Cell Cycle News & Views

Cyclin-dependent kinase 4/6 inhibition in cancer therapy

Comment on: Dean JL, et al. Cell Cycle 2012; 11:2756–61; PMID:22767154
and McClendon AK, et al. Cell Cycle 2012; 11:2747–55; PMID:22751436

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Cyclin-dependent kinases (CDKs) drive cell cycle progression and control transcriptional processes. The dysregulation of multiple CDK family members occurs commonly in human cancer; in particular, the cyclin D-CDK4/6-retinoblastoma protein (RB)-INK4 axis is universally disrupted, facilitating cancer cell proliferation and prompting long-standing interest in targeting CDK4/6 as an anticancer strategy. Most agents that have been tested inhibit multiple cell cycle and transcriptional CDKs and have carried toxicity. However, several selective and potent inhibitors of CDK4/6 have recently entered clinical trial. PD0332991, the first to be developed, resulted from the introduction of a 2-aminopyridyl substituent at the C2-position of a pyrido(2,3-*d*) pyrimidin-7-one backbone, affording exquisite selectivity toward CDK4/6.[1] PD0332991 arrests cells in G_1 phase by blocking RB phosphorylation at CDK4/6-specfic sites and does not inhibit the growth of RB-deficient cells.[2] Phase I studies conducted in patients with advanced RB-expressing cancers demonstrated mild side effects and dose-limiting toxicities of neutropenia and thrombocytopenia, with prolonged stable disease in 25% of patients.[3,4] In *cyclin D1*-translocated mantle cell lymphoma, PD0332991 extinguished CDK4/6 activity in patients' tumors, resulting in markedly reduced proliferation, and translating to more than 1 year of stability or response in 5 of 17 cases.[5]

Two recent papers from the Knudsen laboratory make several important observations that will help guide the continued clinical development of CDK4/6 inhibitors. In the study by Dean et al., surgically resected patient breast tumors were grown on a tissue culture matrix in the presence or absence of PD0332991. Crucially, these cultures retained associated stromal components known to play important roles in cancer pathogenesis and therapeutic sensitivities, as well as key histological and molecular features of the primary tumor, including expression of ER, HER2 and Ki-67. Similar to results in breast cancer cell lines,[6] the authors demonstrate that only

RB-positive tumors have growth inhibition in response to PD0332991, irrespective of ER or HER2 status, while tumors lacking RB were completely resistant. This result underscores RB as the predominant target of CDK4/6 in breast cancer cells and the primary marker of drug response in primary patient-derived tumors. As expected, RB-negative tumors routinely demonstrated robust expression of p16[INK4A]; however, p16[INK4A] expression was not always a surrogate marker for RB loss, supporting the importance of direct screening of tumors for RB expression to select patients appropriate for CDK4/6 inhibitor clinical trials.

In the second study, McClendon et al. investigated the efficacy of PD0332991 in combination with doxorubicin in triple-negative breast cancer cell lines. Again, RB functionality was paramount in determining response to either PD0332991 monotherapy or combination treatment. In RB-deficient cancer cells, CDK4/6 inhibition had no effect in either instance. However, in RB-expressing cancer cells, CDK4/6 inhibition and doxorubicin provided a cooperative cytostatic effect, although doxorubicin-induced cytotoxicity was substantially reduced, assessed by markers for mitotic catastrophe and apoptosis. Additionally, despite cytostatic cooperativity, CDK4/6 inhibition maintained the viability of RB-proficient cells in the presence of doxorubicin, which repopulated the culture after removal of drug. These results reflect previous data demonstrating that ectopic expression of p16[INK4A] can protect cells from the lethal effects of DNA damaging and anti-mitotic chemotherapies.[7] Similar results have been reported in MMTV-c-neu mice bearing RB-proficient HER2-driven tumors, where PD0332991 compromised carboplatin-induced regressions,[8] suggesting that DNA-damaging treatments should not be combined concomitantly with CDK4/6 inhibition in RB-proficient tumors.

To combine CDK4/6 inhibition with cytotoxics, sequential treatment may be considered, in which CDK4/6 inhibition is followed by

DNA damaging chemotherapy; cells relieved of G_1 arrest may synchronously enter S phase, where they may be most susceptible to agents disrupting DNA synthesis. Release of myeloma cells from a prolonged PD0332991-mediated G_1 block leads to S phase synchronization; interestingly, all scheduled gene expression is not completely restored (including factors critical to myeloma survival such as IRF4), further favoring apoptotic responses to cytotoxic agents.[9] Furthermore, in RB-deficient tumors, CDK4/6 inhibitors may be used to maximize the therapeutic window between transformed and non-transformed cells treated with chemotherapy. In contrast to RB-deficient cancer cells, RB-proficient non-transformed cells arrested in G_1 in response to PD0332991 are afforded protection from DNA damaging agents, thereby reducing associated toxicities, including bone marrow suppression.[8]

In summary, the current work provides evidence for RB expression as a determinant of response to CDK4/6 inhibition in primary tumors and highlights the complexity of combining agents targeting the cell cycle machinery with DNA damaging treatments.

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ORCA is regulated by ubiquitin-mediated degradation to control Orc2 function

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Eukaryotic cells ensure that DNA is replicated only once per cell cycle. The initiation of DNA replication is dependent on the formation of the pre-replicative complex (pre-RC) on replication origins. The six components of origin recognition complex (Orc1–6p) are bound to the origins, followed by Cdc6p, Cdt1p and the Mcm2–7p complex, to constitute pre-RC. The pre-RC is then activated by the Dbf kinase-Cdc7p complex to initiate DNA replication, where the Mcm complex acts as a helicase.[1] After initiation, the pre-RC complex is disassembled and/or inactivated in order to inhibit re-initiation until the next cell cycle. The inhibition of the pre-RC assembly can be achieved by protein degradation through ubiquitination, translocation and/or phosphorylation of the pre-RC components. Regulation of the Orc complex across species is diverse. In yeast, the Orc complex is probably associated on the origin throughout the cell cycle, and the Orc proteins are phosphorylated by cyclin/CDK complex to inhibit the function.[2] In humans, Orc1 is ubiquitinated by SCF[skp2] complex and degraded by the proteasome.[3] Orc2 is associated with heterochromatin, centromere and centrosome depending on the cell cycle stage.[3,4]

Previously Shen et al. showed that Orc-associated protein (ORCA) utilizes WD40 repeats to associate with DNA replication factors such as Orc, Cdt1 and Geminin in human cells. The WD40 repeats in ORCA are also required for chromatin binding, suggesting that ORCA may anchor other DNA replication factors to chromatin. ORCA stabilizes Orc to chromatin, indicating that ORCA positively regulates the initiation of DNA replication.[5]
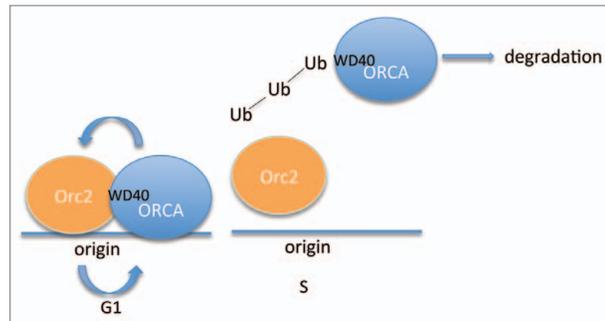

Figure 1. A model: cell cycle-dependent control of ORCA determines Orc2 binding to origin.

The siRNA treatment against Orc2 showed decreased amount of ORCA, suggesting that the stability of ORCA is dependent on Orc2.[6] Therefore, the Orc2-ORCA binding facilitates the stability of this complex. It has been shown that ORCA protein levels are peaked at G_1 phase. In a previous issue of *Cell Cycle*, Shen et al. investigated the molecular mechanism by which ORCA is regulated in a cell cycle-dependent manner.[7] The authors showed that ORCA is polyubiquitinated at the WD40 repeat region, where it is recognized by Orc2. They observed an elevated ORCA ubiquitination level at G_1/S boundary that coincides with ORCA protein degradation. Furthermore, Orc2 is associated only with non-ubiquinated ORCA, indicating that Orc2 prevents ORCA from ubiquitin-mediated degradation. The authors proposed a model that Orc2 releases from chromatin after G_1, which may trigger ORCA ubiquitination and degradation (**Fig. 1**). Therefore, ORCA degradation prevents Orc2 loading on the chromatin after G_1 to allow only one S-phase per cell cycle.

This cell cycle-dependent ORCA regulation may allow Orc2 to obtain diverse functions as well as timely initiation of DNA replication on the specific origins. The novel mechanism to control Orc components through ORCA may be a key mechanism to control DNA re-replication.

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New functions for *ecotropic viral integration site 1 (EVI1)*, an oncogene causing aggressive malignant disease

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The oncogene *Evi1*, which codes for a sequence-specific transcription factor, was cloned as a target of activating retroviral insertions in murine myeloid tumors 24 y ago.[1] Soon thereafter, its human homolog was found to be transcriptionally activated through rearrangements of chromosome band 3q26,[2,3] which are associated with a poor prognosis in acute myeloid leukemia (AML).[4] Since then, *EVI1* has emerged repeatedly as retroviral integration site in different organisms, and a role of its overexpression as an indicator of poor prognosis in a variety of hematopoietic malignancies, as well as some solid tumors, has been firmly established. Because of its well-documented clinical importance, understanding the mechanism(s) of action of *EVI1* is of high significance. However, *EVI1* reveals its secrets only slowly, with merely 475 articles containing the search terms "EVI1," "EVI-1" or "MECOM" (the officially assigned, but still rarely used, gene name) deposited in PubMed at the time of writing.

One of the most recent occasions on which *EVI1* gained negative prominence was in the context of a human gene therapy trial for X-linked chronic granulomatous disease (X-CGD).[5] Transplantation of gene-modified, autologous hematopoietic stem cells initially provided substantial clinical benefit to two young adults with X-CGD. However, over time, hematopoiesis became dominated by clones with vector integrations into, and transcriptional activation of, the *EVI1* locus. These clones finally evolved into myeloid malignancies, acquiring monosomy 7, a chromosome aberration frequently associated with *EVI1* overexpression in AML, during this process. As a preliminary mechanistic explanation for these findings, experimental expression of *EVI1* in human diploid fibroblasts increased the numbers of cells with aberrant centrosome numbers.[5]

In the September 15th issue of *Cell Cycle*, Karakaya and colleagues confirmed and extended these observations.[6] Using U2OS osteosarcoma cells inducibly expressing *EVI1* as a model, they show that only 72 h after induction of *EVI1*, 17% of the cells contained centrosome amplifications vs. 5% of the control cells. Analysis of nuclear morphology and time-lapse video microscopy suggested that supernumerary centrosomes resulted from a cytokinesis defect that is known to activate a p53-dependent tetraploidy checkpoint. Indeed, induction of *EVI1* upregulated p53 and siRNA-mediated p53 depletion increased the percentage of polyploid cells after *EVI1* induction. Furthermore, the vast majority of *EVI1*-overexpressing cells with centrosome amplification had low or undetectable levels of the proliferation marker Ki67, indicating that *EVI1*-induced centrosome aberrations were largely confined to cells in G_0 or early G_1, and confirming that *EVI1*-induced tetraploidization caused a cell cycle arrest.[6]

These data, together with those from the X-CGD study,[5] establish genomic instability as a not previously reported consequence of *EVI1* overexpression, which also might explain its frequent association with monosomy 7. It should be pointed out, though, that chromosome aberrations can also emerge through selection on the background of normal missegregation rates rather than through increased genetic instability.[7] In fact, in the Mitelman database,[8] in ~39% of AML cases with a 3q26 aberration this was the sole cytogenetic anomaly, and for ~82% of 3q26 rearranged cases, only a single clone was reported [compared with ~43% and ~83%, respectively, for the prognostically favorable t(8;21)(q22;q22)]. These data do not argue for greatly increased rates of chromosome instability as a consequence of *EVI1* overexpression. However, excessive instability would likely pose the danger of lethal genetic aberrations and thus not be beneficial to a tumor cell, while low-level instability may become apparent from cytogenetic data only through more detailed analyses. In addition to eventual subtle effects on karyotype at the time of diagnosis, modest *EVI1*-induced chromosome instability raises the intriguing possibility that *EVI1* overexpression may cause poor prognosis at least in part by facilitating acquisition of aberrations that allow tumor cells to escape chemotherapy-induced apoptosis. This hypothesis can be tested through quantitative evaluation of chromosome aberrations and clones at diagnosis and relapse (which is caused by cells that survived the initial therapy) in patients with 3q26 rearranged/*EVI1* overexpressing AML and suitable controls. The work of Karakaya et al., in addition to the novel information it provides as it stands, therefore opens new perspectives that may lead to substantial advances in our understanding of how overexpression of *EVI1* contributes to poor prognosis in AML and other malignancies.

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Addicted to PAR? A closer look at PARP inhibitor sensitivity

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Defects in genome maintenance and repair pathways are common features of human cancers. In some cases, specific DNA repair defects can render cancer cells dependent on back-up pathways for their survival, and targeting these back-up mechanisms is a promising strategy for cancer therapy.[1] One example for such synthetic lethality is the pronounced sensitivity of *BRCA*-deficient cancer cells, defective in DNA repair by homologous recombination (HR), to inhibition of poly(ADP-ribose) polymerases (PARPs).[2] Consequently, PARP inhibitors have entered clinical trials as single agents, but also in combination therapy as chemo- or radiation-sensitizing drugs.[3] Following exciting proof-of-concept results, however, PARP inhibitors recently encountered first difficulties, and the current challenge is to understand why certain cancers respond better to these compounds than others.

In a study now published in *Cell Cycle*, Oplustilova and colleagues used a panel of human cancer cell lines derived from carcinomas of breast, prostate, colon, pancreas and ovary to study the response to PARP inhibition and analyze cellular determinants of sensitivity or resistance.[4]

Acquired resistance by drug efflux can be a major barrier to therapeutic efficacy, and Oplustilova et al. demonstrate that P-glycoprotein drug efflux pumps contribute to inhibitor resistance in colon cancer cells, thereby extending previous findings obtained in a mouse model for BRCA1-associated breast cancer.[5] Importantly, inhibition of P-glycoprotein by verapamil resulted in elevated intracellular levels of the PARP inhibitor KU 58948 and restored inhibitor sensitivity, consolidating the notion that acquired resistance by enhanced drug efflux can, in principle, be overcome.

The authors also investigated a second mechanism of alleviated PARP inhibitor sensitivity based on the recent discovery that loss of the genome caretaker 53BP1 restores HR in BRCA1-deleted cells.[6,7] Consistent with the proposed function of 53BP1 to restrict DNA end resection, exacerbate HR deficiency and enhance PARP inhibitor sensitivity, Oplustilova et al. show that shRNA-mediated depletion of 53BP1 in a human BRCA1-defective breast cancer cell line reduced sensitivity to PARP inhibition. Given the aberrant reduction of 53BP1 in subsets of BRCA-associated breast carcinomas and sporadic triple-negative breast cancers,[7] immunohistochemical analyses of 53BP1 expression, as also performed in the present study, might thus have predictive value when assessing PARP inhibitor sensitivity.

Intrigued by the emerging notion that the concept of synthetic lethality could have wider applicability to other defects in the DNA damage response network, and in line with previous studies demonstrating PARP inhibitor sensitivity associated with BRCA-independent HR defects,[8] Oplustilova et al. show that even partial depletion of the MRN components MRE11 or NBS1 sensitizes to PARP inhibition, whereas ectopic expression in mutant cells had the opposite effect.

Further, arguing that ongoing PARP activity must be a prerequisite for PARP inhibitors to work, Oplustilova et al. went on to assess steady-state poly(ADP-ribose) (PAR) levels in different cell lines and report a correlation between detection of PAR and inhibitor sensitivity. While these results generally support PAR levels as potential candidate biomarker, additional studies are needed to determine whether PAR detection is technically feasible in tissue biopsies, and whether PARP inhibitor sensitivity is inevitably associated with detectable amounts of PAR, especially in light of the limited sensitivity of the available antibodies for shorter PAR chains. Likewise, when Oplustilova et al. assessed RAD51 foci numbers as a surrogate marker for HR, several cell lines were sensitive to PARP inhibition, yet they had normal RAD51 foci levels, suggesting that either specific HR defects do not entail reduced RAD51 loading, or that synthetic lethality can be achieved through HR-independent mechanisms. Together, these results raise the concern that use of single biomarkers could indeed be misleading, and that a combination of markers to assess which cancer cells are likely "addicted to PAR" might be more reliable. The study by Oplustilova et al. evaluates several of such candidate biomarkers and thus contributes to the collective effort to guide targeted cancer therapy to those patients who might benefit most from PARP inhibitor treatment. However, it also illustrates once again that such guidance crucially relies on a more detailed understanding of the complex DNA damage repair network and how the interacting pathways may be rewired in cancers with unstable genomes, as well as on deeper insights into the diverse functions of PARPs and how they contribute to synthetic lethality.

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UnCHKed DNA replication: A stressful matter

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It is easy to conceive that in a toxic environment most cells would abandon the division process. In the past decades, it has indeed become clear that cells have developed complex cell cycle checkpoint mechanisms, which slow-down or stop cells from proliferating under challenging conditions. More recently, however, is the discovery and molecular characterization of signaling cascades capable of integrating classical stress-activated responses and cell cycle checkpoints. A report published in a recent issue of *Cell Cycle*[1] sheds light on the increasing complexity of such pathways. The authors found that the stress-activated protein kinases p38 and JNK cooperate with Chk1 to block progression into mitosis under conditions that alter DNA replication (i.e., treatments of cells with antiproliferative drugs such as hydroxyurea, aphidicolin, camptothecin or etoposide). By using a combination of pharmacological and genetic tools, together with precise protocols of cell cycle synchronization, the study shows compelling evidence that in murine NIH3T3 and embryonic fibroblasts, hydroxyurea (used at concentrations capable of abrogating DNA synthesis) added during progression through S-phase first induces an early acute activation of Chk1 that is immediately followed by a phasic activation of both p38 and JNK. Remarkably, p38 and JNK activities are triggered completely independent of ATM-ATR and Chk1 activation, since their activities were similarly induced in the presence of caffeine and UCN-01, respectively.

Additionally, activation of p38 under DNA replication blockade seems to be mediated by MKK3 and MKK6 (usual kinases turning-on p38 signaling), while interestingly, JNK activation seems to be exclusively caused by MKK4 (one of the regular kinases switching-on the JNK pathway) but not MKK7. Furthermore, downstream of p38 signaling, the authors unveiled activation of MK2 and MK3, kinases classically activated by p38 under many stressors. Strikingly, activation of MKK3/6-p38-MK2/3 and MKK4-JNK cascades in the presence of hydroxyurea is required to suppress entry into mitosis as measured by reduced (Serine 10)-histone H3
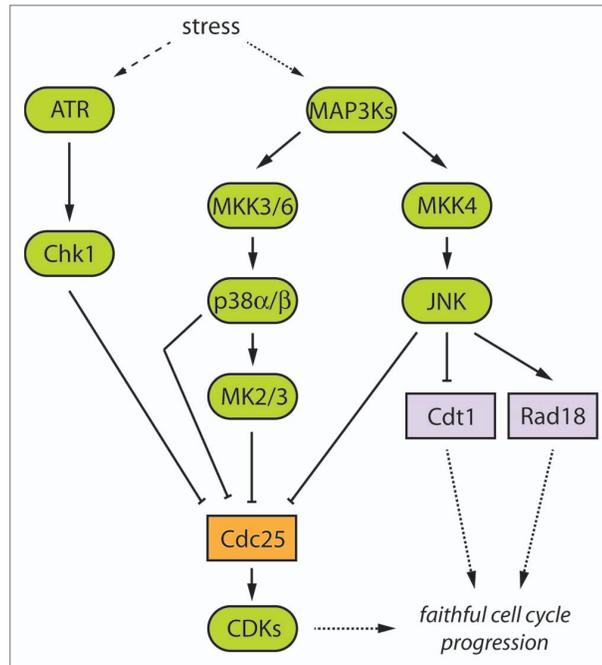

Figure 1. A proposed model of signaling circuitry integrating stress-response and cell cycle checkpoint pathways. Refer to text and references for details.

phospho-signal and impaired (Threonine 14 and Tyrosine 15)-CDK1 dephosphorylation and activation. The effect on CDK1 activity is likely mediated by inhibitory regulation of members of the Cdc25 family of phosphatases (**Fig. 1**), as previously reported by others.[2-4] Finally, the authors establish that at least p38α and p38β are involved in this mechanism; however, it is still unclear whether JNK1 and JNK2 both play significant roles in the process. Also, it remains to be identified which kinase(s) is (are) responsible for the activation of the respective p38 and JNK MKKs, under conditions blocking DNA synthesis/replication.

On the other hand, some progress has been made in the identification of substrates of the stress-activated protein kinases p38 and JNK implicated in DNA replication. Two recent studies found that Cdt1 is phosphorylated, probably at several residues, by both p38 and JNK, in the presence of genotoxic[5] (UV-C) and non-genotoxic[6] (including sorbitol

and anisomycin) stressors. Cdt1 is a key DNA replication licensing factor that contributes with Cdc6 to ensure proper loading of the MCM complex onto chromatin to form the pre-replication complex. Notably, stress-mediated phosphorylation of Cdt1 blocks its degradation during S-phase,[5,6] leads to the dissociation of the histone acetylase HBO1/KAT7 from replication origins[6] and compromises the ability of Cdt1 to instigate loading of the MCM complex,[5] therefore blocking initiation of DNA replication.[5,6]

Moreover, JNK was also found to phosphorylate the RING-finger type E3 ubiquitin ligase Rad18, involved in postreplication repair of damaged DNA (UV-C irradiation). JNK-mediated Rad18 phosphorylation appears to facilitate recruitment of the translesion synthesis DNA polymerase Polη to stalled replication forks.[7] Polη is then presumably capable of DNA synthesis over the damaged DNA, later allowing DNA replication by conventional DNA

polymerases to occur. This intriguing observation rather suggests a role for JNK in DNA damage response tolerance pathways.

Based on those and other studies describing a direct control of cell cycle regulators by stress-response pathways, at least in the case of JNK, it is possible to advocate a general function in cellular genome integrity maintenance, through the regulation of substrates such as Cdt1 and Rad18, or histone H3 and Cdh1/FZR1, under toxic[5-7] or unperturbed[8,9] conditions, respectively. Finally, according to our current understanding of the stress-activated signaling pathways, it is noteworthy that the analytical dynamics, subcellular localization and temporal occurrence of the stress input (directly associated with cell cycle checkpoints or not) would likely determine not only the strength, duration and diffusion of the stress response but more importantly its final functional outcome.[1] In practical terms, the modulability of the stress-response could decide which exact pathways are activated and which substrates need to be accordingly modified. Given the growing involvement of stress responses in the control of cell cycle (checkpoint) mechanisms, we would speculate that in coming years, the cell cycle-related phosphoproteome governed by canonical stress kinases will continue to augment.

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