclin D1 *(8,9)*.

The successful completion of DNA replication during S phase leads to the G_2/M checkpoint, which is controlled by cyclin B and CDK1 (Fig. 1). The cyclin B-CDK1 heterodimer forms during the S to G_2 phase transition but is rendered inactive in early G_2 by phosphorylation. The CDC25 phosphatase dephosphorylates the cyclin B–CDK1

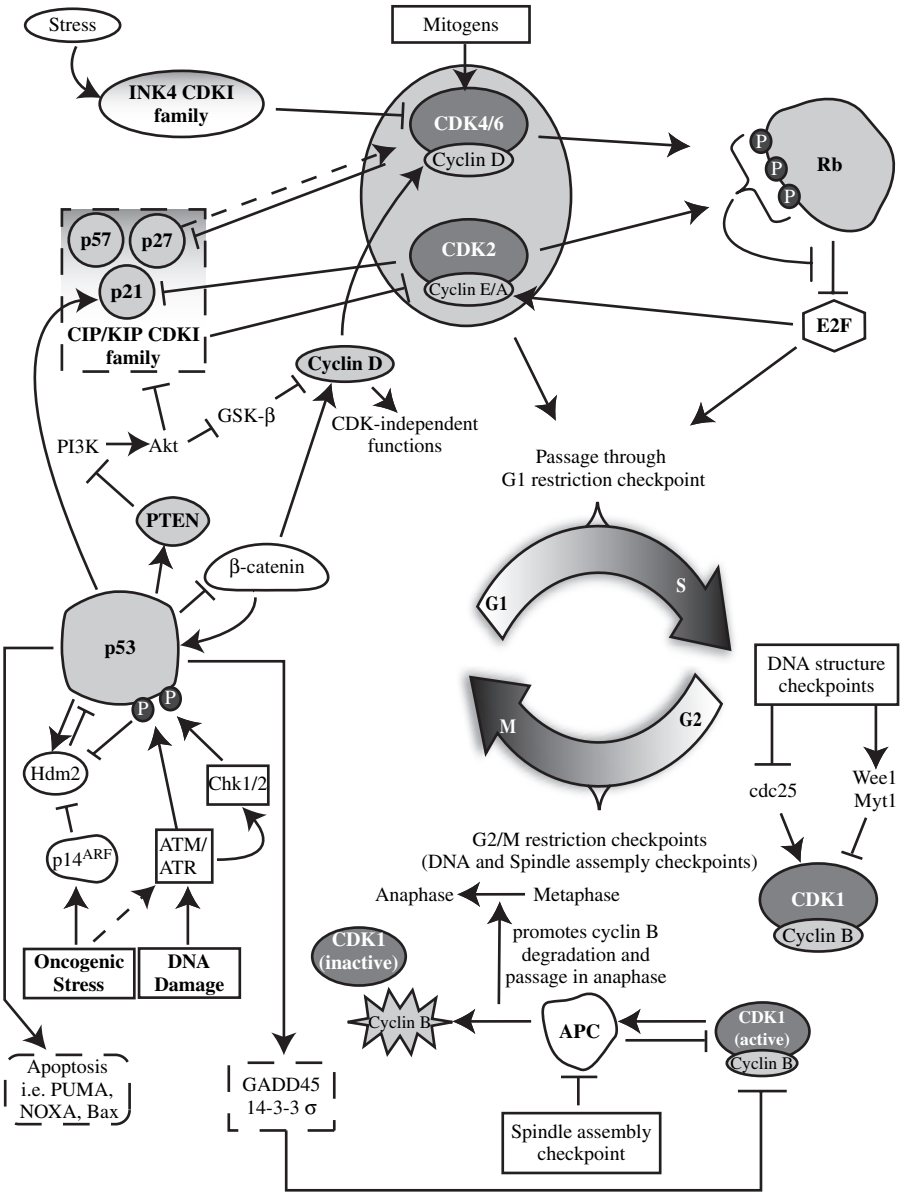

Fig. 1. The checks and balances of the cell cycle. Mitogenic stimuli (e.g., growth factors) and activation of survival pathways (e.g., Akt kinase) enhance the expression of cyclin D family members, which activate cyclin-dependent kinase (CDK) 4/6 holoenzymes, resulting in the phosphorylation and inactivation of retinoblastoma tumor suppressor protein (pRb). The subsequent release of E2F from pRb leads to increased levels of cyclin A/E and CDK2 activity thus perpetuating pRb phosphorylation and progression from G_1 to S phase. The INK4 and CIP/KIP families of CDKIs respond to various stress conditions, including the activation of p53, and work to prevent the activation of cyclin-CDK holoenzymes thereby arresting the cell cycle. Passage through the G_2-DNA structure checkpoint into M phase is accomplished by dephosphorylation of the cyclin B-CDK1 holoenzyme, which results when the activity of the cdc25 phosphatase outpaces that of the Wee/Myt1 kinases. During M phase, the APC is activated by cyclin B-CDK1 and targets cyclin B for degradation allowing passage into anaphase and the subsequent completion of mitosis.

complex resulting in its sustained activation through the completion of metaphase after which cyclin B is ubiquitylated and targeted for degradation by the anaphase promoting complex (APC). In a feedback mechanism, phosphorylation of APC by cyclin B-CDK1 is required for cyclin B proteolysis and transition out of G_2 into interphase (Fig. 1).

In contrast to cyclins, CDKIs were initially described through their ability to negatively regulate CDK function. The CDKIs group into two distinct families—the INK4 family and the CIP/KIP family. The INK4 family ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$) binds directly to and inhibits CDK4 and CDK6. The CIP/KIP family ($p21^{CIP1}$, $p27^{KIP1}$,and $p57^{KIP2}$) share structural homology and can bind and form ternary complexes with cyclin–CDK complexes (cyclin B1-CDK1, cyclin A/E-CDK2). $p21^{CIP1}$ and $p27^{KIP1}$ also promote the assembly and activity of cyclin D–CDK4/6 complexes *(10,11)*. The INK4 and CIP/KIP families of CDKIs thus control pRb phosphorylation indirectly through their effect on CDKs, thereby regulating passage through the G_1 restriction point.

The CIP/KIP family of CDKIs functions in a concentration-dependent manner and are subject to proteasome-mediated degradation through ubiquitin-dependent ($p21^{CIP1}$ and $p27^{KIP1}$) and ubiquitin-independent ($p21^{CIP1}$) pathways (*12–18*). Regulation of $p21^{CIP1}$ and $p27^{KIP1}$ activity and accumulation are primarily controlled by post-translational modifications. In response to mitogenic stimuli, $p27^{KIP1}$ is phosphorylated on threonine 187 by cyclin E-CDK2. This phosphorylation creates a docking site for the substrate recognition factor Skp2, which is part of the larger Skp1-cullin-F-box ubiquitin ligase complex that promotes the ubiquitin-mediated proteolysis of $p27^{KIP1}$. Skp2-independent degradation of $p27^{KIP1}$ involves the Kip1 ubiquitination promoting complex *(19)*. Both $p21^{CIP1}$ and $p27^{KIP1}$ are the target of Akt-induced phosphorylation that results in sequestration of the proteins in the cytoplasm thereby maintaining nuclear CDK2 activity.

3. THE CELL CYCLE IN DEVELOPMENT

The role of cyclins, CDKs, and CDKIs has been analyzed in transgenic mice. Given the importance of each of these components in normal cell cycle progression, the functional redundancy demonstrated in these experiments was surprising. *Cyclin* $D1^{-/-}$ mice are viable with fatty liver, defects in mammary epithelial cell differentiation, retinal apoptosis, and poorly migrating macrophages (*20–29*). *Cyclin* $D2^{-/-}$ mice displayed defective ovarian granulosa cell development and hypoplastic testes and reduced proliferation of B cells in granule neurons *(22,26)*. *Cyclin* $D3^{-/-}$ mice display a hypoplastic thymus *(24)*, and *cyclin D1*, *D2*, and $D3^{-/-}$ mice show a hematopoietic defect reflecting defective proliferative capacity of hematopoietic stem cells, dying at day 16.5 during embryonic development *(2)*. Deletion of either cyclin E1 or cyclin E2 alone has no effect on mouse development or cellular proliferation in vitro *(30,31)*. Deletion of cyclin E1 and cyclin E2 results in placental and cardiac defects, with death at embryonic day 11.5 due to failure of endoreduplication of placental trophoblasts *(30,31)*. Mouse embryonic fibroblasts (MEFs) proliferate more slowly, with a failure to reenter the cell cycle due to failure of loading mini-chromosome maintenance proteins onto prereplication origins *(30,31)*. CDK2-null mice are viable although sterile *(32,33)*. Mice deleted of CDK4 are viable, with mild defects in hematopoiesis and thymic and

splenic hypoplasia *(34)*. *CDK4*$^{-/-}$ mice are viable though sterile (*35–38*). Curiously, the animals demonstrated insulin-dependent diabetes due to abnormal development of β-islet cells. Mice deleted of *cyclin A1 (CCNA1)* have a normal phenotype other than a defect in male meiosis. *Cyclin A2* deletion results in embryonic lethality *(39)*. Collectively, these studies demonstrated an important role for specific cell cycle components in distinct compartments, but were more surprising for their relatively benign effect on cell cycle progression.

Analysis of CDKI function in development also suggested a relatively unimportant role for these proteins individually in normal development. Disruption of individual *INK4* genes in the mouse germ line ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) resulted in viable and fertile mice. Mice developed relatively normally, suggesting no one family member was essential for cell cycle control.

Subsequent analysis of transgenic mice with deleted cell cycle components has provided important insight into cell cycle function in response to oncogenic stimuli. Mice lacking $p16^{INK4a}$ are particularly tumor-prone and develop a wide spectrum of cancers, particularly when exposed to chemical carcinogens or X-rays *(40,41)*. Cyclin D1-deficient mice are resistant to tumorigenesis induced by oncogenic Ras targeted to either the breast or the skin *(42,43)*, but have increased mammary tumors induced by activating β-catenin *(44)*. Mice either completely deficient or heterozygous for *cyclin D1* are resistant to colonic tumorigenesis induced by activation of the *Apc*^{Min} gene mutation *(45)*. Mice haploinsufficient for CDKN1B ($p27^{KIP1}$) develop pituitary tumorigenesis and enhanced tumorigenic response to 7,12-dimethylbenz[a]anthracene *(46)*. In addition, mammary tumorigenesis induced by ErbB2 was accelerated in the CDKN1B heterozygous background *(47)*. MEFs derived from mice deleted of *cyclin* and *CDK* genes demonstrated resistance to oncogenic transformation and reduced ability to enter the cell cycle from quiescence in a subset of experiments. Additionally, *cyclin D1*, *cyclin D2* , and *cyclin D3*$^{-/-}$ cells show reduced induction of DNA synthesis *(2,5)*. *Cyclin D1*, *cyclin D2* , and *cyclin D3*$^{-/-}$ MEFs also show reduced susceptibility to transformation by Ras, Myc, E1A, or dominant negative p53, as do *CDK4*$^{-/-}$ and *cyclin E1 and cyclin E2*$^{-/-}$ MEFs *(31)*. *CDK2*$^{-/-}$ MEFs can be transformed with oncogenic Ras and E1A, but less efficiently than wild-type cells *(32,33)*. Mice deficient in either INK4a or ARF *(48)* are significantly more tumor-prone than wild-type animals, but less tumor-prone than either *p53*$^{-/-}$ or *INK4a/ARF*$^{-/-}$ animals while displaying different tumor spectra suggesting varying roles for the INK4a/ARF proteins. Collectively, these studies are consistent with an important role for the CDKI proteins as tumor suppressors in vivo.

4. THE CELL CYCLE IN CANCER

Tumorigenesis in vivo involves a multi-step process within the primary cell of origin and requires heterotypic signals from the local environment, including angiogenic cues. Inactivation of recessive tumor suppressor genes results from somatic mutations or inherited defects. Tumor suppressor genes include *TP53*, *RB1*, *INK4a*, *ARF*, *APC*, *PTCH*, *PTEN*, *SMAD4*, *DPC4*, *TFC1*, *NF1*, *WT1*, *MSH2*, *MLH1*, *ATM*, *MBS1*, *CHK2*, *BRCA1*, *BRCA2*, *FA* genes, and *VHL (49)*. However, dysregulation of cell cycle components are a common feature. The steps governing initiation and commitment to tumorigenesis may be distinct. Prototypic tumor suppressors are recessive. Their functions are diverse governing a wide range of normal cellular activities, including

cell cycle checkpoint control, mitogenic signaling pathways, protein turnover, DNA damage, hypoxia, and other stress responses (reviewed in ref. *49*). The transition from benign to malignant disease is associated with increases in chromosomal aberrations. Tumor mediators drive bridge fusion breakage cycles that facilitate genomic instability, promoting the molecular genetic aberrations required for full malignant transition *(50)*. The contributions of telomerase activity in the initiation and progression of cancer is complex, and, although a target of cell cycle control, the role of telomerase activity and the therapeutic target role of telomerase as a target in cancer therapy is complex *(51)*.

Deregulation of pRb signaling pathways is a common hallmark found in up to 90% of all human cancers, which leads to unchecked progression into S phase. Deletion of pRb results in tumors of the retina and an increased predisposition to osteosarcomas, while inactivation of CDKIs and/or overexpression of cyclins that predominantly regulate the G_1–S transition are displayed in a broad spectrum of tumors. One paradigm that distills much of the working knowledge of the cell cycle in tumors proposes two parallel pathways of cell cycle surveillance exist. One arm is composed of the CDK/$p16^{INK4A}$/pRb pathway, and the other is composed of the p53/HDM2/$p14^{ARF}$ pathway. Deregulation of any point within a pathway is sufficient to inactivate the pathway. Thus, overexpression of cyclin D1 or deletion of the $p16^{INK4A}$ would inactivate the pRb pathway, resulting in unchecked cell cycle progression. Inactivation of both pathways is required for tumorigenesis by eliminating the checks and balances used by a cell to maintain fidelity of cell cycle control. Inactivation of the p53 arm is commonly accomplished by human double minute 2 (*HDM2*) gene amplification or p53 gene mutations that occur in approximately 50% of all human cancers.

It has been predicted that nearly all human cancers carry at least one alteration in the p53 surveillance pathway. The tumor suppressor p53 protein is an essential regulator of the G_1 checkpoint and can respond to multiple types of cellular stress including DNA damage, oncogenic signaling [possibly via DNA damage *(52)*], and hypoxia *(53)*. Tight regulation of p53 function is required and provided by HDM2, an E3 ubiquitin ligase. HDM2 directs p53 ubiquitylation and subsequent proteasome-mediated degradation, and is a transcriptional target of p53, forming a negative feedback loop. DNA damage dissociates the p53–HDM2 interaction by inducing a kinase cascade that results in the phosphorylation of the HDM2 binding site on the N-terminus of p53 *(54,55)*, while oncogenic stimuli results in the induction of $p14^{ARF}$, which sequesters HDM2 into nucleoli *(56,57)*. The p53/HDM2/$p14^{ARF}$ pathway is disabled by mutation or repression of ARF by other proteins (Twist and TBX2). Although oncogenes such as Myc activate ARF gene expression, $p19^{ARF}$ (murine homolog of $p14^{ARF}$) can also negatively regulate Myc's transcriptional activity through a direct physical interaction independent of Mdm2 and p53 *(58)*.

Phosphorylation regulates the half-life of p53 and facilitates its acetylation and activation of apoptosis. The acetylation of p53, like p53 phosphorylation, is responsible for directing the function of p53 in response to stress *(59,60)*. Acetylation of p53 on lysine 373 and 382 by the histone acetyltransferases (HATs) p300 or CREB-binding protein (CBP) can induce apoptosis, while the acetylation of lysine 320 by p300/CBP-associated factor (P/CAF) has been linked to nonapoptotic stimuli *(61,62)*. Numerous other post-translational modifications have been described to act on p53 including sumoylation and methylation. While highly complex and as yet ill-defined, these various signals are likely to control the diverse functions of the p53 tumor suppressor protein, which include the induction of senescence, the induction of cell

Table 2
Cyclin-Dependent Kinase (CDK)-Independent Functions of Cyclin D1

Signaling targets	*Functional significance*	*Reference*
Transcription factors		
ERα	Cyclin D1 (not D2 or D3) recruits SRC1 to ERα potentiating activation of unliganded ERα	*178,179*
C/EBPβ	Cyclin D1 activates transcriptional activity	*180*
AR	Cyclin D1 represses ligand-bound AR by interfering with P/CAF association and recruiting HDAC1 and HDAC3 to AR	*70,72,181*
TR	Cyclin D1 represses both unliganded TR and liganded TR activity	*182*
PPARγ	Cyclin D1 represses PPARγ induction and transcriptional activity	*29,68*
Myb	Cyclin D1 inhibits transcriptional activity	*183*
	Cyclin D1 inhibits Myb p300-dependent actylation	*184*
DMP1	Cyclin D1 inhibits transcriptional activity	*185*
BETA2/NeuroD	Cyclin D1 represses transcriptional activity	*186*
STAT3	Cyclin D1 represses STAT3 activation	*187*
MyoD	Cyclin D1 represses transcriptional activity	*188*
Sp1	Cyclin D1 represses transcriptional activity	*189*
Brg1	Cyclin D1 and E may regulate SWI/SNF complex through Brg1	*190*
AIB-1	p160 family of co-activators; inhibited	*178*
GRIP-1	p160 family of co-activators; inhibited	*191*
Transcriptional co-factors		
NcoA/SRC1a	Cyclin D1 recruits SRC1 to ERα potentiating activation	*178*
P/CAF	Cyclin D1 represses HAT activity	*70*
p300/CBP	Cyclin D1 represses HAT activity	UO
	Phosphorylated by cyclin E-CDK2 increasing E2F association	*192*
	Cyclin D1 prevents p300 dependent induction of PPARγ	UO
HDAC1	Cyclin D1 recruits HDAC1 to AR and PPARγ	*68* and UO
HDAC3	Cyclin D1 recruits HDAC1 to TR and PPAR	*68,182*
TAF250	Cyclin D1 represses SP-1-mediated transcription	*193*
Additional targets		
BRCA1	Cyclin D1 overcomes BRCA1-mediated inhibition of liganded ERα	*194*
BARD1	Phosphorylated by cyclin E1/A1-CDK2; reduces BRCA1: BRAD ubiquitin ligase activity	*195*
NPAT	Phosphorylated by cyclin E-CDK2; induces histone expression	*196*

UO, Unpublished observation.

cycle arrest, and the induction of apoptosis through both transcriptional-dependent and transcriptional-independent pathways *(63,64)*. p53 primarily induces a G_1 growth arrest through the induction of $p21^{CIP1}$ and a G_2 arrest by promoting the transcription of GADD45 and 14-3-3σ that interfere with cyclin B-CDK1 activation. Furthermore, p53 induction of the PTEN tumor suppressor protein can indirectly regulate cyclin D-CDK4/6 function by inhibiting the PI3K/Akt kinase cascade increasing the activity of the CIP/KIP family of CDKIs and destabilizing cyclin D1.

The cyclin D1 gene is amplified and overexpressed in a broad spectrum of human malignancies ranging from breast carcinomas to soft tissue sarcomas *(65)*. Of interest, cyclin E is also overexpressed in human breast cancer, and a subset of human tumors display a cleaved form of cyclin E that correlates with a poor prognosis *(66)*. CDK-independent functions of cyclins contribute to gene expression, cellular differentiation, and growth *(67)*. Cyclin D1 alters the function of more than 30 transcription factors including v-Myb, MyoD, ERα and STAT3 (Table 2) through physical interaction with co-activators (p300/pCAF) and histone deacetylases (HDACs) *(68)*. Cyclin D1 repression of p300 coactivator function has been linked to the inhibition of p300-autoacetylation by cyclin D1 *(69)*. Alternatively, cyclin D1 can repress the ligand-dependent activity of the androgen receptor (AR) by recruiting HDACs including HDAC3 and by competing with P/CAF for binding to AR (*70–72*). Cyclin D1 represses the transcriptional activity of peroxisome proliferator-activated receptor γ (PPARγ) through recruitment of HDACs, HP1α and the SUV39 methyltransferase to the PPARγ response element to silence transcription in the context of the local chromatin structure *(68)*. In addition to inactivating the tumor suppressor pRb, cyclin D1 blocks the function of BRCA1 and the estrogen receptor *(73)*. Thus, cyclin D1 regulates the cell cycle through CDK activity, regulates chromatin topography at the sites of transcription, and blocks function of tumor suppressors such as BRCA1 and many transcription factors.

5. PHARMACOLOGIC CYCLIN-DEPENDENT KINASE INHIBITORS

Based on the essential functions cyclins and CDKs perform in cell cycle progression and tumorigenesis, pharmacologic inhibitors of the cell cycle machinery have been investigated *(74)*. Inhibitors of CDKs interfere with cyclin binding, compete with ATP for binding to the kinase-ATP binding site, or stimulate natural CDKIs. Of the approximately 50 inhibitors that have been described to date, most are low molecular weight, flat hydrophobic heterocycles that compete for the CDK-ATP binding site. Many of these inhibitors work at nanomolar concentrations and have been co-crystallized with CDK2 or modeled with CDKs *(75)*. While numerous new classes of inhibitors have been characterized (Table 3), flavopiridol and the staurosporine derivative UCN-01 have progressed to clinical trials and demonstrate promise in a wide array of human cancers.

5.1. Flavopiridol

Flavopiridol, which is a semisynthetic flavonoid derived from rohitukine, is a broad spectrum CDKI with activity against CDK1, CDK2, CDK4, and CDK6 [IC_{50} (inhibitory concentration 50%) of ~100nM and against CDK7 [IC_{50} of ~300 nM *(76)*]. The antitumor activities of flavopiridol include inhibition of growth and proliferation, induction of apoptosis, and inhibition of angiogenesis. Flavopiridol competitively and

Table 3
Direct Cyclin-Dependent Kinase (CDK) Modulators

Targeted CDKs	*Compound*	*Targeted CDKs*	*Compound*
CDK1/CDK2/CDK5	Roscovitine and CYC202	Cdk4	Pyrrolo-carbazoles
	Olomucine		Indolocarbazoles
	CVT-313		Tryaminopyrimidine (CINK4) *(202)*
	Butyrolactone I		Fascaplysin
	Purvalanol		PD0183812 *(203)*
	BMS-387032		PD0332991 *(204)*
	Aloisines		Cynnamaldehydes
	Indirubins		Dioxobenzothiazoles
	Hymenialdisine		Pyrazol-3-ylurea (compound 15b)
	Pyrazolo-piridines		Bicyclic 2-anilinopyrimidines (<20 nM) *(203,205)*
	Pyrazolo-quinoxalines		2-Anilinopyrimidines (7 nM) *(206)*
	Indenopyrazoles (9 nM) *(197)*		2,4-bis anilinopyrimidines (10 nM) *(207,208)*
	SU9516		
	Nitrosopirimidines	Nonspecific cdk	Flavopiridol
	Paullones		Staurosporine
	Diaminotriazole (2 nM) *(198)*		UCN-01
	Aminoimadazole (28 nM) *(199)*		Oxyndoles
	Oxindoles (6 nM) *(200,201)*		Quinazolines
		Unknown	Toyocamycin
			Myricetin

References are shown in italics and IC_{50} amounts are shown within parentheses. Reproduced with permission in part from ref. *(95)*.

reversibly inhibits the CDK-ATP-binding site and represses the expression of cyclin D1, cyclin D3, and CDK4 *(77)*. Flavopiridol also inhibits P-TEFb (cyclin T1-CDK9), independently of ATP binding *(78)*, which is critical for the function of RNAP II and transcription elongation. Flavopiridol inhibits proliferation of hematopoietic cells *(79,80)* and human umbilical vein endothelial cells *(81)*, prevents the induction of vascular endothelial growth factor (VEGF) by hypoxia in human monocytes *(82)* and induces apoptosis *(77,79)*.

Initial pharmacokinetic studies of flavopiridol in rodents displayed poor oral bioavailability, so subsequent treatments involved intravenous or intraperitoneal drug administration, where the major toxicities seen involved the bone marrow and gastrointestinal tract *(83)*. Mice treated with boluses of flavopiridol for 5 days or continuously with a 72-h infusion of flavopiridol both demonstrated antitumor activities indicating that repetitive high peak plasma concentrations were desirable for the most effective treatment course *(80,84)*. Synergy is seen with a number of cytotoxic chemotherapeutics and typically requires that the chemotherapeutic treatment precede flavopiridol dosing *(85)*.

Four phase I clinical trials have been completed to date using flavopiridol in monotherapy treatment. The first of these studies was completed by the U.S. National Cancer Institute (NCI) and enrolled 76 patients with refractory malignancies and evidence of prior disease progression *(86)*. Flavopiridol was administered as a 72-h continuous infusion every 2 weeks during which a maximum tolerated dose (MTD) of 50 mg/m^2/day over 3 days was identified with a dose-limiting toxicity (DLT) of secretory diarrhea. In the presence of antidiarrheal prophylaxis, the MTD was escalated to 78 mg/m^2/day and was limited by the occurrence of hypotension and proinflammatory syndrome that included local tumor pain. A second phase I trial that employed a similar treatment regimen corroborated the NCI's findings with an MTD of 40 mg/m^2/day and a DLT of secretory diarrhea *(87)*. Both studies reported patient plasma concentrations between 300 and 500 nM, which can inhibit CDK activity in vitro. Minor responses were observed in patients with non-Hodgkin's lymphoma, colon cancer, renal cell carcinoma (RCC), and prostate cancer, although follow-up phase II trials using a similar regimen demonstrated no significant antitumor effect with only modest activity against metastatic RCC (*88–91*). However, studies of individuals with stage IV non-small-cell lung cancer (NSCLC) and refractory mantle cell lymphoma yielded encouraging results. Patients with refractory mantle cell lymphoma (n = 30) had an overall response rate of 11% with 71% of the patients attaining stable disease with a 3.4 month duration of response *(92)*. In those with NSCLC, the median overall survival for the 20 enrolled patients was approximately 7.5 months *(93)*, which is comparable to the median survival following chemotherapy containing platinum analogs in combination with taxanes or gemcitabine *(94)*. This has prompted the initiation of a phase III clinical trial comparing standard combination chemotherapy versus combination chemotherapy with flavopiridol *(95)*.

In a fourth phase I trial (n = 26), flavopiridol was administered as a 24-h continuous infusion every 2 weeks to patients with previously treated chronic lymphocytic leukemia (CLL) *(96)*. A MTD of 140 mg/m^2 was achieved with thrombocytopenia and diarrhea being the most common toxicities observed. Despite the ability to achieve flavopiridol concentrations capable of inducing apoptosis in cultured CLL cells *(79,97)*, there were no partial or complete responses noted in this phase I trial. From this study, a keen observation has recently been made concerning flavopiridol bioavailability in that flavopiridol has a much higher binding affinity for human plasma proteins compared to fetal calf serum (FCS), which was predominantly used in all of the preclinical studies. Substitution of human serum for FCS in vitro results in a decrease of free drug from 63–100% to 5–8%. Taking this into consideration, the dose schedule of flavopiridol has been optimized and is currently being reevaluated in phase I trials *(96,98)*. Preclinical trials have demonstrated the efficacy of post-flavopiridol treatment in combination with a number of different chemotherapeutic drugs, including the microtubule stabilizing drug paclitaxel *(99)* and irinotecan, which stabilizes DNA–topoisomerase complexes *(100)*.

5.2. UCN-01

UCN-01 (7-hydroxystaurosporine) is a staurosporine analog that induces G_1 cell cycle arrest, and abrogation of the G_2/M checkpoint resulting in apoptosis. Abrogation of the G_2/M checkpoint by UCN-01 in the presence of DNA damage is accomplished by activation of CDK1 and by increasing cdc25 phosphatase activity. The G_1 growth

arrest induced by UCN-01 may involve a loss of CDK2 activity due to increased $p21^{CIP1}/p27^{KIP1}$ interaction with CDK2 *(101)*. Furthermore, UCN-01 can alter the PI3K/Akt survival pathway by inhibiting PDK1, an upstream kinase that is required for sustained Akt activation *(102)*.

Based on preclinical findings, the first phase I trial of UCN-01 was conducted by administering a 72-h continuous infusion every 2 weeks *(103)*. Unexpectedly, UCN-01 displayed a long half-life (30 days), which was approximately 100 times longer than preclinical models suggested. Following this observation, protocols were adjusted to supply UCN-01 once every 4 weeks using a 36-h continuous infusion. Dose-limiting toxicities of nausea/vomiting, hyperglycemia, and pulmonary toxicity were observed and led to the phase II recommendation of 42.5 mg/m^2/day given by a 72-hour continuous infusion. During this trial, a patient with metastatic melanoma had a partial response that lasted approximately 8 months while a patient with refractory anaplastic large-cell lymphoma had a complete regression and was disease-free 4 years after treatment.

5.3. Outlook—Pharmacologic Cyclin-Dependent Kinase Inhibitors

In addition to flavopiridol and UCN-01, CYC202 (R-roscovitine), BMS-387032, and E7070 have demonstrated strong therapeutic potential in preclinical studies (Table 3). CYC202 is a purine analog and BMS-387032 is a 2-aminothiazole that both target CDK2 for inhibition by competing for ATP binding. Both agents have demonstrated antiproliferative effects in a number of tumor cell lines associated with a reduction in pRb phosphorylation, most likely as a result of CDK2 inhibition *(104,105)*. Phase I clinical trials have been initiated for BMS-387032 while phase I trials with CYC202 are under way but have yet to yield an objective response. E7070 is a sulfonamide that has antitumor activity in a range of in vivo and in vitro models and has been shown to inhibit CDK2 activity, upregulate p53, and induce apoptosis. Both phase I and II trials have been conducted using E7070, and while the phase I trials did not demonstrate a therapeutic response, phase II trials have provided more promising results *(98)*. A growing number of selective cyclin or CDKIs have been developed, with selectivity to either CDK2 or CDK4 kinase (Table 3) (reviewed in ref. *106*).

6. THERAPEUTIC TARGETING OF HDACS

The control of histone acetylation through HATs and deacetylases (HDACs) is central to the regulation of gene transcription through the alteration of chromatin topography and promoter accessibility. Non-histone proteins are acetylated, including transcription factors, signal mediators, co-activators, and structural proteins (Table 4). The process of acetylation involves the transfer of an acetyl group to the ε amino group of a lysine residue thereby neutralizing lysine's positive charge within the targeted substrate. Either single or multiple acetylations of protein factors can control a variety of functional activities such as DNA–protein interactions, protein–protein interactions, and subcellular localization, thus altering function *(107)*.

Importantly, like phosphorylation, acetylation of transcription factors has been shown to directly regulate contact-independent growth *(108)*. Proteins involved in controlling the cell cycle are acetylated *(109)* or associate with either HATs or HDACs providing new targets for therapeutic intervention in cancer (*59,70,71,110*). Point

Table 4
Tumor-Associated Proteins Whose Transcriptional Expression is Altered in Response to Histone Deacetylase (HDAC) Inhibitor Treatment of Cells

Regulated protein	*Regulated protein function (oncogeneic or antioncogenic/tumor supressing)*
Downregulated by HDAC inhibitors	
erbB2 (HER2/neu)	Growth factor receptor (EGFR class)
TGF-β	Regulates cell proliferation and differentiation through TGF-b type II receptor
Thioredoxin	Disulfide reductase, cytokine activity, can inhibit apoptosis
Telomerase	Prevents telomere erosion
RECK	Regulates matrix metalloproteinases
VEGF	Angiogenic factor
β-FGF	Angiogenic factor
Myb/c-MyBL2	Oncogenic transcription factor-regulation of transfromation and differentiation
raf-1	Effector of Ras
cyclin A	Cell cycle regulator
cyclin B	Cell cycle regulator
DAF	Complement inhibitory protein
Abl	Growth factor receptor, component of bcr/abl chimeric kinase
DEK	Putative role in regulating chromatin structure and postsplicing events
Proteasome	Degradation of misfolded or oxidized proteins
Upregulated by HDAC inhibitors	
Fas/Fas ligand	Proapoptotic
Bcl2	Proapoptotic
p53	Proapoptotic
Bak, Bax, Bim	Proapoptotic
c-myc	Inhibitor of differentiation
Caspase 3	Cysteine protease involved in apoptosis, proapoptotic
CPA3	Carboxypeptidase, putative role in regulating differentiation
RECK	Negatively regulates matrix metalloproteinases
$p21^{CIP1}$	Cell cycle regulation
Gelsolin	Regulation of cell morphology
ERα	Estrogen-activated nuclear receptor regulates transcription of estrogen responsive genes
TSSC3	Regulates Fas-mediated apoptosis
IGFPB-3	Augments IGF actions, promotes apoptosis, and inhibits cell growth
TBP-2	Inhibits thiol-reducing activity of thioredoxin

Bak, Bcl2 antagonist killer; Bax, Bcl2-associated X protein; CPA3, carboxypeptidase A3; DAF, decay-accelerating factor; TBP-2, thioredoxin binding protein; TSSC3, tumor supressing subtransferable candidate. Reprinted with permission from the Annual Review of Pharmacology and Toxicology (by Annual Reviews, http://www.annualreviews.org) *(115)* and references therein.

mutations have been identified in transcription factors at their site of acetylation, arising as somatic mutations, including the ERα and AR in breast and prostate cancer, respectively (*73,111–113*).

Protein deacetylation is regulated by either trichostatin A (TSA) or nicotinamide-adenine-dinucleotide (NAD)-dependent HDACs (*109,114,115*). HDACs repress transcription through recruitment and association with large multiple protein corepressor complexes. While not required for activity, HDACs commonly associate in larger multi-protein complexes with either mSin3 proteins or Mi-2-NuRD. To date, 18 mammalian HDACs have been identified and are grouped into three class based on their conserved sequence homolog with yeast HDACs. Class I is comprised of HDAC1, 2, 3, 8, and 11 and display homology to the yeast *Rpd3*; Class II is comprised of HDAC4, 5, 6, 7, 9, and 10 and are homologous to the yeast *Hda1*; and Class III is comprised of SIRT1-SIRT7 and share homology with the *Sir2* family of yeast deacetylases. Class I and II HDACs function in a Zn^{2+}-dependent manner, while class III HDACs are dependent on the availability of NAD *(109)*.

HDAC inhibitors inhibit cancer cell growth (through cell cycle arrest at both G_1 and G_2/M checkpoints), induction of differentiation, and/or induction of apoptosis (Table 5). HDAC inhibitors lead to the hyperacetylation of histones of the chromatin around the $p21^{CIP1}$ promoter inducing p21 gene expression and inhibiting CDK activity required for cell cycle progression. HDAC inhibitors repress the expression of growth-promoting genes such as cyclin D1. HDAC inhibitors can be classified into structural groups including hydroxamic acids [e.g., TSA, suberoylanilide hydroxamic acid (SAHA), pyroxamide, and oxamflatin], short chain fatty acids (e.g., valproic acid and sodium butyrate), benzamides (e.g., MS-275), and cyclic tetrapeptides (e.g., trapoxin, apicidin, and depsipeptide). Of these agents, depsipeptide (FR901228, FK228, NSC 630176) has significant preclinical and clinical potential.

6.1. Depsipeptide—Preclinical Studies

Depsipeptide can induce a $p21^{CIP1}$-dependent G_1 arrest associated with repression of cyclin D1 and a $p21^{CIP1}$-independent G_2/M arrest *(116,117)*. In culture, depsipeptide effectively inhibited the proliferation of human tumor cell lines and had less effect on non-transformed cultured cells *(118)*, inhibiting human B-cell CLL (B-CLL) cells and B-cell prolymphocytic leukemia (B-PLL) cells while sparing peripheral blood mononuclear cells *(119,120)*. In addition, the same B-PLL cells failed to respond to treatment with F-araA, gemcitabine, flavopiridol, or UCN-01. The mean lethal concentration to 50% (LC_{50}) in B-CLL and B-PLL cells after 96 h of in vitro exposure to depsipeptide ranged between 0.2 and 15 nM.

Two phase I dose escalation trials of depsipeptide have been completed *(121,122)*. Depsipeptide was administered as a 4-hour infusion either biweekly (day 1 and 5) every 3 weeks, or 3 times a week every 4 weeks. These studies used a starting dose of 1.0 mg/m^2, which was defined as 1/3 the toxic dose low in preclinical rat studies. The MTD achieved was 13.3 mg/m^2 and 17.8 mg/m^2, respectively. The most common DLTs were thrombocytopenia and progressive fatigue. In a phase II study with peripheral or cutaneous T-cell lymphoma, objective responses were observed in 11 patients including one complete response in a peripheral T-cell lymphoma at a dose level of 12.7 mg/m^2.

Patients with CLL and acute myeloid leukemia in a phase I trial of depsipeptide *(123)* using a dose of 13.3 mg/m^2 administered by a 4-hour infusion three times every

Table 5
Non-Histone Acetyl-Transferase Substrates and Their Accompaning Factor Acetyltransferases (FATs)

Substrates for FAT	*FAT*	*Possible effects on transcription*
General transcriptional factors		
TFIIF	P300/CBP, P/CAF	Unknown
TFIIEB	P300/CBP, P/CAF, TAFII 250	Unknown
TAF(I)68	P/CAF	Up
UBF	CBP	Up
CIITA	P/CAF	Up
Transcriptional effectors		
P53	P300/CBP, P/CAF	Up
GATA-1,-3	P300/CBP, P/CAF	Up
EKLF	P300/CBP	Up
TCF	P300/CBP	Down
C-Myb	P300/CBP, GCN5	Up
HIV-1tat	P300/CBP, P/CAF	Up
E2F1,2	P300/CBP, P/CAF	Up
E2F,4	TRRAP, P/CAF	Up
TR-RXR	P300/CBP	Unknown
MyoD	P300/CBP, P/CAF	Up
TAL1/SCL	P300/CBP, P/CAF	Up
AR	P300/CBP, P/CAF, TIP6 0	Down
SF-1	GCN5	Up
ERα	P300/CBP	Down
Sp1	P300/CBP	Up
E1A	P300/CBP	Up
YY1	P300/CBP, P/CAF	Down
RelA	P300/CBP	Nuclear import
STAT6	P300/CBP	Up
IRF-1,2	P300/CBP, P/CAF	Up
NF-E2	P300/CBP	Up
pRb	P300/CBP	Up
Nuclear receptor coactivators		
P300/CBP	P300/CBP	Down
P/CAF	P/CAF	Unknown
ACTR	P300/CBP	Down
SRC-1	P300/CBP	Unknown
TIF2	P300/CBP	Unknown
Rip140	P300/CBP	Up
PC4	P300	Up
Nonhistone chromatin proteins		
HMG1	P300/CBP	Unknown
HMG2	–	Unknown
HMG14	P300/CBP	Down
HMG17	P/CAF	Unknown
HMGI (Y)	P300/CBP,P/CAF	Up (P/CAF), Down (p300)
Sin1	GCN5	Unknown
Fen-1	P300	Reduce DNA binding and nuclease activity
Others		
α-Tubulin	P300	Unknown
Importin-α7	P300/CBP	Unknown
CDP/cut	P300/CBP,P/CAF	Reduce DNA binding

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