Cell-cycle dysregulation is a hallmark of tumor cells. The ability of normal cells to undergo cell-cycle arrest after damage to DNA is crucial for the maintenance of genomic integrity. The biochemical pathways that stop the cell cycle in response to cellular stressors are called checkpoints. Defective checkpoint function results in genetic modifications that contribute to tumorigenesis. The regulation of checkpoint signaling also has important clinical implications because the abrogation of checkpoint function can alter the sensitivity of tumor cells to chemotherapeutics. Here, we provide an overview of the mechanisms that regulate the cell cycle, current anticancer therapies that target checkpoint signaling pathways, and strategies for the development of novel chemotherapeutic agents.

Eukaryotic cells have evolved signaling pathways to coordinate cell-cycle transitions and ensure faithful replication of the genome before cell division. Cell-cycle progression is stimulated by protein kinase complexes, each of which consists of a cyclin and a cyclin-dependent kinase (CDK) [1]. CDKs are expressed constitutively throughout the cell cycle, whereas cyclin levels are restricted by transcriptional regulation of cyclin-encoding genes and by ubiquitin-mediated degradation [2]. CDK activation requires the binding of a cyclin partner in addition to site-specific phosphorylation [1].

The complexes cyclin-D–CDK4, cyclin-D–CDK6, cyclin-E–CDK2 and cyclin-A–CDK2 regulate the progression from G1 phase to S phase [1]. The retinoblastoma protein (pRB) is a crucial substrate of activated cyclin–CDK complexes in the G1 phase. pRB is sequentially phosphorylated by cyclin-D–CDK4,6 and cyclin-E–CDK2 during G1 phase progression, and either represses or activates transcription, depending on its phosphorylation state and associated proteins [3]. Hypophosphorylated pRB binds to and inactivates members of the E2F family of transcription factors [3]. Because E2F family members mediate transcription of genes required for DNA synthesis, binding of hypophosphorylated pRB to E2F represses progression into the S phase. During G1-phase progression, CDK-mediated hyperphosphorylation of pRB results in the dissociation of pRB and E2F and entry into the S phase (Fig. 1).

Progression from G2 to M phase is regulated by the cyclin-B–CDK1 complex. Inactive cyclin-B–CDK1 complexes accumulate during the G2 phase of the cell cycle because phosphorylation of CDK1 by Wee1 and Myt1 inhibits CDK1 activity [4]. Entry into mitosis requires that cyclin-B–CDK1 complexes are activated by CDC25C phosphatase, which removes the inhibitory phosphorylation of CDK1 [4]. Mitotic exit occurs after ubiquitination and proteolytic degradation of cyclin B by the anaphase-promoting complex (APC), which inactivates CDK1 (Fig. 2) [2].

Cell-cycle checkpoints
Eukaryotic cells have developed control mechanisms that restrain cell-cycle transitions in response to stress. These
regulatory pathways are termed cell-cycle checkpoints [5]. Cells can arrest transiently at cell-cycle checkpoints to allow for the repair of cellular damage. Alternatively, if damage is irreparable, checkpoint signaling might activate pathways that lead to programmed cell death. Loss of checkpoint integrity can allow the propagation of DNA lesions and result in permanent, genomic alterations [5].

**G1–S checkpoint**

Inhibition of G1-phase cyclin–CDK complexes plays a key role in the function of the G1–S checkpoint. CDKs are negatively regulated by a group of functionally related proteins called CDK inhibitors (CDKIs) of which there are two families, the INK4 inhibitors and the Cip/Kip inhibitors [1]. The INK4 family has four members: p16INK4A (p16), p15INK4B (p15), p18INK4C (p18) and p19INK4D (p19); and the Cip/Kip family has three members: p21Waf1/Cip1 (p21), p27Kip1 (p27) and p57Kip2 (p57). The INK4 family inhibits CDK4 and CDK6 activity during G1 phase specifically, whereas the Cip/Kip family can inhibit CDK activity during all phases of the cell cycle [1]. Both families of CDKI have important roles in the G1–S checkpoint (Fig. 1). After normal cells are exposed to genotoxic agents, transcription of p21 is activated by the p53 tumor suppressor protein. The p21 protein binds to and inactivates cyclin-E–CDK2 complexes, which results in pRB hypophosphorylation and cell-cycle arrest at the G1–S transition [6]. p16 mediates the p53-independent G1 arrest in response to DNA damage in several cell types through abrogation of cyclin-D–CDK4 and cyclin-D–CDK6-mediated phosphorylation of pRB [7].

**G2 checkpoint**

Genotoxic stress also triggers checkpoint pathways that initiate cell-cycle arrest in the G2 phase. After DNA damage, ATM (ataxia telangiectasia mutated)- and ATR (ATM and Rad3-related)-dependent signaling induces G2 cell-cycle arrest by inhibiting CDK1 (Fig. 2). ATM activates human checkpoint kinase 2 (Chk2) in cells that are exposed to ionizing radiation, whereas ATR signaling mediates activation of Chk1 in cells treated with ultraviolet radiation [8]. Chk1 and Chk2 phosphorylate CDC25C, which generates a consensus binding site for 14–3–3 proteins. Binding of 14–3–3 proteins to CDC25C results in nuclear export and cytoplasmic sequestration of the phosphatase, with subsequent G2 arrest caused by inhibition of CDK1 [8]. Recent studies indicate that p53 is required to sustain the G2-phase arrest induced by DNA damage in tumor cells [9,10]. p53 maintains the G2 checkpoint by upregulating transcription of 14–3–3σ and p21, which inhibit G2 progression by sequestrating CDK1 in the cytoplasm and by inactivating
cyclin-B–CDK1 complexes, respectively [10–13]. Furthermore, p21 can disrupt the interaction between proliferating cell nuclear antigen (PCNA) and CDC25C to induce G2 cell-cycle arrest [14].

**Mitotic spindle checkpoint**

The mitotic spindle checkpoint monitors the microtubule structure and chromosome attachments of the mitotic spindle and delays chromosome segregation during anaphase until defects in the mitotic spindle apparatus are corrected (Fig. 3). The kinetochore-associated MAD2, BUBR1, BUB1 and BUB3 proteins are crucial constituents of the spindle-checkpoint pathway. MAD2 and BUBR1 regulate mitotic progression by direct interaction and inhibition of the APC machinery, which prevents anaphase entry in the presence of mitotic spindle dysfunction [15]. BUB1 and BUB3 also mediate mitotic arrest after disruption of microtubules, because cells that lack either BUB1 or BUB3 do not undergo mitotic arrest when treated with spindle-disrupting agents [15].

**Dysregulation of the cell cycle in human cancers**

Loss of cell-cycle checkpoints is a hallmark of human cancers. Alterations in components of the cell-cycle machinery and checkpoint signaling pathways occur in most human tumors. Ultimately, these genetic modifications result in the dysregulation of oncogenes and tumor suppressor genes, which has important implications for the optimization of current therapeutic regimens and the selection of novel cell-cycle targets.

**Alterations in the constitutive cell-cycle machinery**

Cell-cycle regulation by the tumor suppressor protein pRB plays an integral role in preventing human tumors because oncogenic alterations in cyclins, CDKs and other upstream regulators of pRB occur in a variety of human tumors, including breast and lung cancers, retinoblastoma and osteosarcoma [3]. In tumors with a normal pRB-encoding gene, modifications in components of the signaling pathways that regulate pRB are frequently noted, such as increased levels of cyclin D and cyclin E, amplification of the genes that encode CDK4 and CDK6 and deletion of p16 [3]. For example, ~50% of invasive breast cancers have elevated cyclin D expression, compared with surrounding normal breast epithelium [16], and transgenic mice that overexpress human cyclin D1 or cyclin E in mammary cells develop mammary adenocarcinomas [17,18]. Similarly, amplification of the genes that encode CDK4 and CDK6 occurs in sarcomas, gliomas, melanomas and breast cancers [19].

**Alterations in checkpoint signaling proteins**

Genetic alterations of genes encoding cell-cycle checkpoint signaling molecules are common in human tumors. Mutation of p53 is the most frequently observed genetic lesion in human tumors [6]. The importance of p53-dependent signaling in tumor suppression is underscored by the
finding that germ-line mutations of p53 result in Li-Fraumeni syndrome, a highly penetrant familial syndrome that is associated with significantly increased incidence of brain tumors, breast cancers and sarcomas [20]. In human tumors in which p53 is normal, p53 function might be disrupted by alterations in cellular proteins that modulate the expression, localization and activity of the protein. For example, during normal cellular growth, Mdm2 binds p53 and targets it for ubiquitin-mediated degradation [21]. In some tumors with wild-type p53, the gene that encodes Mdm2 is amplified, which results in overexpression of Mdm2 and subsequent inactivation of p53 [22].

Modifications in CDKI function are also common in human tumors. p27 is often expressed aberrantly in human breast cancers and low concentrations of p27 are associated with more aggressive breast tumors [23,24]. p27 haplo-insufficiency also renders murine mammary epithelium more susceptible to oncogene-dependent transformation [25]. Likewise, decreased expression of p57 is reported in human bladder cancers [26] and either deletion of p15 and p16 or inactivation through methylation is linked to the pathogenesis of human melanomas, lymphomas, mesotheliomas and pancreatic cancers [19].

Mutations in other components of pathways associated with responses to DNA damage could lead to enhanced tumorigenesis. For example, ATM mutations occur in ataxia telangiectasia, a familial disease that is associated with an elevated incidence of leukemias, lymphomas and breast cancer [27]. Mutations of Chk2 and Chk1 also arise in human cancers. Chk2 mutations are reported in human lung cancer [28], whereas Chk1 mutations are observed in human colon and endometrial cancers [29]. In addition, heterozygous mutations in CHK2 occur in a subset of individuals with Li-Fraumeni syndrome that lack p53 mutations [30].

Disruption of spindle checkpoints is also linked to the pathogenesis of several human tumors. In human colon carcinoma cells, BUB1 mutations have been identified [31] that facilitate the transformation of cells that lack the breast cancer susceptibility gene, BRCA2 [32]. Recent studies by Michel et al. demonstrate that MAD2 haplo-insufficiency significantly elevates the rate of lung tumor development in MAD2+/− mice compared with age-matched wild-type mice [33].

Therapeutic manipulation of cell-cycle checkpoints

Preclinical studies indicate that cells with defective checkpoint function are more vulnerable to some anticancer agents. Thus, research efforts are focused on identifying compounds that disrupt cell-cycle checkpoints. These investigations include: (1) the development of chemical inhibitors through structure-based, rational, drug design; (2) the use of high-throughput screening assays; and (3) the manipulation of genetic-based screening technologies to identify novel anticancer therapies.

Chemical approaches

Because the activity of CDKs is often deregulated in tumors, compounds that inhibit CDK function might be effective anticancer agents. Flavopiridol, a chemical CDKI, arrests cancer cells at the G1–S and G2–M transitions by inhibiting CDK2, CDK4 and CDK1 [34]. Flavopiridol has potent antiproliferative activity in several human cancer cell lines, and xenograft tumor studies in mice indicate that it acts synergistically with other anticancer agents [34]. Flavopiridol produced favorable clinical responses in Phase 1 and Phase 2 studies of patients with renal, colorectal, gastric, lung and esophageal carcinomas, and current clinical trials are evaluating the drug in non-Hodgkin’s lymphoma, breast and prostate cancers [19]. The clinical tests of flavopiridol and related, chemical CDKIs has spawned further research efforts to design mechanism-based CDKIs through manipulation of the phosphorylation and cyclin-binding sites of CDK proteins. However, the specificity of chemical CDKIs remains a limiting factor because patients in Phase 1 and Phase 2 studies experienced severe side-effects. Several studies suggest that flavopiridol might act through cell-cycle-independent mechanisms because it binds to and inactivates cytosolic aldehyde dehydrogenase and glycogen phosphorylase [35,36] and inhibits transcription globally [37]. Recent studies by Knockaert et al. addressed the issue of the specificity of chemical CDKIs using an affinity chromatography approach in which cellular extracts are passed over chemical CDKIs immobilized on a matrix. Microsequencing was then used to identify the cellular proteins that bind the chemical CDKIs [38,39]. Studies such as these will help determine the specificity of an inhibitor and identify other targets that might be either beneficial or antagonistic to therapeutic strategies with newly developed compounds.

Numerous studies indicate that the chemotherapeutic efficacy of agents such as ionizing radiation and cisplatin can be enhanced by the abrogation of the DNA-damage-induced G2 arrest in cancer cells. Tumor cells treated with either caffeine or pentoxifylline, compounds that disrupt the G2 checkpoint, are sensitized to ionizing radiation [40,41]. Caffeine inhibits ATM and ATR kinases, which prevents activation of Chk2 and Chk1 [8]. Caffeine also inhibits homologous recombination, which provides an additional mechanism by which it abrogates G2 block [42]. The effectiveness of targeting Chk1 to ablate the G2 checkpoint is exemplified by the use of the Chk1 inhibitors UCN01 (7-hydroxystaurospongine) and SB2180708 (a staurospongine-related compound; see Chemical names) to increase the cytotoxic effect of DNA-damaging agents in tumor cells [43,44]. Promising preclinical results that show that pentoxifylline and UCN01 inhibit tumor growth in vivo have prompted clinical trials to evaluate the efficacy of these compounds against a variety of tumors [45–47].

As with chemical CDKIs, UCN01 might disrupt tumor growth by multiple mechanisms. For example, several recent studies link mitogenic signaling to abrogation of the G1 cell-cycle checkpoint in breast cancers [48–50]. These studies demonstrate that protein kinase B (PKB) phosphorylates p27 and causes it to accumulate in the cytoplasm. PKB is a downstream target of the phosphatidylinositol 3-kinase (PI 3-kinase) signaling pathway and phosphorylation and activation of PKB after PI 3-kinase signaling is mediated by the 3-phosphoinositide-dependent protein kinase 1 (PDK1) [51]. A study by Sato et al. shows that inhibition of PDK1 by UCN01 induces
apoptosis by preventing the phosphorylation and activation of PKB [52]. Thus, in addition to modulating the activity of Chk1, UCN01 might inhibit the survival signals generated by kinases such as PDK1.

Although many anticancer drugs target kinases that are involved in checkpoint signaling pathways, others regulate checkpoint function by inhibiting either histone deacetylases or proteasome-dependent ubiquitination. Histone deacetylase inhibitors alter chromatin structure and gene expression, which produces G2 checkpoint arrest in normal human cells and mitotic catastrophe in tumor cells [53]. In transformed cells inhibitors of histone deacetylase can induce either differentiation or apoptosis, but the precise mechanisms by which histone deacetylase inhibitors induce growth arrest, differentiation and apoptosis is a focus of current research [54,55]. The histone deacetylase inhibitors FR901228 and MS27275 have potent anticancer activity in vitro [56] and in vivo [57], and FR901228 has demonstrated efficacy against T-cell lymphoma in clinical trials [58]. The ubiquitin–proteasome pathway is also an attractive target for manipulation by anticancer drugs because it mediates the degradation of cyclins and CDKIs [2]. PS341 is a proteasome-specific inhibitor with significant in vitro cytotoxicity against a variety of human tumor cell lines that has shown favorable patient responses in Phase 2 clinical trials for melanoma, lung cancer and sarcomas [59].

**Screens for new compounds**

Several screening strategies have been employed to identify novel anticancer agents. In one such effort, the National Cancer Institute (NCI) evaluated the anticancer activity of 70 000 compounds against a panel of 60 cell lines from human tumors [60]. Subsequent studies found that the p53 status of these tumor cell lines was a crucial determinant of cellular sensitivity to chemotherapeutic agents because the growth of cell lines with mutant p53 was inhibited less than those with wild-type [61]. Recently, Amundson et al. used the NCI cell line to evaluate the basal expression of transcripts of genes associated with checkpoint pathways and correlated these with the sensitivity of the cells to standard chemotherapy agents [62]. Similarly, cDNA microarray analyses have been used to study the gene-expression profiles of these cell lines in response to standard chemotherapeutic drugs [63]. Such studies provide valuable insight into the correlation between the expression of specific genes and drug sensitivity.

High-throughput screens have also been used to identify novel compounds that disrupt checkpoint function. In one study, breast cancer cells that express mutant p53 were grown in microtiter plates, irradiated to induce G2 arrest, and then cotreated with the microtubule inhibitor nocodazole and extracts from marine invertebrates [64]. This assay identified isogranulatimide, a novel G2 inhibitor that has synergistic cytotoxicity in combination therapy with ionizing radiation [64]. A similar microtiter-plate assay was used recently to screen 24 000 extracts from marine invertebrates and terrestrial plants for novel antimitotic agents. This identified eight novel chemicals with antimitotic activity [65]. In another study, isogenic human colorectal cancer cell lines that differ only at the K-Ras locus were stably transfected with an expression vector for either a yellow or a blue fluorescent protein [66]. These isogenic cell lines were used to screen 30 000 compounds to identify compounds that are selectively toxic to the mutant Ras genotype. The latter approach has identified a novel cytidine nucleoside analog that is selective to tumor xenografts that contain mutant Ras [66]. Recently, Bykov et al. screened a library of low-molecular-weight compounds to identify agents that restore wild-type function to mutant p53. The investigators isolated the compound p53 reactivation and induction of massive apoptosis (PRIMA-1), a 2,2-bis(hydroxy-methyl)-1-azabicyclo[2,2,2]octan-3-one that restores sequence-specific DNA binding and an ‘active’ conformation to p53 derived from tumors. In mice, PRIMA-1 has antitumor effects with little toxicity [67]. The identification of small molecules that either inhibit or restore biochemical activity of key, cell-cycle-regulatory proteins opens exciting possibilities for the future of cancer therapy.

**Genetic approaches**

*Saccharomyces cerevisiae* provides an attractive model system to evaluate chemotherapeutic agents because of the conservation of cell-checkpoint processes between yeast and mammals, the availability of yeast genomic sequences [68] and the ease of genetic manipulation in yeast. In a recent study, anticancer drugs were screened using a panel of isogenic strains of *S. cerevisiae* that contain defined mutations in cell-cycle-checkpoint pathways [69]. The toxicity profiles of several chemotherapeutic agents and ionizing radiation differed between strains, which indicates that the particular mutation in DNA repair and cell-cycle-checkpoint systems in individual tumors might influence the outcome of a particular chemotherapeutic regimen. Similarly, *Schizosaccharomyces pombe* has been employed to select peptide inhibitors and identify novel cellular targets for anticancer drugs [70]. In this study, peptides were selected using phenotypic analyses, followed by genetic dissection of candidate target pathways to identify putative targets of the selected inhibitors [70]. The identification of Ydr617w, a previously unknown component of the spindle checkpoint, exemplifies the power of genetic approaches for identifying novel components of known biological pathways that are potential targets for drug discovery. Yeast genomics can also be combined with analyses of cDNA microarrays to examine genome-wide changes in gene expression after treatment with anticancer regimens [71]. In a recent study, this approach generated a database of expression profiles from cell-cycle mutants of *S. cerevisiae* treated with a panel of chemotherapeutic compounds and ionizing radiation [72], and analysis of these profiles revealed novel components of cell-cycle-signaling pathways.

**Concluding remarks**

Identifying the molecular differences between cancer cells and normal cells is crucial for the continued development of anticancer agents that preferentially target cancer cells and minimize the toxicity to normal tissues. Information generated from genomic and proteomic studies of prokaryotes and eukaryotes will continue to reveal
new cell-cycle-regulatory molecules. As our knowledge of cell-cycle checkpoints increases, novel signaling molecules will be identified that can be targeted for rational drug design. This will allow mechanism-based approaches to cancer treatment that exploit the molecular defects in tumors. To fully realize this potential, we need to continue to develop technologies that define precisely the cell-cycle-checkpoint defects in individual tumors, and treatment regimens that are tailored to the cell-cycle phenotypes of tumors.

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Chemical names
FR901228 (FK228 or depsipeptide): (E)-(1S,4S,10S,21R)-7-[(2-ethylidene)-4,21-diisopropyl-2-oxa-12,13-dithia-5,8, 20, 23-tetraazabic cyclo-[8,7,6]-tricos-16-ene-3,6,9,19,22-pentanone
MS27257: N-(2-amino phenyl)-4-[N-(pyridin-3-yl-methoxy-carbonyl)amino]methyl]benzamide
PS341: N-pyrazincarbonyl-L-phenylalanine-L-leucine boronic acid
SB2180708: 9,10,11,12-tetrahydro-9,12-epoxy-1H-diindolol[1,2,3-fg:3’,2’,1’-kl]pyrrolo[3,4-i][1,6]benzodiazocine-1,3(2H)-dione
UCN01: 7-hydroxyasturosporine

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