รายงานวิจัยฉบับสมบูรณ์

โครงการ บทบาทของไซคลิน ดี1 และเครือข่ายโปรตีนก่อมะเร็งของไซคลิน ดี1 ในการก่อโรคมะเร็งและการรักษาแบบมุ่งเป้า

Roles of Cyclin D1 Oncogenic Network in Tumorigenesis and Cancer Targeted Therapy

โดย อ. ดร. ภก.ศิวนนท์ จิรวัฒโนทัย

มิถุนายน 2561

สัญญาเลขที่ RSA5880038

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มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและต้นสังกัด
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Investigator : ชื่อนักวิจัย และสถาบัน

(ชื่อนักวิจัย)

ด.ร. ศิวนนท์ จิรวัฒโนทัย ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

E-mail Address : Siwanon.jir@mahidol.ac.th

Project Period :

(ระยะเวลาโครงการ) ตั้งแต่วันที่ 1 กรกฎาคม 2558 ถึงวันที่ 30 มิถุนายน 2561

เนื้อหางานวิจัยประกอบด้วย

1. วัตถุประสงค์

   1) To study role(s) and collaboration between cyclin D1 and cyclin D1 oncogenic network during tumorigenesis and tumor survival

   2) To study the consequence(s) of cyclin D1/cyclin D1 network inhibition or modification in cancer cells

   3) To study the possible mechanism to block cyclin D1 in cancer cells.

2. วิธีทดลอง

   Please see below.

3. ผลการทดลอง

   For the last three years, our team has discovered a significant role of cyclin D1 and interactor in the cancer network.

   The major discovery stemmed from our project is a discovery of a novel function of cyclin D1 in controlling cancer cell homeostasis during cell cycle (Ref). Our results support the notion that cyclin D1, as a hub for oncogenic activity, works not only in the cell proliferation, but also may act as a master regulator that controls and smoothens the whole process of cell division (including, replication, DNA repair, gene expression, and the novel function in oxidative stress response).

   Our project showed for the first time the role of cyclin D1 in suppressing oxidative stress at the low, non-lethal level, allowing cancer cell to continue proliferation. This novel is entirely independent of its cell cycle role.

   Our team carefully observed the cancer cell, after cyclin D1 was removed from the cells, and found that the cell transformed into a senescence-like phenotype. We observed that the cells expanded, became non-proliferative, and contained very high cytoplasm/nuclear ratio. That was when we hypothesized about the novel function of this protein.

   In detail, please see below.

ABSTRACT

Expression of cyclin D1 is required for cancer cell survival and proliferation. This is presumably due to the role of cyclin D1 in RB inactivation. Here we investigated the prosurvival function of cyclin D1 in a number of cancer cell lines. We found that cyclin D1 depletion facilitated cellular senescence in several cancer cell lines tested. Senescence triggered by cyclin D1 depletion was more extensive than that caused by the prolonged CDK4 inhibition. Intriguingly, the senescence caused by cyclin D1 depletion was independent of RB status of the cancer cell. We identified a buildup of intracellular reactive oxygen species, in the cancer cells that underwent senescence upon cyclin D1 depletion, but not in CDK4 inhibition, and that ROS buildup was responsible for the senescence. Lastly, the senescence was found to be instigated by the p38/JNK-FOXO3a-p27 pathway. Therefore, expression of cyclin D1 prevents cancer cells from undergoing senescence, at least partially, by keeping the level of intracellular oxidative stress at a tolerable sub-lethal level. Depletion of cyclin D1 promotes the RB-independent pro-senescence pathway, and cancer cell succumbing to the endogenous oxidative stress.

Introduction

Cyclin D1 is a cell cycle regulatory protein, that is amplified and overexpressed in a large number of human cancer (Sukov et al., 2009, Musgrove et al., 2011, Lee et al., 2016). Expression of cyclin D1 is essential for oncogenic transformation, as well as, for cancer cell survival. Activation of Kras or HER2 pathways targeted in mouse breast tissue resulted in breast cancer, but failed to induce any tumor in cyclin D1-deficient breast(Yu et al., 2001). In addition, shutdown of cyclin D1 expression in breast tumor resulted in tumor cessation associated with cancer cell senescence (Choi et al., 2012).

The cancer supporting role of cyclin D1 in cancer formation and survival represents a curious circumstance, in that although it is required for cancer formation, cyclin D1 does not appear to be a strong cancer driver. Forced expression of cyclin D1 in mouse model did not promote cancer formation until after a very long latency(Wang et al., 1994, Casimiro and Pestell, 2012). Thus, expression of cyclin D1 may be required to support oncogenic transformation, possibly by creating permissive cellular condition for cancer cell transformation.

The best described function of cyclin D1 is to forms complexes and activates cyclin-dependent kinase 4 or 6 (CDK4/6). Cyclin D1-CDK4/6 complex phosphorylates and inactivates retinoblastoma (RB) protein which allows the cell to enter S phase from G1. Emerging evidence suggests that cyclin D1 also holds several cancer related roles beyond the canonical RB inactivation. These proposed additional roles include functions in transcriptional control, homologous-mediated DNA repair, differentiation, migration, and mitochondria regulation, etc. (Hydbring et al., 2016). It remains unclear, whether each of these non-canonical roles may have any role in cancer formation.

Cellular redox status has been shown to influence several biological processes (Trachootham et al., 2009). Oxidative equilibrium relies on a well-regulated rate of production versus elimination of oxidative species in the cell. Oxidative imbalance has been demonstrated to influence cellular behavior, and to underline several pathological conditions, including cancer (Vurusaner et al., 2012).

Herein, we investigated detail of the anti-senescence role of cyclin D1 in cancer cells.

We showed that cyclin D1 expression is essential for cancer cells to remain in the proliferative and non-senescence stage, by maintaining oxidative stress in cancer cell at a low/sub-lethal level, and hindering cancer cell succumbing to the oxidative stress-induced senescence.

Results

Cyclin D1 depletion resulted in cancer senescence in several types of human cancer

Expression of cyclin D1 was shown to be indispensable for preventing cell senescence in cancer cells in vivo and in vitro (Choi et al., 2012, Brown et al., 2012). However, it is not known, whether the anti-senescence role of cyclin D1 is universal in every cellular context, as in a number cell types and certain circumstances, cell division may take place without cyclin D1(Sherr and Roberts, 2004). Thus, we examined the role of cyclin D1 in cancer cell senescence by using cyclin D1-specific RNA interference to deplete cyclin D1 in the following human cancer cell lines: MDA-MB-175, ZR-75-1, MDA-MB-134, T47D, SKBR3, and MCF7 (breast cancer), KKU-156, KKU-214, TYBDC-1, (cholangiocarcinoma), and UMSCC2 (squamous cell carcinoma). After 4 days of cyclin D1 depletion, 6 of 10 cell lines demonstrated clear signs of cellular senescence, gauged by marked increase in the number of cells with an established senescent marker, senescence associated β-galactosidase (SA-β-Gal) expression (Fig. 1A and Fig. S1A).

The senescent cells were from three tissues of origin. We noticed that these cell lines expressed varying levels of endogenous cyclin D1 (Fig. S1B). Therefore, the anti-senescence function of cyclin D1 was not ubiquitous among all of the cancer cell lines, and this role was not correlated with the pre-existing level of expression of cyclin D1 in each cancer cell line.

We carefully verified the senescence induced by cyclin D1 depletion by examining the characteristics of these cells. Depletions of cyclin D1 by two independent cyclin D1-specific short-hairpin RNAs (shRNAs) produced the same outcomes. They inhibited cancer cell division. Cyclin D1-depleted cells remained non-proliferative after 5 days in culture (Fig. 1B, C), and did not re-enter into cell cycle after long-term culture (Fig. 1D). Cyclin D1-depleted cells appeared flatten with large cytoplasm (Fig. 1E), and they were positive for SA-β-Gal staining (Fig. 1F, G). Cyclin D1-depleted cells also showed significant increase in senescence-associated heterochromatin foci (SAHF), another putative marker for cellular senescence(Narita et al., 2003) (Fig. 1H, I). Lastly, expression of CDKN2A mRNA was upregulated(Rayess et al., 2012), and LMNB1 mRNA was down-regulated(Freund et al., 2012)(Fig. 1J), confirming that these cells underwent cellular senescence after cyclin D1 depletion. These results indicate that cyclin D1 depletion facilitated cancer cell to undergo authentic cellular senescence. In addition, we found that re-expressions of cyclin D1 neither restored cell proliferation, nor reduced SAHF in the cyclin D1-depleted cells (Fig. S1C-E), indicated that, once triggered, the senescence was irreversible. To understand the nature of the senescence observed in cyclin D1-depleted cells, we studied the timing when the senescence was initiated. We found that a significant number of SAHF-positive cell was detected at 48 hours after transfection of cyclin D1-specific small interfering RNA (siD1), which correlated with the depletion of cyclin D1 protein (Fig. 2 A, B). We also observed a moderate increase of cancer cell apoptosis upon cyclin D1 depletion. The increased apoptosis was not observed in control cells, or in cancer cells treated with PD0332991, a specific CDK4/6 inhibitor (Fig. S2A). Apoptosis associated with cyclin D1 depletion might be a result of DNA damage, since cyclin D1 depletion-induced senescent cells contained increased number of a DNA damage marker □H2AX, at the level comparable to 50-100 μM H2O2 treatments (Fig. S2B).

Previous study demonstrated that prolonged inhibition of CDK4 activity by CDK4/6 inhibitor also resulted in cancer cell senescence (Otto and Sicinski, 2017). We treated MCF7 cells with PD0332991 and observed that CDK4/6 inhibition also resulted in growth

arrest, and senescence (Fig. S3A, B, respectively). However, prolonged inhibition caused a delayed type of senescence that became significant only at 96 hours after the 0.5 μM PD0332991 treatment. In agreement with that, suppression of CDK4 activity by CDK4-specific siRNA also resulted in the delayed type of senescence (Fig. S3C,-D). These results implicated that depletion of cyclin D1 maybe a more robust trigger for cancer cell senescence, than inhibition of CDK4/6 kinase activity. In addition, we showed that expression of the kinase-dead version of cyclin D1 (K112E), significantly reduced senescence in cyclin D1-depleted cells at 96 hours after cyclin D1 depletion (Fig.), therefore, CDK4 activity was not involved with the early-onset senescence observed in cyclin D1-depleted cells.

Anti-senescence role of cyclin D1 is independent of its RB-inactivation function

In order to investigate whether the senescence observed in cyclin D1-depleted cancer cells was mediated by RB, we established a RB-deficient MCF7 cell line by stably expressing RB-specific shRNA (Fig. 3A). We verified that these RB-deficient cells were oblivious to an inhibition of CDK4 kinase activity by treating the cells with PD0332991. MCF7-shRB cells continued to proliferate under treatment, indicating that without RB, cyclin D1-CDK4 kinase was no longer required for these cells to proliferate (Fig. 3B). Consistent with the preceding, MCF7-shRB cells did not undergo senescence under a prolonged 0.5 μM PD0332991 treatment (Fig. 3C, and Fig. S3EE).

Interestingly, we found that cyclin D1 depletion effectively inhibited MCF7-shRB cell proliferation (Fig. 3A, D), and triggered extensive cellular senescence in these cells (Fig. 3E-I). We also confirmed that cyclin D1 depletion was able to inhibit cell proliferation and to promote senescence in another RB-deficient cancer cell line TYBDC-1-shRB (Fig. 3J-L, K). Thus, the observation was not specific to only MCF7 cells.

We also established a pocket protein-deficient MCF7 cell line (MCF7-E7), in which all pocket proteins (RB, p107, p130) were destabilized by an expression of HPV16 E7(Helt and Galloway, 2001). We verified that MCF7-E7 cells were unresponsive to CDK4 inhibition and did not submit to senescence upon 5 day PD0332991 treatment (Fig. 3L, M). We found that cyclin D1 depletion effectively inhibited MCF7-E7 cell proliferation (Fig. 3N, O), and promoted widespread senescence in these pocket protein-deficient cells (Fig. 3P, M). Finally, we showed that expression of the kinase-dead version of cyclin D1 (K112E) significantly reduced senescence in cyclin D1-depleted cells under

cyclin D1 depletion condition (Fig. S2F, G), therefore, CDK4 activity was not involved with the early-onset senescence observed in cyclin D1-depleted cells.

Therefore, inhibition of CDK4 resulted in RB-mediated cellular senescence. On the other hand, inhibition of cyclin D1 resulted in cellular senescence was RB-independent.

Elevated ROS level in cyclin D1-depleted cells triggered cancer cell senescence

Senescence could be triggered by various cellular stressors, including oxidative stress(Salama et al., 2014). To elucidate the cause of senescence associated with cyclin D1 depletion, we examined levels of reactive oxygen species (ROS) between the responders (cell lines that responded to cyclin D1 depletion by undergoing senescence: KKU-156, TYBDC-1, SKBR3, MCF7, KKU-214, UMSCC2), and the non-responders (cell lines that did not undergo senescence: MDA-MB-175, MDA-MB-134, T47D, ZR-75-1). Interestingly, there was a significant increase in endogenous ROS in all responder cell lines after cyclin D1 depletion, whereas no change in level of endogenous ROS in non-responder cell lines (Mann-Whitney U test, $p = 0.0095$) (Fig. 4A, upper panel versus lower panel). We noticed that the upregulation of ROS, and the senescence response were not related to average basal levels of ROS in each cell line (Fig. 4A, shconts).

The upregulation of ROS was detected at approximately 48 hours after cyclin D1 siRNA transfection, coinciding with cyclin D1 depletion, and it intensified by the 72 hours time point (Fig. 4B, C). Interestingly, PD0332991 treatment, although resulting in cell cycle arrest and senescence, did not cause ROS elevation (Fig. S3A, B, and Figure 4B).

To investigate, whether the elevated ROS was responsible for the senescence phenotypes, first, we promoted an accumulation of ROS by treating MCF7 cells with H2O2. Majority Many of MCF7 cells responded to H2O2 treatment by undergoing senescence, suggested that senescence is a primary response to oxidative stress in these cells (Fig. S4A).

Next, we treated cyclin D1-depleted cells with an anti-oxidant N-acetylcysteine (NAC) to suppress intracellular ROS. We found that NAC treatment significantly reduced the number of cells that underwent senescence, gauged by SA-β-Gal and SAHF staining (Fig. 4D-F). NAC treatment did not reduce delayed senescence mediated by PD0332991 treatment, indicating that ROS played a limited role in the PD0332991-mediated senescence (Fig. S4B).

Of note, although NAC effectively suppressed senescence caused by cyclin D1 depletion, it was unable to entirely abolish the senescence. This suggested that

senescence in cyclin D1-depleted cells may also be influenced by another cause other than ROS. We and others have previously reported that cyclin D1 participates in homologous recombinant-mediated DNA repair (HR) and cyclin D1 depletion reduces the efficiency of HR (Li et al., 2010, Jirawatnotai et al., 2011). Reduced efficiency of HR may also contribute to cancer cell senescence observed in the cyclin D1-depleted cells.

To this end, we employed a model cell line Capan-1, in which the HR is not operative, because of a protein-truncating mutation on BRCA2 gene (Yuan et al., 1999). Therefore, in this cell line, we would be able to measure the anti-oxidative stress function of cyclin D1, without interference from the HR-related role of cyclin D1. We depleted cyclin D1 from Capan-1 and found that, cyclin D1 depletion resulted in increased senescence (Fig. S4C, D). This result implicated an existing of an HR-independent anti-senescence role of cyclin D1. Then, we treated the cyclin D1-depleted cells with NAC and found that, NAC treatments were able to abolish senescence in cyclin D1-depleted Capan-1 to the level that is comparable to that in the control Capan-1 cells (Fig. S4C, D). Therefore, we believed that in cells with normal HR, cyclin D1 plays roles to protect cell from senescence by facilitating efficient HR repair, and by keeping level of intracellular oxidative stress at a tolerable sub-lethal level.

Cyclin D1 was shown to negatively modulate the number of mitochondria, an important endogenous source of ROS (Sakamaki et al., 2006, Wang et al., 2006, Tchakarska et al., 2011). Consistent with previous reports, we found that cyclin D1-depleted cells exhibited an increased number of total and functioning mitochondria, as measured by transmission electron microscopy (TEM), expression of total mitochondria-specific DNA (mtND5), and MitoTracker staining (Fig. S4E-I).

We also measured levels of mitochondrial superoxide using a specific fluoroprobe MitoSOX, and detected an upregulation of the mitochondrial superoxide in cyclin D1-depleted cells, but not in the cells treated with PD0332991 (Fig. S4J, K). An increase in total and functioning mitochondria was also found in RB-deficient cells, suggesting that the increase in ROS was independent of RB (Fig. S4L-P). These evidences collectively supported that, cyclin D1 depletion-promoted cancer cell senescence is mediated by the ROS build-up in the cancer cells, which is possibly a consequence of the increased number of functioning mitochondria number in the cells.

High level of endogenous ROS activated p38/FOXO3a/p27 pathway in cyclin D1-depleted cancer cells

To investigate the consequences of elevated ROS in cyclin D1-depleted cells, we studied activation of p38 and JNK, the MAP kinases involved in oxidative stress response(Ho et al., 2012, Essers et al., 2004). We detected upregulation of phospho-p38 MAPK (Thr180/Tyr182) and JNK-mediated c-JUN phosphorylation at Ser73 in cyclin D1-depleted cells around 48-72 hours after cyclin D1 depletion, but not in control cells (Fig. 5A, Fig. S5A). We did not detect activation of AKT in cyclin D1-depleted cell (data not shown). Activations of p38 and JNK were abolished, when cyclin D1-depleted cells were treated with NAC (Fig. 5B, Fig. S5B). FOXO3a is a transcription factor that activates genes in response to oxidative stress in order to mediate oxidative detoxification, cell cycle arrest, and apoptosis. When it senses cellular stress, FOXO3a relocalizes to the nucleus and binds to promoters of target genes such as, SOD2, and catalase (Kops et al., 2002, Bartell et al., 2014). In case that the stress level is overwhelming, FOXO3a can activate genes that promote senescence or apoptosis, such as CDKN1B (encoded for a cell cycle inhibitor p27), or GADD45A and BIM(Lam et al., 2013).

We detected upregulation of FOXO3a protein in association with increased FOXO3a Ser7 phosphorylation, a phosphorylation involved in the stress-induced activation of FOXO3a by p38(Ho et al., 2012) (Fig. 6A, B), and translocalization of FOXO3a from cytoplasm to nucleus, after cyclin D1 depletion (Fig. 6C-E). This translocalization of FOXO3a was abolished by NAC treatment (Fig. S5C). We detected recruitments of endogenous FOXO3a to several downstream promoters, such as CDKN1B, SOD2, GADD45a, PrxIII, and BCL10, by FOXO3a chromatin immunoprecipitation. All together these results confirmed an activation of FOXO3a induced by cyclin D1 depletion (Fig. S6). Activation of FOXO3a was also detected when cyclin D1 was depleted in RB-deficient MCF7 cells (Fig. 6E). We also confirmed the activation of FOXO3a by cyclin D1 depletion in cell lines TYBDC-1, and KKU-214 (Fig. 6F). Lastly, we detected upregulations of several genes known to be regulated by FOXO3a (Fig. 6G). Therefore, cyclin D1 depletion was sufficient to fully activate FOXO3a.

To elucidate the cause of RB-independent cellular senescence in cyclin D1-depleted cells, we focused on upregulated p27 (encoded by CDKN1B), since it is a gene downstream of FOXO3a known to regulate cellular senescence through multiple mechanisms, including RB-independent mechanisms(Majumder et al., 2008, Young et

al., 2008). We found that p27 protein level was also upregulated in cyclin D1-depleted cells (Fig. 6H). The upregulation of p27 coincided with p38 and c-JUN phosphorylations (Fig. S7A5C). In addition, we detected increased binding between p27 and CDK2 in cyclin D1-depleted cells, implicating a downregulation of CDK2 activity. We measured senescence levels in MCF7 and MCF7-shRB cells co-transfected with siRNAs against cyclin D1 and p27. We found that p27 siRNA significantly increased the numbers of EdU-positive cells, and reduced the number of SAHF-positive cells in both cyclin D1-depleted MCF7 and MCF7-shRB cells, indicating that p27 depletion prevented the cell cycle arrest and senescence observed in cyclin D1-depleted cells (Fig. 6I-L). Therefore, p27 mediated, at least partially, the cell cycle arrest and cellular senescence found in cyclin D1 depleted cells.

Discussion

Overexpression of cyclin D1 is commonly found in many types of cancer. However, how precisely overexpressed cyclin D1 contributes to cancer formation is not entirely known. In this study, we investigated a novel anti-senescence role of cyclin D1 in human cancer. We found that cyclin D1 expression maintains endogenous ROS at the sub-lethal level, likely by inhibiting mitochondria number and function. In the absence of cyclin D1, cancer cells accumulate intolerable levels of endogenous ROS, which subsequently triggers cancer cell senescence and possibly cell death. The senescence triggered by cyclin D1 depletion appeared to be independent of cell cycle regulatory function of cyclin D1, since the senescence induced by cyclin D1 depletion fully occurred in RB-deficient cells. In addition, ROS-induced senescence was specific to cyclin D1 depletion, and not observed in cells treated with a CDK4/6-specific inhibitor. We, therefore, conclude that cyclin D1 has a CDK4-, RB-independent role in keeping oxidative balance for the cancer cells (Fig. 7A)

We elucidated that the senescence was mediated by the p38/JNK-FOXO3a-p27 pathway, which was activated by elevated ROS after cyclin D1 depletion (Fig. 7B). FOXO3a is a tumor suppressor, which facilitates apoptosis and senescence upon stress (Nestal de Moraes et al., 2016). It was shown that FOXO3a functions at both transcriptional and post-transcriptional levels to promote p27 expression(Lam et al., 2013, Wu et al., 2013). p27 is a CDK2 inhibitor, capable of blocking non-RB processes of cell division controlled by CDK2 (Chae et al., 2004, Luscher-Firzlaff et al., 2006, Voit et al., 1999). We found that significant higher amount of p27 was co-precipitated with CDK2, in cyclin D1-depleted cells (Fig. S7B), promoting a downregulation of CDK2

activity. This finding supported that p27 may be a key regulator for the senescence in cyclin D1-depleted cells. We have also examined expressions of GADD45A and SOD2, downstream genes of FOXO3a, which also known to be involved in senescence. Although, in cyclin D1-depleted cells, FOXO3a localized at the promoters of these two genes (Fig. S6), and mRNA levels were upregulated (Fig. 6G), we found that GADD45A and SOD were not upregulated at the protein level (both in RB-negative and RB-positive cells, Fig. S7C). From these results, we believe that role of GADD45A and SOD2 might be limited in cyclin D1-depleted cells. Although, we found that depletion of p27 significantly suppressed senescence, it is possible that there are other FOXO3a target genes that also contribute to the senescence induced by cyclin D1 depletion.

Previous studies demonstrated that cyclin D1 represses mitochondria number and that cyclin D1 functions by two possible mechanisms. First, cyclin D1 and CDK4 phosphorylates and represses nuclear respiratory factor-1 (NRF-1), a transcription factor that induces nuclear-encoded mitochondrial genes(Wang et al., 2006). Second, cyclin D1, independent of CDK4, competes with hexokinase 2 to bind to a voltage-dependent anion channel and inhibit mitochondrial function (Tchakarska et al., 2011). However, biological role of these findings in disease such as cancer is not entirely clear. Our results demonstrated that, in addition to role of cyclin D1 in cell cycle control, the new role of cyclin D1 in maintaining oxidative balance is critical for RB-positive and RB-negative cancer cell survival.

It remains to be addressed why some cancer cells managed to avoid ROS accumulation, thus escaped senescence, upon cyclin D1 depletion. These cells may rely on different type of redox modulations to cope with the consequence of excessive ROS.

Controls of cell cycle and cellular energy level were shown to be coordinated. Disruption of energy balance may disrupt normal cell cycle, and vice versa (Salazar-Roa and Malumbres, 2017). Thus, it may be sensible when a cell cycle machinery such as cyclin D1 also plays a dual role in controlling energy and oxidative balance during cell cycle. Cancer cells do not invent new pathways for survival, but instead using pre-existing machineries to operate. Consequently, they may become reliance on the adaptation. Our findings offered a view of cyclin D1 as a regulator that controls the cancerous oxidative balance, and describe the consequences when the balance is altered by cyclin D1 depletion. The role of cyclin D1 in other contexts and setting requires further investigation.

Altered redox status has long been observed in cancer cells. Recent study suggests that this adopted property of cancer cells can be exploited for therapeutic benefits(Trachootham et al., 2009). For the therapeutic use, inhibition of cyclin D1 may make cancer cells hypersensitive to ROS-modulating agents.

Materials and methods

Cell culture and treatment

The MDA-MB-175, ZR-75-1, MDA-MB-134, T47D, SKBR3, and MCF7 cell lines were from ATCC (Manassas, VA, USA). UMSCC2 was from University of Michigan cell bank. KKU-156, KKU-214, and TYBDC-1 cell lines were from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). Cell line authentication were done by Short Tandem Repeat (STR) profiling at the cell line banks indicated. The MDA-MB-175, ZR-75-1, and T47D were maintained in RPMI1640; SKBR3 in McCoy's 5A Medium; MDA-MB-134 in L-15; and MCF7, UMSCC2, KKU-156, KKU-214, and TYBDC-1 in DMEM, supplemented with 10% FBS, and 100 U/ml penicillin/streptomycin under standard conditions. shRNA expressing cells were maintained in selection mediums with one of the antibiotics, puromycin, G418, or hygromycin (Thermo Fisher Scientific, MA, USA). NAC was from Amresco (Solon, OH, USA). PD0332991 was a gift from Dr. P. Sicinski, Dana Farber Cancer Institute (DFCI), Boston MA, USA. Carboxy-H2DCFDA was from Sigma-Aldrich (St. Louis, MO, USA (D6883)).

Plasmids and siRNA

The shD1- pLKO.1#A(5'-GCCAGGATGATAAGTTCCTTT-3'), #B (5'-ATTGGAATAGCTTCTGGAAT-3'), and a control non-targeting shRNA (5'-CAACAAGATGAAGAGCACCAA-3') (each contains puromycin resistance gene) were from Sigma-Aldrich. The cyclin D1-shRNA (pBabe-neo) #C (5'-CCACAGATGTGAAGTTCATTT-3') (contains neomycin resistance gene), was a gift from Dr. E. Sicinska, DFCI. The pGIPZ RB1-shRNA (5'-CGCAGTTCGATATCTACTGAAA-3') was provided by Dr. Sunkyu Kim, Novartis Institutes for Biomedical Research. pBabe-hygro-E7 was provided by Dr. D.A. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA, USA. Cyclin D1-specific siRNAs (SI02654540, SI02654547), CDK4 (SI00604744), and control siRNA (1027310) were from Qiagen (Valencia, CA, USA). p27 siRNA (sc-29429) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cyclin D1 re-expression in senescent cells

The inducible system (Takara Bio Inc., Mountain view, CA, USA), composed of pEF1α-Tet3G and pTRE3G-cyclin D1-HA plasmids was co-transfected with GFP expressing plasmid pEGFP (a gift from Dr. P. Sicinski, DFCI) into MCF7 cells, followed by cyclin D1 depletion using cyclin D1-specific shRNA targeting 5'UTR of cyclin D1 mRNA (shD1#A). Expression of cyclin D1-HA was achieved by addition of 1 μM doxycycline. Analyses of SAHF, and EdU-positive cells were performed only in the cells expressing cyclin D1-HA (GFP expressing cells) by immunofluorescence, at 48 hours after doxycycline induction.

Detection of DNA damage

Cells were transduced with non-targeted shRNA control or cyclin D1-specific shRNA. After 4 day, □H2AX foci were analyzed as described (Jirawatnotai et al., 2011)

Growth curve and colony-forming assay

For growth curves, cells were seeded into 24-well plates at a density of 5,000 cells/well. Cell numbers were counted at indicated time points. For colony forming assay, 200 cells/well were seeded into 6-well plate. After 14 days, colonies were fixed with 10% neutral buffered formalin for 30 minutes and stained with 0.5% crystal violet solution for visualization.

SA-β-gal assay

SA-β-gal assay was performed as previously described (Debacq-Chainiaux et al., 2009). Percentage of SA-β-gal-positive cells was calculated from at least 500 cells.

Semi-quantitative real-time PCR

Total RNA was extracted using an RNA extraction Kit (Thermo Fisher Scientific). Complementary DNA were generated by Superscript III rev transcriptase and oligo-dT primers (Thermo Fisher Scientific). Transcript levels were normalized by expression of GAPDH. The mitochondrial DNA (mtDNA), mtND5 was analyzed against HBB. All primers used in this experiment were synthesized by bioDesign, Bangkok, Thailand. The primers used were: p16-for: 5'-CCGAATAGTTACGGTCGGAGG-3', p16-rev: 5'-CACCAGCGTGTCCAGGAAG-3', LMNB1-for: 5'-AGCGGAAGAGGGTTGATGTG-3', LMNB1-rev: 5'-CCAGCCTCCCATTGGTTGAT-3', mtND5-for: 5'-GCCTAGCATTAGCAGGAATAC-3', mtND5-rev: 5'-GGGGAAGCGAGGTTGACCTG-3', HBB-for: 5'-GCTTCTGACACAACTGTGTTCACTAGC-3', HBB-rev: 5'-CACCAACTTCATCCACGTTCACC-3', GADD45A-for: 5'-TCCTGCTCTTGGAGACCGA-3', GADD45A-rev: 5'-ATCCATGTAGCGACTTTCCCG-3', BIM-for: 5'-CAAGAGTTGCGGCGTATTGG-3'

, BIM-rev: 5'- TGTCTGCATGGTATCTCGGC-3', SOD2-for: 5'-TGGCCAAGGGAGATGTTACAG-3', SOD2-rev: 5'-CTTCCAGCAACTCCCCTTTG-3', p27-for: 5'- TCTGAGGACACGCATTTGGT-3', p27-rev: 5'-ACAGAACCGGCATTTGGGG-3', GAPDH-for: 5'-CCTCCAAAATCAAGTGGGGCGATG-3', GAPDH-rev: 5'-CGAACATGGGGGCATCAGCAGA-3'

Apoptosis detection

Cells were analyzed for apoptosis using an Annexin-V Apoptosis Detection Kit according to manufacturer's instruction (Thermo Fisher Scientific,). Forty eight hours after siRNA transfection, 10,000 cells from each condition were analyzed by flow cytometry (CytoFLEX, Beckman Coulter, Brea, CA, USA)

ROS measurement

ROS measurement was performed in 96-well plates. Cells were seeded at a density of 8 × 103 cells/well. At the detection time, cells were washed twice with PBS, and carboxy-DCFDA dye in serum free media was added at a final concentration of 5 μM. Plates were incubated at 37°C for 30 minutes, before removal of the dye. Cells were then washed twice with PBS, stained with nuclear staining dye and immediately analyzed by high content imaging system (PerkinElmer, Bangkok, Thailand). Results are presented as a median fluorescent intensity. For mitochondrial-specific superoxide detection, cells were transduced with either non-targeting shRNA (shcont), or cyclin D1-specific shRNA. After 4 day, cells were incubated with MitoSOX Red (Thermo Fisher Scientific), fixed and counterstained with DAPI, according to the manufacturer's instruction. Mean integrated fluorescence intensities were analyzed by high content imaging system.

Western blotting and immunoprecipitation

Western blotting and immunoprecipitation were performed as previously described (Jirawatnotai et al., 2011) using specific antibodies.

Immunofluorescence staining

Cells were seeded in microplates (#3904) (Corning, Corning, NY, USA), and fixed in 4% paraformaldehyde, permeabilized in methanol, and blocked in Odyssey® Blocking Buffer (PBS) (LI-COR, Lincoln, NE, USA). Cells were then incubated with specific primary antibody overnight, followed by 1:1000 dilution fluorescent-conjugated secondary antibody prior to nuclear staining with DAPI. For detection of cells in S-phase, cells were pulse with EdU (5-ethynyl-2-deoxyuridine) for 90 mins, fixed and stained by Click-iT reaction according to the manufacturer's protocol (C10337) (Thermo Fisher Scientific). Samples were evaluated by the high content imaging system.

Mitochondria staining

Cells were stained with 250 nmol/L MitoTracker® Red CMXRos (#9082) (Cell Signaling, Danvers, MA, USA) and DAPI, before subjected to the high content imaging system.

Antibodies

Rabbit Anti-cyclin D1 (sc-753), mouse anti-actin (sc-47778), mouse anti-HSP90 (sc-13119), mouse anti-total p38 (sc-535), and mouse anti-total c-JUN (sc-74543) were purchased from Santa Cruz Biotechnology. Rabbit anti-FOXO3a (07-702), rabbit anti-trimethyl-histoneH3(Lys9); H3K9 (07-442), and mouse anti-γH2AX Ser139 (05-636) were purchased from Merck Millipore (Bangkok, Thailand). Rabbit anti-FOXO3a (#2497), mouse anti-Rb (#9309), rabbit anti-phospho-p38 (Thr180/Tyr182, #4511), rabbit anti-phospho-c-JUN (Ser73, #3270) were from Cell Signaling. Generation of sheep anti-phospho FOXO3a (Ser7) was described previously [REF]. The antibody was used in dilution 1:500 for immunoprecipitation. All antibodies were used in dilution 1:1000 for Western blotting, 1:300 for immunofluorescence staining.

Transmission electron microscopy (TEM)

Sample preparation and analyses were performed according to a standard method as previously described (Parameyong et al., 2013).

Image processing and analysis

All images from the high content imaging system were analyzed using Columbus™ Image Data Storage and Analysis System (PerkinElmer), CellProfiler 2.1.1 and MATLAB R2015b. ImageStudio® program was used for quantification of Western blot signals.

Statistical analysis

The Mann-Whitney U test was used to evaluate significance between the median of control (shcont) and the cyclin D1 knockdown groups (shD1) in ROS measurement analysis and to compare ROS changes in the non-responder versus the responder group, deltas of ROS changes from all cell lines in each group were calculated for medians. Comparison between two groups was performed using two-tailed Student's t-test. Where *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

Figure 1 Depletion of cyclin D1 caused cellular senescence in various cancer cell lines.

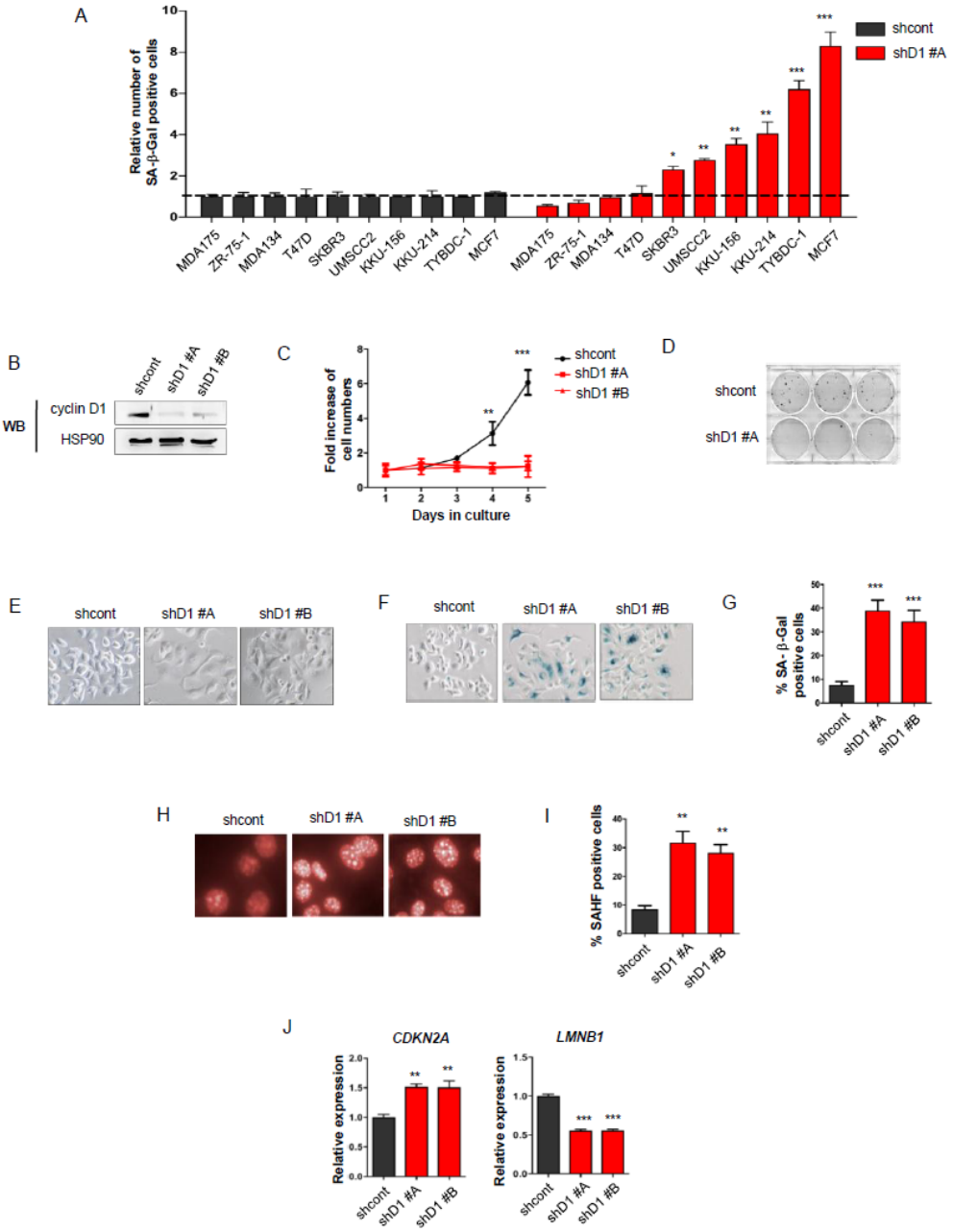

Figure 1. Depletion of cyclin D1 caused cellular senescence in cancer cell lines

(A) Cancer cell lines were transduced with lentivirus expressing non-targeting shRNA (shcont), or shRNA against cyclin D1 (shD1#A) and SA-β-Gal-positive cells were counted on day 4 after the shRNA transductions. Bars show averaged relative numbers ± s.d. of SA-β-Gal-positive cells (n = 500). (B) Western blot (WB) analysis of cyclin D1 expressions by 2 independent shRNAs (shD1#A, and shD1#B). Heat shock protein 90 (HSP90) was used as a loading control. (C) Growth curves of cyclin D1-depleted MCF7 cells (shD1#A and shD1#B). (D) Colony-forming assay of cyclin D1-depleted MCF7 cells. (E) Cellular morphology and (F) SA-β-Gal expression of cyclin D1-depleted MCF7 cells at day 4 after cyclin D1 depletion. Positive cells appear in greenish-blue. (G) Percentages of SA-β-Gal-positive cells from (F). Bars represent average numbers of SA-β-Gal-positive cells ± s.d. (H) Immunofluorescence staining (IF) of SAHF in cyclin D1-depleted cells. The results showed trimethyl-histoneH3 at Lys9 (H3K9) foci (bright foci) in cyclin D1-depleted MCF7 cells. Cells with more than 5 foci/ nucleus are considered positive. (I) Percentages of SAHF-positive cell from (H). (J) Upregulations of CDKN2A (p16) and downregulations of LMNB1 (lamin B) in cyclin D1-depleted cells, as measured by qRT-PCR. Bars represent the averages of 3 independent experiments ± s.d. Statistical significance was determined by Student's t-test (**p ≤0.01 and ***p ≤0.001).

Figure 2 Association between cyclin D1 downregulation and senescence.

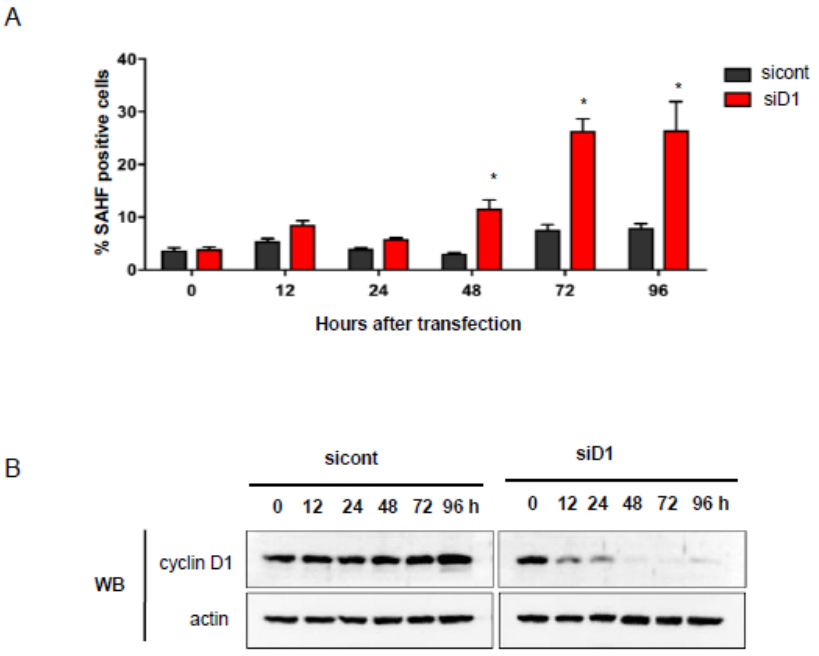

Figure 2 Association between cyclin D1 downregulation and senescence.
(A) Cellular senescence formations in cyclin D1-depleted cancer cells at various time points. MCF7 cells were transfected with either control non-targeting siRNA (sicont) or cyclin D1-specific siRNA (siD1), and SAHF-positive cell numbers were determined at indicated time points. Bars represent percentages of SAHF-positive cells. (B) Expressions of cyclin D1 after siRNA-mediated depletion. MCF7 cells were transfected with either sicont or siD1. Protein lysates were harvested at indicated time points after siRNA transfection. Western blot (WB) analysis showed cyclin D1 depletion efficiency. Actin was used as a loading control. Statistical significance was determined by Student's t-test (*$p \leq 0.05$).

Figure 3 Cyclin D1 depletion induced senescence is independent of RB status

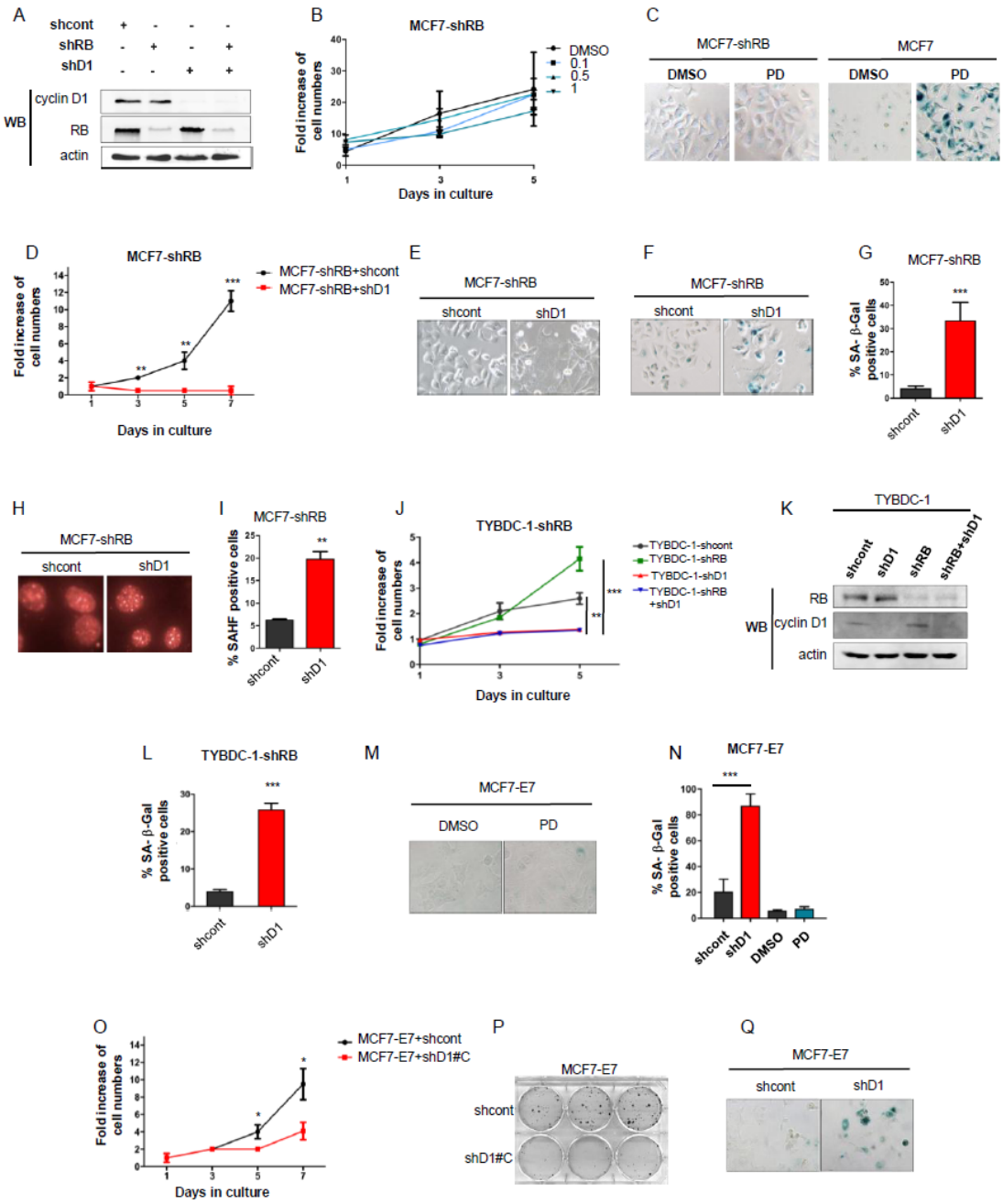

Figure 3 Cyclin D1 depletion induced senescence is independent of RB status.

(A) Western blot (WB) analysis of cyclin D1 and retinoblastoma protein (RB) in MCF7 cells. Actin was used as a loading control. (B) Growth curves of MCF7 cells expressing RB-specific shRNA (MCF7-shRB) treated with indicated concentrations of PD0332991 (0.1, 0.5, or 1 μM) or vehicle (DMSO). (C) SA-β-Gal expression in MCF7-shRB cells was determined following a 5-day treatment of 0.5 μM PD0332991. MCF7 cells were used as a senescence control. (D) Growth curves of RB-deficient MCF7 cells expressing shcont, or cyclin D1-specific shRNA (shD1#C). (E) Cellular morphology and (F) SA-β-Gal expressions of MCF7-shRB cells with non-targeting (shcont) or shD1. (G) Percentages ± s.d. of SA-β-Gal-positive cells from (F). (H) Immunofluorescence of SAHF in cyclin D1-depleted MCF7-shRB cells. (I) Percentages of SAHF-positive cells from (H). (J) Growth curves of TYBDC-1 expressing non-targeting shRNA control (shcont; grey), RB-specific shRNA (shRB; green), cyclin D1-specific shRNA (shD1#C; red), and shRB+shD1 (purple). (K) Percentages ± s.d. of SA-β-Gal-positive cells in RB-deficient TYBDC-1 cells expressing shcont, or shD1. (L) SA-β-Gal expressions in DMSO and PD0332991 (PD) treated E7-expressing MCF7 cells (MCF7-E7) at day 5 after treatment. (M) Percentages of SA-β-Gal-positive cells were determined after MCF7-E7 was transduced with the indicated shRNAs (shcont or shD1), or treated with DMSO or PD0332991. (N) Growth curves of MCF7-E7 cells expressing either shcont or shD1 (shD1#C). (O) Colony forming assay of MCF7-E7 cells expressing shcont or shD1 (shD1#C). (P) SA-β-Gal expressions in MCF7-E7 expressing shcont or shD1 cells.

Bars represent averages of three independent experiments. Statistical significance was determined by Student's t-test (**$p \leq 0.01$ and ***$p \leq 0.001$).

Figure 4 Reactive oxygen species (ROS) was upregulated in cyclin D1-depleted cancer cells and responsible for senescence.

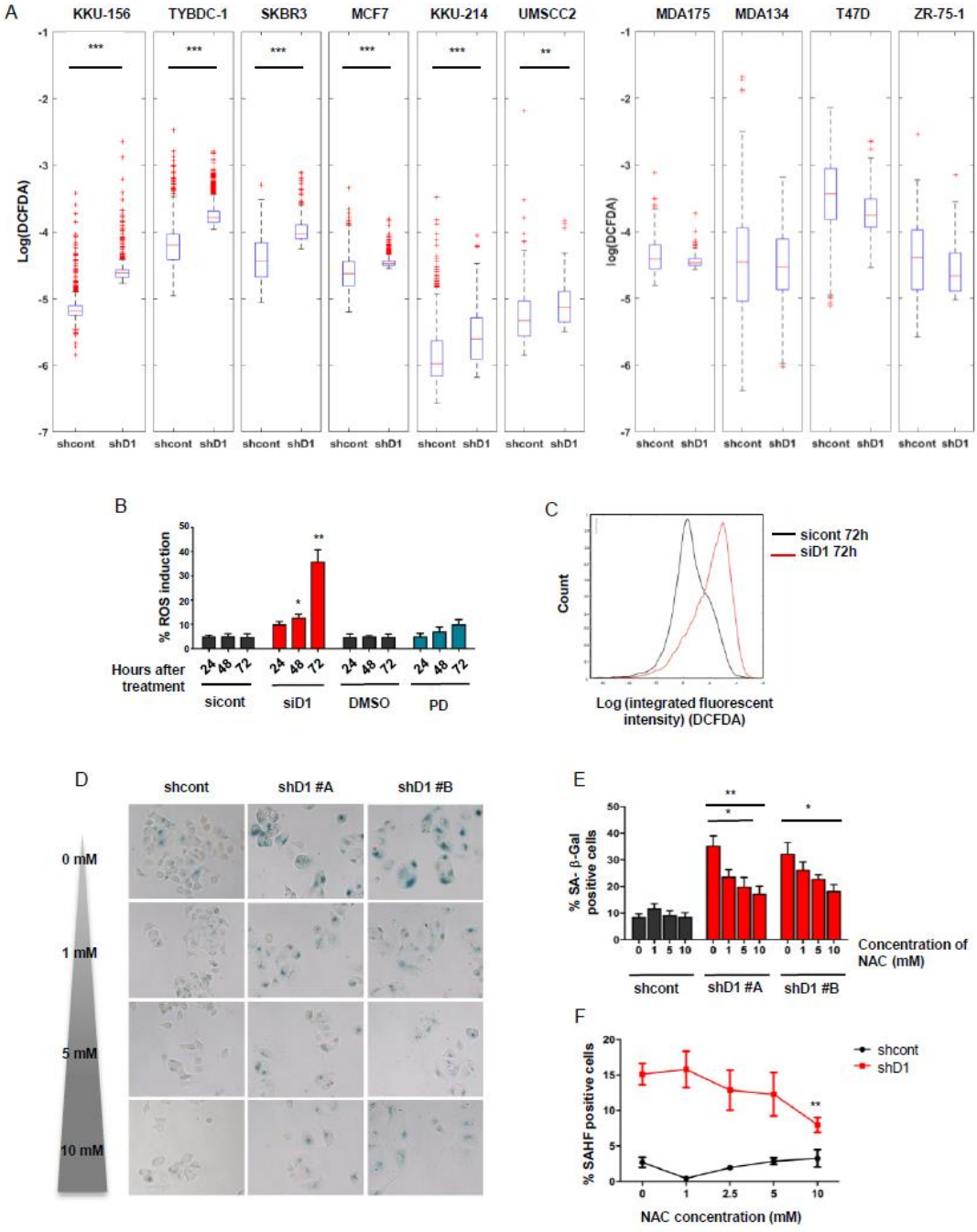

Figure 4 Reactive oxygen species (ROS) was upregulated in cyclin D1-depleted cancer cells and was responsible for senescence.

(A) Cancer cells were transduced with either lentivirus expressing non-targeting shRNA (shcont) or cyclin D1-specific shRNA (shD1). After 4 days ROS levels were measured using carboxy-DCFDA. Box plots show distributions of ROS in the indicated cell lines. Statistical significance was determined by Mann-Whitney U test. (B) ROS levels measured in MCF7 cells transfected with non-targeting siRNA (sicont) or cyclin D1 siRNA (siD1), or treated with vehicle (DMSO) or PD0332991 (PD) at indicated time points (24, 48, and 72 hours). Bars represent percentages of DCFDA-positive cells. (C) Histograms of intracellular ROS levels in MCF7 cells transfected with sicont or siD1 at 72 hour post-transfection. (D) SA-β-Gal expressions in cyclin D1-depleted MCF7 cells treated with indicated NAC concentrations. (E) Quantification of SA-β-Gal-positive cells after being treated with indicated concentrations of NAC. Bars represent average percentages of positive cells ± s.d. (F) Percentages of SAHF-positive cells after NAC treatments. Statistical significance was determined by Student's t-test. (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

Figure 5 Upregulation of ROS in cyclin D1-depleted cells activated p38 and JNK pathways

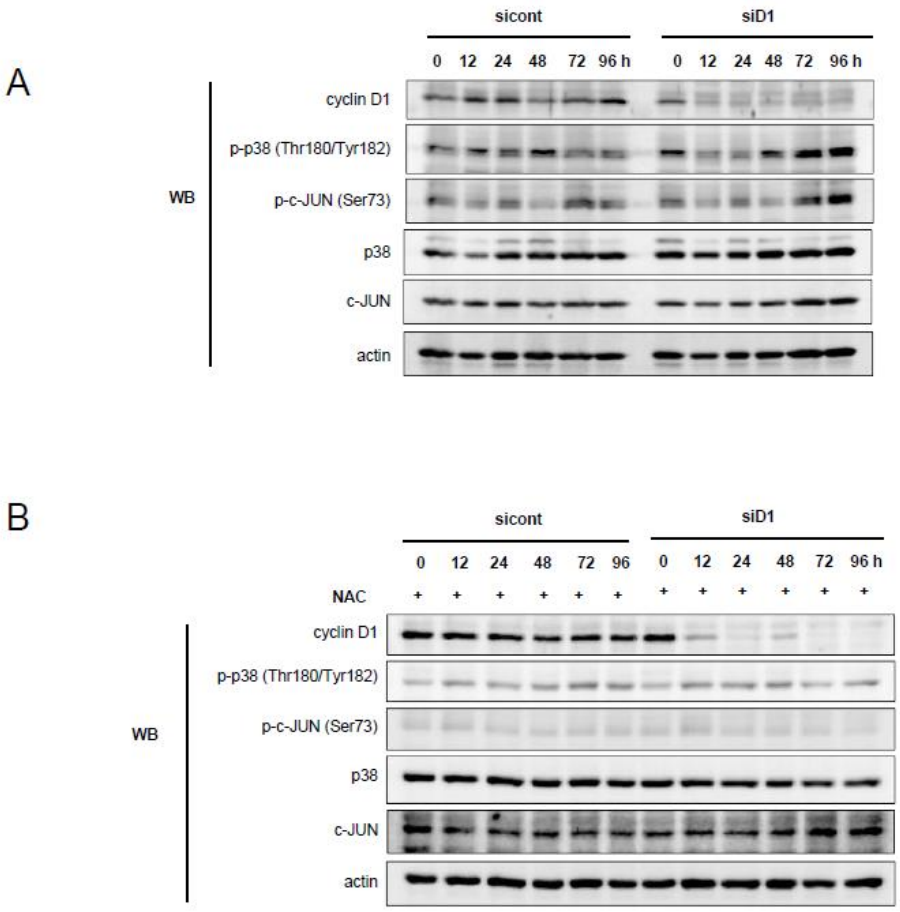

Figure 5 Upregulation of ROS in cyclin D1-depleted cells activated p38 and JNK pathways

Western blot (WB) analysis of p38, and c-JUN activations. (A) Protein lysates were prepared at 0, 12, 24, 48, 72, and 96 hours after non-targeted siRNA (sicont) or cyclin D1 siRNA (siD1) transfection. (B) WB analysis was performed similarly as in (A), except that freshly prepared NAC was added to the cells every other day from the 0 hour until the cells were harvested.

Figure 6 Elevated ROS induced hyper-functional FOXO3a.

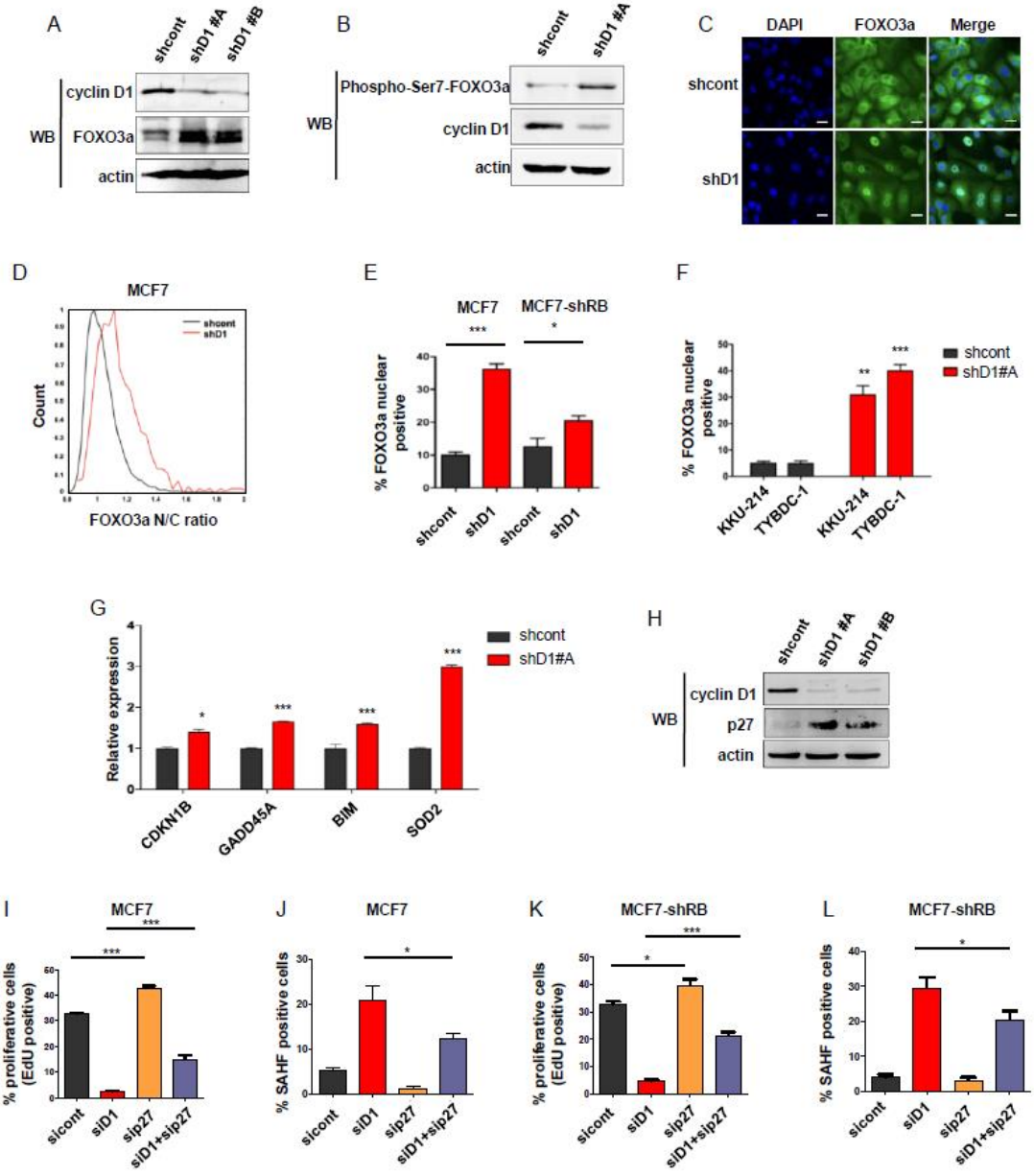

Figure 6 Elevated ROS induced hyper-functional FOXO3a.

(A) Immunoprecipitation (IP) of phospho-Ser7 FOXO3a, followed by total FOXO3a Western blotting. Isotype IgG was used as a control antibody for IP. (B) Western blot (WB) analysis of total FOXO3a in MCF7 cells expressing non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1). (C) Immunofluorescence of FOXO3a localization (green) in cyclin D1-depleted MCF7 cells. Nuclei were counterstained with DAPI (blue). Scale bars: 5 μm. (D) Histograms of FOXO3a nuclear-to-cytoplasmic (N/C) ratio showed intracellular distribution of FOXO3a in MCF7 cells expressing shcont (black) compared to shD1 (red). (E) Percentages of FOXO3a-nuclear-positive in MCF7, and MCF7-shRB cells. (F) Percentages of FOXO3a nuclear-positive KKU-214 and TYBDC-1 cells, after cyclin D1 depletion. (G) Expressions of CDKN1B (p27), GADD45A, BIM, and manganese-SOD (SOD2) gene were determined by qRT-PCR. The mRNAs were harvested after cyclin D1 depletion in MCF7 cells. Bars represent averages ± s.d. (H) WB analysis of p27 protein expressions in cyclin D1-depleted cells. (I) Analysis of proliferative cells by 5-ethynyl-2'-deoxyuridine (EdU) incorporation and (J) percentages of SAHF-positive cells in MCF7 transfected with sicont, siD1, or sip27, as indicated. Analyses of proliferative cell by 5-ethynyl-2'-deoxyuridine (EdU) incorporation (K) and percentages of SAHF-positive cells in MCF7-shRB cells (L) transfected with sicont, siD1, sip27, as indicated. Bars represent averages of three independent experiments. Statistical significance was determined by Student's t-test (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

Figure 7 Anti-senescent role of cyclin D1 in cancer cells.

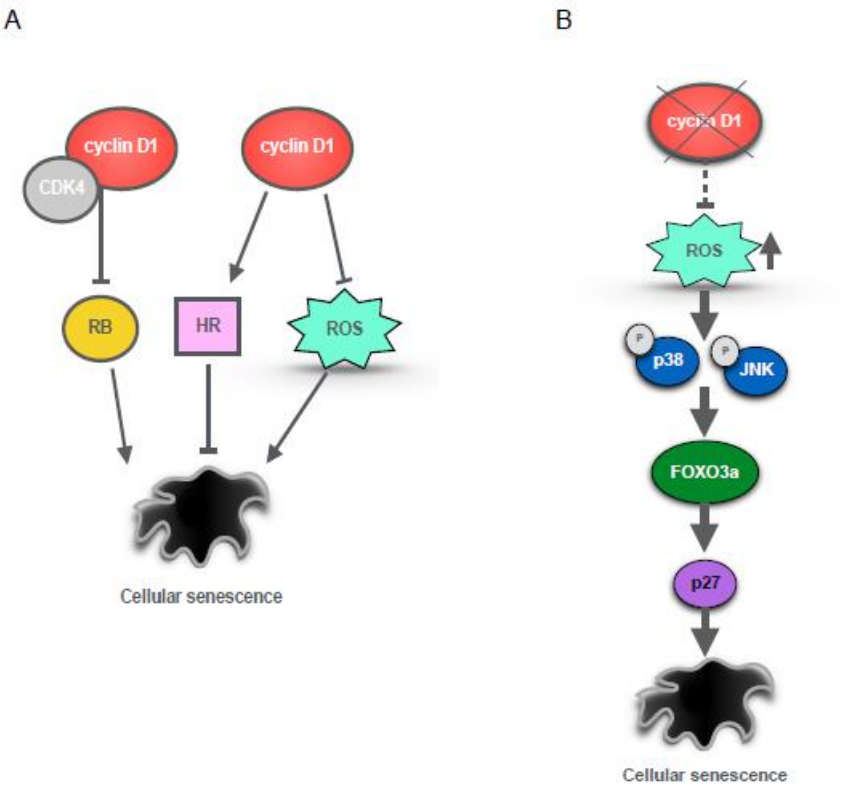

Figure 7 Anti-senescence role of cyclin D1 in cancer cells.

(A) Cyclin D1 is required for cancer cell survival and proliferation by promoting CDK4-mediated cell cycle entry. Cancer cells are known to have a high level of oxygen consumption, thus adopts vigorous oxidative stress defense mechanisms(Ward and Thompson, 2012). We showed that cyclin D1 also has additional role in maintaining the level of reactive oxygen species (ROS) at the sub-lethal level (right). (B) Depletion of cyclin D1 overwhelms cancer cells with increased level of ROS. Consequently, elevated ROS broadcasts its signal via several intracellular pathways, including p38/JNK-FOXO3a-p27, to promote cancer cell senescence.

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4. สรุปและวิจารณ์ผลการทดลอง

   This project was successfully showing a novel function of cyclin D1 as a hub for the oncogenic network. The new function appeared to be an anti-senescence function which is independent of pRB or CDK4/6 function. We found that cyclin D1 appears to suppress oxidative stress for the cancer cells, thus keeping the cancer cell in the proliferative stage. Therefore, it is a novel and unexplored function. With this discovery, we proposed using cyclin D1 as a therapeutic target for cancer treatment, i.e. modulating cyclin D1 level of teasing oxidative level in the cells is a novel approach to cancer treatment. Unfortunately, part of the project, such as the generating a large batch of the novel blocking HuScFV against cyclin D1 could not be practically performed. The protein aggregate and did not generating in the level that can be tested in animal model. We had to drop this part.

5. ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

   None

Keywords : จำนวน **3-5** คำ
(คำหลัก) cyclin D1, targeted therapy, oncogene, cancer, systems biology

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

   1. Cyclin d1 Depletion Interferes with Cancer Oxidative Balance and Sensitizes Cancer Cells to Senescence

   Phatthamon Laphanuwat1, Pornlada Likasitwatanakul1, Gunya Sittithumcharee1, Araya Thaphaengphan1, Nussara Chomanee2, Orawan Suppramote1, Nuttavadee Ketaroonrut1, Komgrid Charngkaew2, Eric W.-F Lam3, Seiji Okada4, Uraiwan Panich1, Somponnat Sampattavanich1, Siwanon Jirawatnotai1

   1Laboratory for Systems Pharmacology, Department of Pharmacology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand, 2Department of Pathology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand, 3Department of Surgery and Cancer, Imperial College London, Hammersmith Hospital Campus, London, UK, 4Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, Kumamoto, Japan

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   2. An Overview and Perspective: Non-Cell Cycle Functions of Cell 1 Cycle Regulators

      *Phatthamon Laphanuwat1, Siwanon Jirawatnotai1 **

   1Laboratory for Systems Pharmacology, Department of Pharmacology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand, 2Department of Pathology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand

   Frontier in Cell and Developmental Biology, submitted

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2. การนำผลงานวิจัยไปใช้ประโยชน์

   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
     ไม่มี
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
     ไม่มี
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
     Importance of this work was highlighted by an invitation for an oral presentation at **2017 Gordon Research Conference on Cell Growth and Proliferation; Mechanisms of Cell Division with Special Emphasis on Cancer**, Vermont, USA.
     (Only invitation based only)

The output of the project demonstrated a research capability in basic discovery for Thailand, as part of the cancer discovery exclusive circle.

- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)
  1. A publication in a highly prestigious journal such as Journal of Cell Science
  2. Proceeding; A Novel ROS-Suppressive Role of A Cell Cycle Protein, Cyclin D1 in Breast Cancer Cells, 37th Congress on Pharmacology of Thailand meeting “Genomics medicine and novel cancer therapy
  3. 7 conference presentations (7, international level)
  4. 1 Ph.D. student, and 3 medical student (on a research program) participated in this project

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

1. Oral presentation at 2017 Gordon Research Conference on Cell Growth and Proliferation; Mechanisms of Cell Division with Special Emphasis on Cancer, Vermont, USA.
2. Oral presentation: An anti-senescence role of a cell cycle protein, cyclin D1 in breast cancer cells, Conference: 2nd AMDI International Biohealth Sciences Conference 2016 (IBSC 2016) "Updates in Multidisciplinary Approach in Cancer Research and Therapy” at Advanced Medical & Dental, Institute, University Sains Malaysia, Penang, Malaysia
3. Oral presentation: A Novel ROS-Suppressive Role of A Cell Cycle Protein, Cyclin D1 in Breast Cancer Cells Conference: ASEAN Congress on Medical Biotechnology and Molecular Biosciences 2015 at Arnoma Grand Hotel, Bangkok, Thailand
4. Poster presentation at 2017 Gordon Research Conference on Cell Growth and Proliferation; Mechanisms of Cell Division with Special Emphasis on Cancer, Vermont, USA.
5. Poster presentation, the International conference in medicine and public health 2016 (SICMPH2016), May 18-19, 2016.
6. Poster presentation: A novel anti-senescence role of a cell cycle protein, cyclin D1 by reactive oxygen species suppression, Conference: 36th Pharmacological and Therapeutic Society of Thailand meeting “Advanced Pharmacology in Drug Development: Towards The ASEAN Union” at Siriraj Hospital, Bangkok, Thailand
7. Proceeding and poster presentation: A Novel ROS-Suppressive Role of A Cell Cycle Protein, Cyclin D1 in Breast Cancer Cells, Conference: 37th Congress on Pharmacology of Thailand meeting “Genomics medicine and novel cancer therapy : Challenges and Oppotunities” at Sunee Grand Hotel & Convention centre, Ubon
   Ratchathani, Thailand

ภาคผนวก 1

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Cyclin D1 depletion interferes with oxidative balance and promotes cancer cell senescence.

Laphanuwat P[1], Likasitwatanakul P[1], Sittithumcharee G[1], Thaphaengphan A[1], Chomanee N[2], Suppramote O[1], Ketaroonrut N[1], Charngkaew K[2], Lam EW[3], Okada S[4], Panich U[1], Sampattavanich S[1], **Jirawatnotai** S[5].

Author information

Abstract

Expression of cyclin D1 (*CCND1*) is required for cancer cell survival and proliferation. This is presumably due to the role of cyclin D1 in inactivation of the RB tumor suppressor. Here, we investigated the pro-survival function of cyclin D1 in a number of cancer cell lines. We found that cyclin D1 depletion facilitated cellular senescence in several cancer cell lines. Senescence triggered by cyclin D1 depletion was more extensive than that caused by the prolonged CDK4 inhibition. Intriguingly, the senescence caused by cyclin D1 depletion was independent of RB status of the cancer cell. We identified a build-up of intracellular reactive oxygen species in the cancer cells that underwent senescence upon depletion of cyclin D1 but not in those cells where CDK4 was inhibited. The higher ROS levels were responsible for the cell senescence, which was instigated by the p38-JNK-FOXO3a-p27 pathway. Therefore, expression of cyclin D1 prevents cancer cells from undergoing senescence, at least partially, by keeping the level of intracellular oxidative stress at a tolerable sub-lethal level. Depletion of cyclin D1 promotes the RB-independent pro-senescence pathway and the cancer cells then succumb to the endogenous oxidative stress levels.This article has an associated First Person interview with the first author of the paper.

KEYWORDS: CDK4; Cyclin D1; FOXO3a; Oxidative stress; Retinoblastoma protein; Senescence

Cyclin d1 Depletion Interferes with Cancer Oxidative Balance and Sensitizes Cancer Cells to Senescence

Phatthamon Laphanuwat[1], Pornlada Likasitwatanakul[1], Gunya Sittithumcharee[1], Araya Thaphaengphan[1], Nussara Chomanee[2], Orawan Suppramote[1], Nuttavadee Ketaroonrut[1], Komgrid Charngkaew[2], Eric W.-F Lam[3], Seiji Okada[4], Uraiwan Panich[1], Somponnat Sampattavanich[1], Siwanon Jirawatnotai[1]

[1] Siriraj Center of Research for Excellence (SiCORE) for Systems Pharmacology, Department of Pharmacology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand, [2]Department of Pathology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand, [3]Department of Surgery and Cancer, Imperial College London, Hammersmith Hospital Campus, London, UK, [4]Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, Kumamoto, Japan

Running title: *Cyclin D1 controls oxidative balance*

To whom correspondence should be addressed: Siwanon Jirawatnotai, Ph.D., Siriraj Center of Research for Excellence (SiCORE) for Systems Pharmacology, Department of Pharmacology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand 10700; E-mail: siwanon.jir@mahidol.ac.th

Keywords: CDK4, cyclin D1, FOXO3a, retinoblastoma protein, oxidative stress, senescence

Summary statement:

A cell cycle protein cyclin D1, independent of its CDK4/RB function, plays role as a controller for oxidative balance in cancer cell. This function is required for cancer cell survival.

ABSTRACT

Expression of cyclin D1 is required for cancer cell survival and proliferation. This is presumably due to the role of cyclin D1 in RB inactivation. Here we investigated the prosurvival function of cyclin D1 in a number of cancer cell lines. We found that cyclin D1 depletion facilitated cellular senescence in several cancer cell lines tested. Senescence triggered by cyclin D1 depletion was more extensive than that caused by the prolonged CDK4 inhibition. Intriguingly, the senescence caused by cyclin D1 depletion was independent of RB status of the cancer cell. We identified a buildup of intracellular reactive oxygen species, in the cancer cells that underwent senescence upon cyclin D1 depletion, but not in CDK4 inhibition, and that ROS buildup was responsible for the senescence. Lastly, the senescence was found to be instigated by the p38/JNK-FOXO3a-p27 pathway. Therefore, expression of cyclin D1 prevents cancer cells from undergoing senescence, at least partially, by keeping the level of intracellular oxidative stress at a tolerable sub-lethal level. Depletion of cyclin D1 promotes the RB-independent pro-senescence pathway, and cancer cell succumbing to the endogenous oxidative stress.

Introduction

Cyclin D1 is a cell cycle regulatory protein, which is amplified and overexpressed in a large number of human cancer (Sukov et al., 2009, Musgrove et al., 2011, Lee et al., 2016). Expression of cyclin D1 is essential for oncogenic transformation, as well as, for cancer cell survival. Activation of Kras or HER2 pathways targeted in mouse breast tissue resulted in breast cancer, but failed to induce any tumor in cyclin D1-deficient breast(Yu et al., 2001). In addition, shutdown of cyclin D1 expression in breast tumor resulted in tumor cessation associated with cancer cell senescence (Choi et al., 2012).

The cancer supporting role of cyclin D1 in cancer formation and survival represents a curious circumstance, in that although it is required for cancer formation, cyclin D1 does not appear to be a strong cancer driver. Forced expression of cyclin D1 in mouse model did not promote cancer formation until after a very long latency(Wang et al., 1994, Casimiro and Pestell, 2012). Thus, expression of cyclin D1 may be required to support oncogenic transformation, possibly by creating permissive cellular condition for cancer cell transformation.

The best described function of cyclin D1 is to form complexes and activate cyclin-dependent kinase 4 or 6 (CDK4/6). Cyclin D1-CDK4/6 complex phosphorylates and inactivates retinoblastoma (RB) protein which allows the cell to enter S phase from G1. Emerging evidence suggests that cyclin D1 also holds several cancer related roles beyond the canonical RB inactivation. These proposed additional roles include functions in transcriptional control, homologous-mediated DNA repair, differentiation, migration, and mitochondria regulation, etc. (Hydbring et al., 2016). It remains unclear, whether each of these non-canonical roles may have any role in cancer formation.

Cellular redox status has been shown to influence several biological processes (Trachootham et al., 2009). Oxidative equilibrium relies on a well-regulated rate of production versus elimination of oxidative species in the cell. Oxidative imbalance has been demonstrated to influence cellular behavior, and to underline several pathological conditions, including cancer (Vurusaner et al., 2012).

Herein, we investigated detail of the anti-senescence role of cyclin D1 in cancer cells.

We showed that cyclin D1 expression is essential for cancer cells to remain in the proliferative and non-senescence stage, by maintaining oxidative stress in cancer cell at a low/sub-lethal level, and hindering cancer cell succumbing to the oxidative stress-induced senescence.

Results

Cyclin D1 depletion resulted in cancer senescence in several types of human cancer

Expression of cyclin D1 was shown to be indispensable for preventing cell senescence in cancer cells *in vivo* and *in vitro* (Choi et al., 2012, Brown et al., 2012). However, it is not known, whether the anti-senescence role of cyclin D1 is universal in every cellular context, as in a number cell types and certain circumstances, cell division may take place without cyclin D1(Sherr and Roberts, 2004). Thus, we examined the role of cyclin D1 in cancer cell senescence by using cyclin D1-specific RNA interference to deplete cyclin D1 in the following human cancer cell lines: MDA-MB-175, ZR-75-1, MDA-MB-134, T47D, SKBR3, and MCF7 (breast cancer), KKU-156, KKU-214, TYBDC-1, (cholangiocarcinoma), and UMSCC2 (squamous cell carcinoma). After 4 days of cyclin D1 depletion, 6 of 10 cell lines demonstrated clear signs of cellular senescence, gauged by marked increase in the number of cells with an established senescent marker, senescence associated β-galactosidase (SA-β-Gal) expression (Fig. 1A and Fig. S1A). The senescent cells were from three tissues of origin. We noticed that these cell lines expressed varying levels of endogenous cyclin D1 (Fig. S1B). Therefore, the anti-senescence function of cyclin D1 was not ubiquitous among all of the cancer cell lines, and this role was not correlated with the pre-existing level of expression of cyclin D1 in each cancer cell line.

We carefully verified the senescence induced by cyclin D1 depletion by examining the characteristics of these cells. Depletions of cyclin D1 by two independent cyclin D1-specific short-hairpin RNAs (shRNAs) produced the same outcomes. They inhibited cancer cell division. Cyclin D1-depleted cells remained non-proliferative after 5 days in culture (Fig. 1B, C), and did not re-enter into cell cycle after long-term culture (Fig. 1D). Cyclin D1-depleted cells appeared flatten with large cytoplasm (Fig. 1E), and they were positive for SA-β-Gal staining (Fig. 1F, G). Cyclin D1-depleted cells also showed significant increase in senescence-associated heterochromatin foci (SAHF), another putative marker for cellular senescence(Narita et al., 2003) (Fig. 1H, I). Lastly, expression of *CDKN2A* mRNA was upregulated(Rayess et al., 2012), and *LMNB1* mRNA was down-regulated(Freund et al., 2012)(Fig. 1J), confirming that these cells underwent cellular senescence after cyclin D1 depletion. These results indicate that cyclin D1 depletion facilitated cancer cell to undergo authentic cellular senescence. In

addition, we found that re-expressions of cyclin D1 neither restored cell proliferation, nor reduced SAHF in the cyclin D1-depleted cells (Fig. S1C-E), indicated that, once triggered, the senescence was irreversible. To understand the nature of the senescence observed in cyclin D1-depleted cells, we studied the timing when the senescence was initiated. We found that a significant number of SAHF-positive cell was detected at 48 hours after transfection of cyclin D1-specific small interfering RNA (siD1), which correlated with the depletion of cyclin D1 protein (Fig. 2 A, B). We also observed a moderate increase of cancer cell apoptosis upon cyclin D1 depletion. The increased apoptosis was not observed in control cells, or in cancer cells treated with PD0332991, a specific CDK4/6 inhibitor (Fig. S2A). Apoptosis associated with cyclin D1 depletion might be a result of DNA damage, since cyclin D1 depletion-induced senescent cells contained increased number of a DNA damage marker γH2AX, at the level comparable to 50-100 μM H_2O_2 treatments (Fig. S2B).

Previous study demonstrated that prolonged inhibition of CDK4 activity by CDK4/6 inhibitor also resulted in cancer cell senescence (Otto and Sicinski, 2017). We treated MCF7 cells with PD0332991 and observed that CDK4/6 inhibition also resulted in growth arrest, and senescence (Fig. S3A, B, respectively). However, prolonged inhibition caused a delayed type of senescence that became significant only at 96 hours after the 0.5 μM PD0332991 treatment. In agreement with that, suppression of CDK4 activity by CDK4-specific siRNA also resulted in the delayed type of senescence (Fig. S3C, D). These results implicated that depletion of cyclin D1 maybe a more robust trigger for cancer cell senescence, than inhibition of CDK4/6 kinase activity. Finally, we showed that expression of the kinase-dead version of cyclin D1 (K112E) significantly reduced senescence in cyclin D1-depleted cells under cyclin D1 depletion condition (Fig. S3E, F), therefore, CDK4 activity was not involved with the early-onset senescence observed in cyclin D1-depleted cells.

Anti-senescence role of cyclin D1 is independent of its RB-inactivation function

In order to investigate whether the senescence observed in cyclin D1-depleted cancer cells was mediated by RB, we established a RB-deficient MCF7 cell line by stably expressing RB-specific shRNA (Fig. 3A). We verified that these RB-deficient cells were oblivious to an inhibition of CDK4 kinase activity by treating the cells with PD0332991. MCF7-shRB cells continued to proliferate under treatment, indicating that without RB, cyclin D1-CDK4 kinase was no longer required for these cells to proliferate (Fig. 3B). Consistent with the preceding, MCF7-shRB cells did not undergo senescence under a prolonged 0.5 μM PD0332991 treatment (Fig. 3C, and Fig. S3G).

Interestingly, we found that cyclin D1 depletion effectively inhibited MCF7-shRB cell proliferation (Fig. 3A, D), and triggered extensive cellular senescence in these cells (Fig. 3E-I). We also confirmed that cyclin D1 depletion was able to inhibit cell proliferation and to promote senescence in another RB-deficient cancer cell line TYBDC-1-shRB (Fig. 3J-L). Thus, the observation was not specific to only MCF7 cells.

We also established a pocket protein-deficient MCF7 cell line (MCF7-E7), in which all pocket proteins (RB, p107, p130) were destabilized by an expression of HPV16 E7(Helt and Galloway, 2001). We verified that MCF7-E7 cells were unresponsive to CDK4 inhibition and did not submit to senescence upon 5 day PD0332991 treatment (Fig. 3M, N). We found that cyclin D1 depletion effectively inhibited MCF7-E7 cell proliferation (Fig. 3O, P), and promoted widespread senescence in these pocket protein-deficient cells (Fig. 3Q).

Therefore, inhibition of CDK4 resulted in RB-mediated cellular senescence. On the other hand, inhibition of cyclin D1 resulted in cellular senescence was RB-independent.

Elevated ROS level in cyclin D1-depleted cells triggered cancer cell senescence

Senescence could be triggered by various cellular stressors, including oxidative stress(Salama et al., 2014). To elucidate the cause of senescence associated with cyclin D1 depletion, we examined levels of reactive oxygen species (ROS) between the responders (cell lines that responded to cyclin D1 depletion by undergoing senescence: KKU-156, TYBDC-1, SKBR3, MCF7, KKU-214, UMSCC2), and the non-responders (cell lines that did not undergo senescence: MDA-MB-175, MDA-MB-134, T47D, ZR-75-1). Interestingly, there was a significant increase in endogenous ROS in all responder cell lines after cyclin D1 depletion, whereas no change in level of endogenous ROS in non-responder cell lines (Mann-Whitney U test, $p = 0.0095$) (Fig. 4A, left panel versus right panel). We noticed that the upregulation of ROS, and the senescence response were not related to average basal levels of ROS in each cell line (Fig. 4A, shconts).

The upregulation of ROS was detected at approximately 48 hours after cyclin D1 siRNA transfection, coinciding with cyclin D1 depletion, and it intensified by the 72 hours time point (Fig. 4B, C). Interestingly, PD0332991 treatment, although resulting in cell cycle arrest and senescence, did not cause ROS elevation (Fig. S3A, B, and Figure 4B).

To investigate, whether the elevated ROS was responsible for the senescence phenotypes, first, we promoted an accumulation of ROS by treating MCF7 cells with H_2O_2. Many of MCF7 cells responded to H_2O_2 treatment by undergoing senescence, suggested that senescence is a primary response to oxidative stress in these cells (Fig. S4A).

Next, we treated cyclin D1-depleted cells with an anti-oxidant *N*-acetylcysteine (NAC) to suppress intracellular ROS. We found that NAC treatment significantly reduced the number of cells that underwent senescence, gauged by SA-β-Gal and SAHF staining (Fig. 4D-F). NAC treatment did not reduce delayed senescence mediated by PD0332991 treatment, indicating that ROS played a limited role in the PD0332991-mediated senescence (Fig. S4B).

Of note, although NAC effectively suppressed senescence caused by cyclin D1 depletion, it was unable to entirely abolish the senescence (Fig. 4E, F). This suggested that senescence in cyclin D1-depleted cells may also be influenced by another cause other than ROS. We and others have previously reported that cyclin D1 participates in homologous recombinant-mediated DNA repair (HR), and cyclin D1

depletion reduces the efficiency of HR (Li et al., 2010, Jirawatnotai et al., 2011). Reduced efficiency of HR may also contribute to cancer cell senescence observed in the cyclin D1-depleted cells. To this end, we employed a model cell line Capan-1, in which the HR is not functioning, because of a protein-truncating mutation on BRCA2 gene (Yuan et al., 1999). Therefore, in this cell line, we would be able to measure the anti-oxidative stress function of cyclin D1, without interference from the HR-related role of cyclin D1. We depleted cyclin D1 from Capan-1 and found that, cyclin D1 depletion resulted in increased senescence (Fig. S4C, D). This result implicated an existing of an HR-independent anti-senescence role of cyclin D1. Then, we treated the cyclin D1-depleted cells with NAC and found that, NAC treatments were able to abolish senescence in cyclin D1-depleted Capan-1 to the level that is comparable to that in the control Capan-1 cells (Fig. S4C, D). Therefore, we believed that in the cells with normal HR, cyclin D1 plays non-cell cycle roles to protect cell from senescence by facilitating efficient HR repair, and by keeping level of intracellular oxidative stress at a tolerable sub-lethal level.

Cyclin D1 was shown to negatively modulate the number of mitochondria, an important endogenous source of ROS (Sakamaki et al., 2006, Wang et al., 2006, Tchakarska et al., 2011). Consistent with previous reports, we found that cyclin D1-depleted cells exhibited an increased number of total and functioning mitochondria, as measured by transmission electron microscopy (TEM), expression of total mitochondria-specific DNA (*mtND5*), and MitoTracker staining (Fig. S4E-I).

We also measured levels of mitochondrial superoxide using a specific fluoroprobe MitoSOX, and detected an upregulation of the mitochondrial superoxide in cyclin D1-depleted cells, but not in the cells treated with PD0332991 (Fig. S4J, K). An increase in total and functioning mitochondria was also found in RB-deficient cells, suggesting that the increase in ROS was independent of RB (Fig. S4L-P). These evidences collectively supported that, cyclin D1 depletion-promoted cancer cell senescence is mediated by the ROS build-up in the cancer cells, which is possibly a consequence of the increased number of functioning mitochondria number in the cells.

High level of endogenous ROS activated p38/FOXO3a/p27 pathway in cyclin D1-depleted cancer cells

To investigate the consequences of elevated ROS in cyclin D1-depleted cells, we studied activation of p38 and JNK, the MAP kinases involved in oxidative stress response(Ho et al., 2012, Essers et al., 2004). We detected upregulation of phospho-p38 MAPK (Thr180/Tyr182) and JNK-mediated c-JUN phosphorylation at Ser73 in cyclin D1-depleted cells around 48-72 hours after cyclin D1 depletion, but not in control cells (Fig. 5A, Fig. S5A). We did not detect activation of AKT in cyclin D1-depleted cell (Fig. S5B). Activations of p38 and JNK were abolished, when cyclin D1-depleted cells were treated with NAC (Fig. 5B, Fig. S5C). FOXO3a is a transcription factor that activates genes in response to oxidative stress in order to mediate oxidative detoxification, cell cycle arrest, and apoptosis. When it senses cellular stress, FOXO3a relocalizes to the nucleus and binds to promoters of target genes such as, *SOD2*, and catalase (Kops et al., 2002, Bartell et al., 2014). In case that the stress level is

overwhelming, FOXO3a can activate genes that promote senescence or apoptosis, such as *CDKN1B* (encoded for a cell cycle inhibitor p27), or *GADD45A* and *BIM*(Lam et al., 2013).

We detected an upregulation of FOXO3a protein in association with increased FOXO3a Ser7 phosphorylation, which is involved in the stress-induced activation of FOXO3a by p38(Ho et al., 2012) (Fig. 6A, B), and a translocalization of FOXO3a from cytoplasm to nucleus, after cyclin D1 depletion (Fig. 6C-E). This translocalization of FOXO3a was abolished by NAC treatment (Fig. S5D), indicated that the translocalization was involved with ROS. We detected recruitments of endogenous FOXO3a to several downstream promoters, such as *CDKN1B*, *SOD2*, *GADD45a*, *PrxIII*, and *BCL10*, by FOXO3a chromatin immunoprecipitation (Fig. S6), in cyclin D1-depleted cells. Activation of FOXO3a was also detected when cyclin D1 was depleted in RB-deficient MCF7 cells (Fig. 6E). We also confirmed the activation of FOXO3a by cyclin D1 depletion in cell lines TYBDC-1, and KKU-214 (Fig. 6F). Lastly, we detected upregulations of several genes known to be regulated by FOXO3a (Fig. 6G). Therefore, cyclin D1 depletion was sufficient to fully activate FOXO3a. All together these results confirmed an activation of FOXO3a induced by ROS in cyclin D1-depleted cells.

To elucidate the cause of RB-independent cellular senescence in cyclin D1-depleted cells, we focused on upregulated p27 (encoded by *CDKN1B)*, since it is a gene downstream of FOXO3a known to regulate cellular senescence through multiple mechanisms, including RB-independent mechanisms(Majumder et al., 2008, Young et al., 2008). We found that p27 protein level was also upregulated in cyclin D1-depleted cells (Fig. 6H), while GADD45a and SOD2 were not upregulated at the protein level (Fig. S7A). The upregulation of p27 coincided with p38 and c-JUN phosphorylations (Fig. S7B). In addition, we detected increased binding between p27 and CDK2 in cyclin D1-depleted cells, by CDK2 immunoprecipitation, implicating an increased p27 function (Fig. S7C). We measured senescence levels in MCF7 and MCF7-shRB cells co-transfected with siRNAs against cyclin D1 and p27. We found that p27 siRNA significantly increased the numbers of EdU-positive cells, and reduced the number of SAHF-positive cells in both cyclin D1-depleted MCF7 and MCF7-shRB cells, indicating that p27 depletion prevented the cell cycle arrest and senescence observed in cyclin D1-depleted cells (Fig. 6I-L). Therefore, p27 mediated, at least partially, the cell cycle arrest and cellular senescence found in cyclin D1 depleted cells.

Discussion

Overexpression of cyclin D1 is commonly found in many types of cancer. However, how precisely overexpressed cyclin D1 contributes to cancer formation is not entirely known. In this study, we investigated a novel anti-senescence role of cyclin D1 in human cancer. We found that cyclin D1 expression maintains endogenous ROS at the sub-lethal level, likely by inhibiting mitochondria number and function. In the absence of cyclin D1, cancer cells accumulate intolerable levels of endogenous ROS, which subsequently triggers cancer cell senescence and possibly cell death. The senescence triggered by cyclin D1 depletion appeared to be independent of cell cycle regulatory function of cyclin

D1, since the senescence induced by cyclin D1 depletion fully occurred in RB-deficient cells. In addition, ROS-induced senescence was specific to cyclin D1 depletion, and not observed in cells treated with a CDK4/6-specific inhibitor. We, therefore, conclude that cyclin D1 has a CDK4-, RB-independent role in keeping oxidative balance for the cancer cells. This novel function, along other cancer-promoting functions such as function in HR, make cyclin D1 as a multifunctional oncoprotein that facilitates overall cancer cell survival (Fig. 7A).

We elucidated that the senescence was mediated by the p38/JNK-FOXO3a-p27 pathway, which was activated by elevated ROS after cyclin D1 depletion (Fig. 7B). FOXO3a is a tumor suppressor, which facilitates apoptosis and senescence upon stress (Nestal de Moraes et al., 2016). It was shown that FOXO3a functions at both transcriptional and post-transcriptional levels to promote p27 expression(Lam et al., 2013, Wu et al., 2013). p27 is a CDK2 inhibitor, capable of blocking non-RB processes of cell division controlled by CDK2 (Chae et al., 2004, Luscher-Firzlaff et al., 2006, Voit et al., 1999). We found that higher amount of p27 was co-precipitated with CDK2, in cyclin D1-depleted cells, promoting a downregulation of CDK2 activity. This finding supported that p27 may be a key regulator for the senescence in cyclin D1-depleted cells. We have also examined expressions of GADD45A and SOD2, downstream genes of FOXO3a, which also known to be involved in senescence. Although, in cyclin D1-depleted cells, FOXO3a localized at the promoters of *GADD45A* and *SOD2* genes (Fig. S6), and the mRNAs were upregulated (Fig. 6G), we found that GADD45A and SOD were not upregulated at the protein level (both in RB-negative and RB-positive cells. From these results, we believe, that role of GADD45A and SOD2 might be limited in this context. Although, we found that depletion of p27 significantly suppressed senescence, it is possible that there are other FOXO3a target genes that also contribute to the senescence induced by cyclin D1 depletion.

Previous studies demonstrated that cyclin D1 represses mitochondria number and that cyclin D1 functions by two possible mechanisms. First, cyclin D1 and CDK4 phosphorylates and represses nuclear respiratory factor-1 (NRF-1), a transcription factor that induces nuclear-encoded mitochondrial genes(Wang et al., 2006). Second, cyclin D1, independent of CDK4, competes with hexokinase 2 to bind to a voltage-dependent anion channel and inhibit mitochondrial function (Tchakarska et al., 2011). However, biological role of these findings in disease such as cancer is not entirely clear. Our results demonstrated that, in addition to role of cyclin D1 in cell cycle control, the new role of cyclin D1 in maintaining oxidative balance is critical for RB-positive and RB-negative cancer cell to survive.

It remains to be addressed why some cancer cells managed to avoid ROS accumulation, thus escaped senescence, upon cyclin D1 depletion. These cells may rely on different type of redox modulations to cope with the consequence of excessive ROS.

Controls of cell cycle and cellular energy level were shown to be coordinated. Disruption of energy balance may disrupt normal cell cycle, and *vice versa* (Salazar-Roa and Malumbres, 2017). Thus, it may be sensible when a cell cycle machinery such as cyclin D1 also plays a dual role in controlling energy and oxidative balance during cell cycle. Cancer cells do not invent new pathways for survival,

but instead using pre-existing machineries to operate. Consequently, they may become reliance on the adaptation. Our findings offered a view of cyclin D1 as a regulator that controls the cancerous oxidative balance, and describe the consequences when the balance is altered by cyclin D1 depletion. The role of cyclin D1 in other contexts and setting requires further investigation.

Altered redox status has long been observed in cancer cells**.** Recent study suggests that this adopted property of cancer cells can be exploited for therapeutic benefits(Trachootham et al., 2009). For the therapeutic use, inhibition of cyclin D1 may make cancer cells hypersensitive to ROS-modulating agents.

Materials and methods

Cell culture and treatment

The MDA-MB-175, ZR-75-1, MDA-MB-134, T47D, SKBR3, and MCF7 cell lines were from ATCC (Manassas, VA, USA). UMSCC2 was from University of Michigan cell bank. KKU-156, KKU-214, and TYBDC-1 cell lines were from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). Cell line authentication were done by Short Tandem Repeat (STR) profiling at the cell line banks indicated. The MDA-MB-175, ZR-75-1, and T47D were maintained in RPMI1640; SKBR3 in McCoy′s 5A Medium; MDA-MB-134 in L-15; and MCF7, UMSCC2, KKU-156, KKU-214, and TYBDC-1 in DMEM, supplemented with 10% FBS, and 100 U/ml penicillin/streptomycin under standard conditions. shRNA expressing cells were maintained in selection mediums with one of the antibiotics, puromycin, G418 , or hygromycin (Thermo Fisher Scientific, MA, USA). NAC was from Amresco (Solon, OH, USA). PD0332991 was a gift from Dr. P. Sicinski, Dana Farber Cancer Institute (DFCI), Boston MA, USA. Carboxy-H2DCFDA was from Sigma-Aldrich (St. Louis, MO, USA (D6883)).

Plasmids and siRNA

The shD1- pLKO.1#A(5’-GCCAGGATGATAAGTTCCTTT-3’), #B (5’-ATTGGAATAGCTTCTGGAAT-3’), and a control non-targeting shRNA (5’-CAACAAGATGAAGAGCACCAA-3’) (each contains puromycin resistance gene) were from Sigma-Aldrich. The cyclin D1-shRNA (pBabe-neo) #C (5’-CCACAGATGTGAAGTTCATTT-3’) (contains neomycin (geneticin) resistance gene), was a gift from Dr. E. Sicinska, DFCI. The pGIPZ *RB1*-shRNA (5’- CGCAGTTCGATATCTACTGAAA-3’) was provided by Dr. Sunkyu Kim, Novartis Institutes for Biomedical Research. pBabe-hygro-E7 was provided by Dr. D.A. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA, USA. pRc/CMV-cyclin D1 K112E-HA was a gift from Philip Hinds (Addgene plasmid #8952). The pcDNA plasmid was purchased from Invitrogen. Cyclin D1-specific siRNAs (SI02654547), CDK4 (SI00604744), and control siRNA (1027310) were from Qiagen (Valencia, CA, USA). Cyclin D1-UTR targeted siRNA used in K112E experiment (#s229) was from

Thermo Fisher Scientific (MA, USA). The p27 siRNA (sc-29429) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cyclin D1 re-expression in senescent cells, and expression of K112E cyclin D1

The inducible system (Takara Bio Inc., Mountain view, CA, USA), composed of pEF1α-Tet3G and pTRE3G-cyclin D1-HA plasmids was co-transfected with GFP expressing plasmid peGFP (a gift from Dr. P. Sicinski, DFCI) into MCF7 cells, followed by cyclin D1 depletion using cyclin D1-specific shRNA targeting 5'UTR of cyclin D1 mRNA (shD1#A). Expression of cyclin D1-HA was achieved by addition of 1 μM doxycycline. Analyses of SAHF, and EdU-positive cells were performed only in the cells expressing cyclin D1-HA (GFP expressing cells) by immunofluorescence, at 48 hours after doxycycline induction. The pRc/CMV-K112E-cyclin D1 was transiently co-transfected with peGFP to MCF7 cells for 24 hour, then the cells were transfected with non-targeting siRNA or cyclin D1-UTR targeted siRNA. Seventy two hour after siRNA transfection, cells were fixed and GFP expressing cells were analyzed for SAHF.

Detection of DNA damage

Cells were transduced with non-targeted shRNA control or cyclin D1-specific shRNA. After 4 day, γH2AX foci were analyzed as described (Jirawatnotai et al., 2011)

Growth curve and colony-forming assay

For growth curves, cells were seeded into 24-well plates at a density of 5,000 cells/well. Cell numbers were counted at indicated time points. For colony forming assay, 200 cells/well were seeded into 6-well plate. After 14 days, colonies were fixed with 10% neutral buffered formalin for 30 minutes and stained with 0.5% crystal violet solution for visualization.

SA-β-gal assay

SA-β-gal assay was performed as previously described (Debacq-Chainiaux et al., 2009). Percentage of SA-β-gal-positive cells was calculated from at least 500 cells.

Semi-quantitative real-time PCR

Total RNA was extracted using an RNA extraction Kit (Thermo Fisher Scientific). Complementary DNA were generated by Superscript III rev transcriptase and oligo-dT primers (Thermo Fisher Scientific). Transcript levels were normalized by expression of *GAPDH*. The mitochondrial DNA (mtDNA), *mtND5* was analyzed against *HBB*. All primers used in this experiment were synthesized by bioDesign, Bangkok, Thailand. The primers used were: p16-for: 5'-CCGAATAGTTACGGTCGGAGG-3', p16-rev: 5'-CACCAGCGTGTCCAGGAAG-3', *LMNB1*-for: 5'-AGCGGAAGAGGGTTGATGTG-3', *LMNB1*-rev: 5'-CCAGCCTCCCATTGGTTGAT-3', *mtND5*-for: 5'-GCCTAGCATTAGCAGGAATAC-3', *mtND5*-rev: 5'-GGGGAAGCGAGGTTGACCTG-3', *HBB*-for: 5'-GCTTCTGACACAACTGTGTTCACTAGC-3', *HBB*-rev: 5'-CACCAACTTCATCCACGTTCACC-3', *GADD45A*-for: 5'-TCCTGCTCTTGGAGACCGA-3', *GADD45A*-rev: 5'-ATCCATGTAGCGACTTTCCCG-3', *BIM*-for: 5'- CAAGAGTTGCGGCGTATTGG-3'

, *BIM*-rev: 5'- TGTCTGCATGGTATCTCGGC-3', *SOD2*-for: 5'-TGGCCAAGGGAGATGTTACAG-3', *SOD2*-rev: 5'-CTTCCAGCAACTCCCCTTTG-3', *p27*-for: 5'- TCTGAGGACACGCATTTGGT-3', *p27*-rev: 5'- ACAGAACCGGCATTTGGGG-3', *GAPDH*-for: 5'-CCTCCAAAATCAAGTGGGGCGATG-3', *GAPDH*-rev: 5'-CGAACATGGGGGCATCAGCAGA-3'

Apoptosis detection

Cells were analyzed for apoptosis using an Annexin-V Apoptosis Detection Kit according to manufacturer's instruction (Thermo Fisher Scientific,). Forty eight hours after siRNA transfection, 10,000 cells from each condition were analyzed by flow cytometry (CytoFLEX, Beckman Coulter, Brea, CA, USA)

ROS measurement

ROS measurement was performed in 96-well plates. Cells were seeded at a density of 8×10^3 cells/well. At the detection time, cells were washed twice with PBS, and carboxy-DCFDA dye in serum free media was added at a final concentration of 5 µM. Plates were incubated at 37°C for 30 minutes, before removal of the dye. Cells were then washed twice with PBS, stained with nuclear staining dye and immediately analyzed by high content imaging system (PerkinElmer, Bangkok, Thailand). Results are presented as a median fluorescent intensity. For mitochondrial-specific superoxide detection, cells were transduced with either non-targeting shRNA (shcont), or cyclin D1-specific shRNA. After 4 day, cells were incubated with MitoSOX Red (Thermo Fisher Scientific), fixed and counterstained with DAPI, according to the manufacturer's instruction. Mean integrated fluorescence intensities were analyzed by high content imaging system.

Western blotting and immunoprecipitation

Western blotting and immunoprecipitation were performed as previously described (Jirawatnotai et al., 2011) using specific antibodies.

Immunofluorescence staining

Cells were seeded in microplates (#3904) (Corning, Corning, NY, USA), and fixed in 4% paraformaldehyde, permeabilized in methanol, and blocked in Odyssey® Blocking Buffer (PBS) (LI-COR, Lincoln, NE, USA). Cells were then incubated with specific primary antibody overnight, followed by 1:1000 dilution fluorescent-conjugated secondary antibody prior to nuclear staining with DAPI. For detection of cells in S-phase, cells were pulse with EdU (5-ethynyl-2-deoxyuridine) for 90 mins, fixed and stained by Click-iT reaction according to the manufacturer's protocol (C10337) (Thermo Fisher Scientific). Samples were evaluated by the high content imaging system.

Mitochondria staining

Cells were stained with 250 nmol/L MitoTracker® Red CMXRos (#9082) (Cell Signaling, Danvers, MA, USA) and DAPI, before subjected to the high content imaging system.

Chromatin Immunoprecipitation (ChIP)

MCF7 cells were transduced with either non-targeting control shRNA (shcont) or cyclin D1-specific shRNA (shD1) for 4 days. Chromatin immunoprecipitation was performed as described earlier (Jirawatnotai et al., 2011). Briefly, samples were immunoprecipitated overnight at 4 °C with anti-FOXO3a antibody ChIP-grade. The immune complexes were captured using protein Protein G beads (Santa cruz). After a series of washing steps, the beads were extracted in 120 µl of elution buffer (0.1 M $NaHCO_3$, 1% SDS) and analyzed by qPCR. Primers used for PCR amplification (5′ to 3′) were as follows: p27 F, CAACCAATGGATCTCCTCCT, p27 R, GCCTCTCTCGCACTCTCAAA, BCL10 F, AAGCTGCAGTGAGCCGAGAA, BCL10 R, TAGGGCTGCCGGGATCATA, PrxIII F, CGGACTAAAACTGCATTTGTAATTA, PrxIII R, CACGTGGTTTTCCTACTGTC, MnSOD F, GTTCCTCTTCGCCTGACTGTT, MnSOD R, CTGAACCGTTTCCGTTGCTT, GADD45A F, CACTCTCGGGACTTCTCACG, GADD45A R, GCACCTGGGCTCTACGAAAA

Antibodies

Rabbit Anti-cyclin D1 (sc-753), mouse anti-actin (sc-47778), mouse anti-HSP90 (sc-13119), mouse anti-total p38 (sc-535), mouse anti-total c-JUN (sc-74543), mouse anti-GADD45 (sc-6850), and mouse anti-Cdk2 (sc-6248) were purchased from Santa Cruz Biotechnology. Rabbit anti-FOXO3a (07-702), rabbit anti-trimethyl-histoneH3 (Lys9); H3K9 (07-442), mouse anti-γH2AX Ser139 (05-636), and mouse anti-SOD2 (MAB4081) were purchased from Merck Millipore (Bangkok, Thailand). Rabbit anti-FOXO3a (#2497), mouse anti-Rb (#9309), rabbit anti-phospho-p38 (Thr180/Tyr182, #4511), rabbit anti-phospho-c-JUN (Ser73, #3270), rabbit anti-phospho-AKT (Ser473, #4060), rabbit anti-phospho-FOXO3a (Ser7, #14724S) were from Cell Signaling. The rabbit anti-FOXO3a ChIP-grade (ab12162) was from Abcam, UK. The antibody was used in dilution 1:500 for immunoprecipitation. All antibodies were used in dilution 1:1000 for Western blotting, 1:300 for immunofluorescence staining.

Transmission electron microscopy (TEM)

Sample preparation and analyses were performed according to a standard method as previously described (Parameyong et al., 2013).

Image processing and analysis

All images from the high content imaging system were analyzed using Columbus™ Image Data Storage and Analysis System (PerkinElmer), CellProfiler 2.1.1 and MATLAB R2015b. ImageStudio® program was used for quantification of Western blot signals.

Statistical analysis

The Mann-Whitney U test was used to evaluate significance between the median of control (shcont) and the cyclin D1 knockdown groups (shD1) in ROS measurement analysis and to compare ROS changes in the non-responder versus the responder group, deltas of ROS changes from all cell lines in each group were calculated for medians. Comparison between group of cell numbers in TYBDC-1 experiment was performed using Two-way ANOVA. Comparison between two groups was performed

using two-tailed Student's t-test, except when another method was specified. Experiments were performed in biological triplicates. The positive SA-β-Gal cells were counted from at least 500 cells. The positive SAHF and proliferative cells were counted from at least 3,000 cells. The number of sample in other experiments were specified. Bars represent the mean ± s.d. The level of statistical significance was determined by *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

Footnotes

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Competing interests

The authors declare no competing or financial interests.

Author contributions

P.La., P.Li., G.S., A.T., N.C., N.K.; performed experiments and analyzed results. O.S., E.W.F.L., S.O.; provided unique reagents, construct, or cell lines. K.C., E.W.F.L., S.O., U.P., S.S., S.J.; provided expert advice and helps with data analysis. P.La. and S.J.; prepared manuscripts. S.J.; directed the project. All authors read and approved the manuscript.

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Figure 1. Depletion of cyclin D1 caused cellular senescence in cancer cell lines

(A) Cancer cell lines were transduced with lentivirus expressing non-targeting shRNA (shcont), or shRNA against cyclin D1 (shD1#A) and SA-β-Gal-positive cells were counted on day 4 after the shRNA transductions. Bars show averaged relative numbers ± s.d. of SA-β-Gal-positive cells (n = 500). **(B)** Western blot (WB) analysis of cyclin D1 expressions by 2 independent shRNAs (shD1#A, and shD1#B). Heat shock protein 90 (HSP90) was used as a loading control. **(C)** Growth curves of cyclin D1-depleted MCF7 cells (shD1#A and shD1#B). **(D)** Colony-forming assay of cyclin D1-depleted MCF7 cells. **(E)** Cellular morphology and (**F)** SA-β-Gal expression of cyclin D1-depleted MCF7 cells at day 4 after cyclin D1 depletion. Positive cells appear in greenish-blue. **(G)** Percentages of SA-β-Gal-positive cells from **(F)**. Bars represent average numbers of SA-β-Gal-positive cells ± s.d. (**H)** Immunofluorescence staining (IF) of SAHF in cyclin D1-depleted cells. The results showed trimethyl-histoneH3 at Lys9 (H3K9) foci (bright foci) in cyclin D1-depleted MCF7 cells. Cells with more than 5 foci/ nucleus are considered positive. **(I)** Percentages of SAHF-positive cell from **(H)**. **(J)** Upregulations of *CDKN2A* (p16) and downregulations of *LMNB1* (lamin B) in cyclin D1-depleted cells, as measured by qRT-PCR. Bars represent the averages of 3 independent experiments ± s.d. Statistical significance was determined by Student's t-test *(*p ≤0.05, **p ≤0.01 and ***p ≤0.001).*

Figure 2 Association between cyclin D1 downregulation and senescence.

(A) Cellular senescence formations in cyclin D1-depleted cancer cells at various time points. MCF7 cells were transfected with either control non-targeting siRNA (sicont) or cyclin D1-specific siRNA (siD1), and SAHF-positive cell numbers were determined at indicated time points. Bars represent percentages of SAHF-positive cells. **(B)** Expressions of cyclin D1 after siRNA-mediated depletion. MCF7 cells were transfected with either sicont or siD1. Protein lysates were harvested at indicated time points after siRNA transfection. Western blot (WB) analysis showed cyclin D1 depletion efficiency. Actin was used as a loading control. Statistical significance was determined by Student's t-test *(*$p \leq 0.05$*).*

Figure 3 Cyclin D1 depletion induced senescence is independent of RB status. **(A)** Western blot (WB) analysis of cyclin D1 and retinoblastoma protein (RB) in MCF7 cells. Actin was used as a loading control. **(B)** Growth curves of MCF7 cells expressing RB-specific shRNA (MCF7-shRB) treated with indicated concentrations of PD0332991 (0.1, 0.5, or 1 μM) or vehicle (DMSO). **(C)** SA-β-Gal expression in MCF7-shRB cells was determined following a 5-day treatment of 0.5 μM PD0332991. MCF7 cells were used as a senescence control. (**D)** Growth curves of RB-deficient MCF7 cells expressing shcont, or cyclin D1-specific shRNA (shD1#C). **(E)** Cellular morphology and **(F)** SA-β-Gal expressions of MCF7-shRB cells with non-targeting (shcont) or shD1. (**G)** Percentages ± s.d. of SA-β-Gal-positive cells from **(F)**. **(H)** Immunofluorescence of SAHF in cyclin D1-depleted MCF7-shRB cells. **(I)** Percentages of SAHF-positive cells from **(H)**. **(J)** Growth curves of TYBDC-1 expressing non-targeting shRNA control (shcont; grey), RB-specific shRNA (shRB; green), cyclin D1-specific shRNA (shD1#C; red), and shRB+shD1 (purple). Statistical significance was determined by two-way ANOVA (**p≤0.01; shcont VS shD1 and *** p≤0.001; shRB VS shRB+shD1).* **(K)** WB analysis of cyclin D1 and retinoblastoma protein (RB) in RB-deficient TYBDC-1 cells expressing shcont, or shD1. **(L)** Percentages ± s.d. of SA-β-Gal-positive cells in RB-deficient TYBDC-1 cells expressing shcont, or shD1. (**M)** SA-β-Gal expressions in DMSO and PD0332991 (PD) treated E7-expressing MCF7 cells (MCF7-E7) at day 5 after treatment. **(N)** Percentages of SA-β-Gal-positive cells were determined after MCF7-E7 was transduced with the indicated shRNAs (shcont or shD1), or treated with DMSO or PD0332991. **(O)** Growth curves of MCF7-E7 cells expressing either shcont or shD1 (shD1#C). **(P)** Colony forming assay of MCF7-E7 cells expressing shcont or shD1 (shD1#C). (**Q)** SA-β-Gal expressions in MCF7-E7 expressing shcont or shD1 cells.

Bars represent averages of three independent experiments. Statistical significance was determined by Student's t-test *(*p≤0.05, **p≤0.01 and ***p≤0.001).*

Figure 4 Reactive oxygen species (ROS) was upregulated in cyclin D1-depleted cancer cells and was responsible for senescence.

(A) Cancer cells were transduced with either lentivirus expressing non-targeting shRNA (shcont) or cyclin D1-specific shRNA (shD1). After 4 days ROS levels were measured using carboxy-DCFDA. Box plots show distributions of ROS in the indicated cell lines. Statistical significance was determined by Mann-Whitney U test. **(B)** ROS levels measured in MCF7 cells transfected with non-targeting siRNA (sicont) or cyclin D1 siRNA (siD1), or treated with vehicle (DMSO) or PD0332991 (PD) at indicated time points (24, 48, and 72 hours). Bars represent percentages of DCFDA-positive cells. **(C)** Histograms of intracellular ROS levels in MCF7 cells transfected with sicont or siD1 at 72 hour post-transfection. At least 500 DCFDA-positive cells were analyzed**. (D)** SA-β-Gal expressions in cyclin D1-depleted MCF7 cells treated with indicated NAC concentrations. **(E)** Quantification of SA-β-Gal-positive cells after being treated with indicated concentrations of NAC. Bars represent average percentages of positive cells ± s.d. **(F)** Percentages of SAHF-positive cells after NAC treatments. Statistical significance was determined by Student's t-test. *(*p≤0.05, **p≤0.01, and ***p≤0.001).*

Figure 5 Upregulation of ROS in cyclin D1-depleted cells activated p38 and JNK pathways

Western blot (WB) analysis of p38, and c-JUN activations. **(A)** Protein lysates were prepared at 0, 12, 24, 48, 72, and 96 hours after non-targeted siRNA (sicont) or cyclin D1 siRNA (siD1) transfection. **(B)** WB analysis was performed similarly as in **(A)**, except that freshly prepared NAC was added to the cells every other day from the 0 hour until the cells were harvested.

Figure 6 Elevated ROS induced hyper-functional FOXO3a.

(A) Western blot (WB) analysis of total FOXO3a in MCF7 cells expressing non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1). **(B)** WB analysis of phospho-Ser7 FOXO3a in MCF7 cells expressing shcont, or shD1. Actin was used as a loading control. (**C)** Immunofluorescence of FOXO3a localization (green) in cyclin D1-depleted MCF7 cells. Nuclei were counterstained with DAPI (blue). Scale bars: 5 μm. (**D)** Histograms of FOXO3a nuclear-to-cytoplasmic (N/C) ratio showed intracellular distribution of FOXO3a in MCF7 cells expressing shcont (black) compared to shD1 (red). **(E)** Percentages of FOXO3a-nuclear-positive in MCF7, and MCF7-shRB cells**. (F)** Percentages of FOXO3a nuclear-positive KKU-214 and TYBDC-1 cells, after cyclin D1 depletion. The FOXO3a nuclear positive cells were counted from at least 5000 cells. **(G)** Expressions of *CDKN1B* (p27), *GADD45A, BIM,* and *SOD2* genes were determined by qRT-PCR. The mRNAs were harvested after cyclin D1 depletion in MCF7 cells. Bars represent averages ± s.d. **(H)** WB analysis of p27 protein expressions in cyclin D1-depleted cells. **(I)** Analysis of proliferative cells by 5-ethynyl-2'-deoxyuridine (EdU) incorporation and **(J)** percentages of SAHF-positive cells in MCF7 transfected with sicont, siD1, or sip27, as indicated**.** Analyses of proliferative cell by 5-ethynyl-2'-deoxyuridine (EdU) incorporation **(K)** and percentages of SAHF-positive cells in MCF7-shRB cells **(L)** transfected with sicont, siD1, sip27, as indicated. Bars represent averages of three independent experiments. Statistical significance was determined by Student's t-test *(*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).*

Figure 7 Anti-senescence role of cyclin D1 in cancer cells.

(A) Cyclin D1 is required for cancer cell survival and proliferation by promoting cell cycle entry and efficient homologous recombination mediated DNA repair (HR). Cancer cells are known to have a high level of oxygen consumption, thus adopts vigorous oxidative stress defense mechanisms(Ward and Thompson, 2012). We showed that cyclin D1 also has additional role in maintaining the level of reactive oxygen species (ROS) at the sub-lethal level (right). **(B)** Depletion of cyclin D1 overwhelms cancer cells with increased level of ROS. Consequently, elevated ROS broadcasts its signal via several intracellular pathways, including p38/JNK-FOXO3a-p27, to promote cancer cell senescence.

Figure 1 Depletion of cyclin D1 caused cellular senescence in various cancer cell lines.

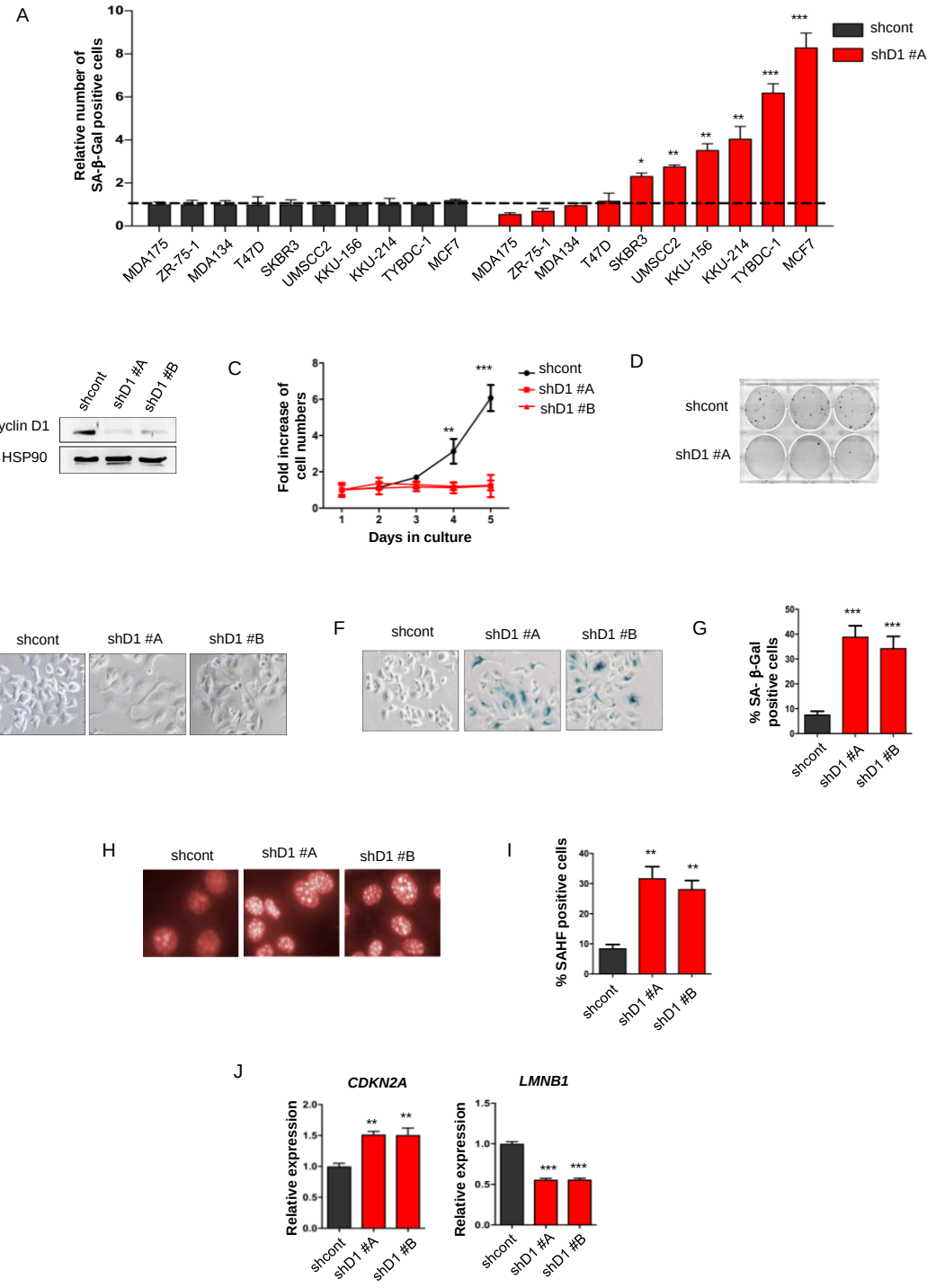
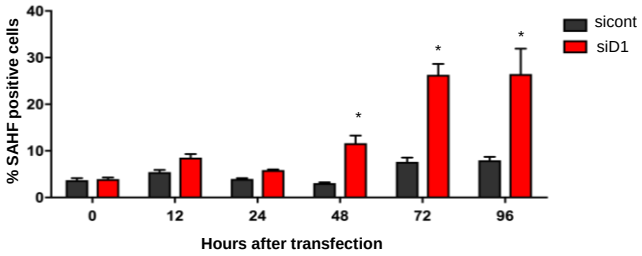


Figure 2 Association between cyclin D1 downregulation and senescence.

A



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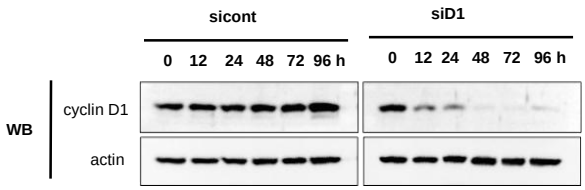


Figure 3 Cyclin D1 depletion induced senescence is independent of RB status

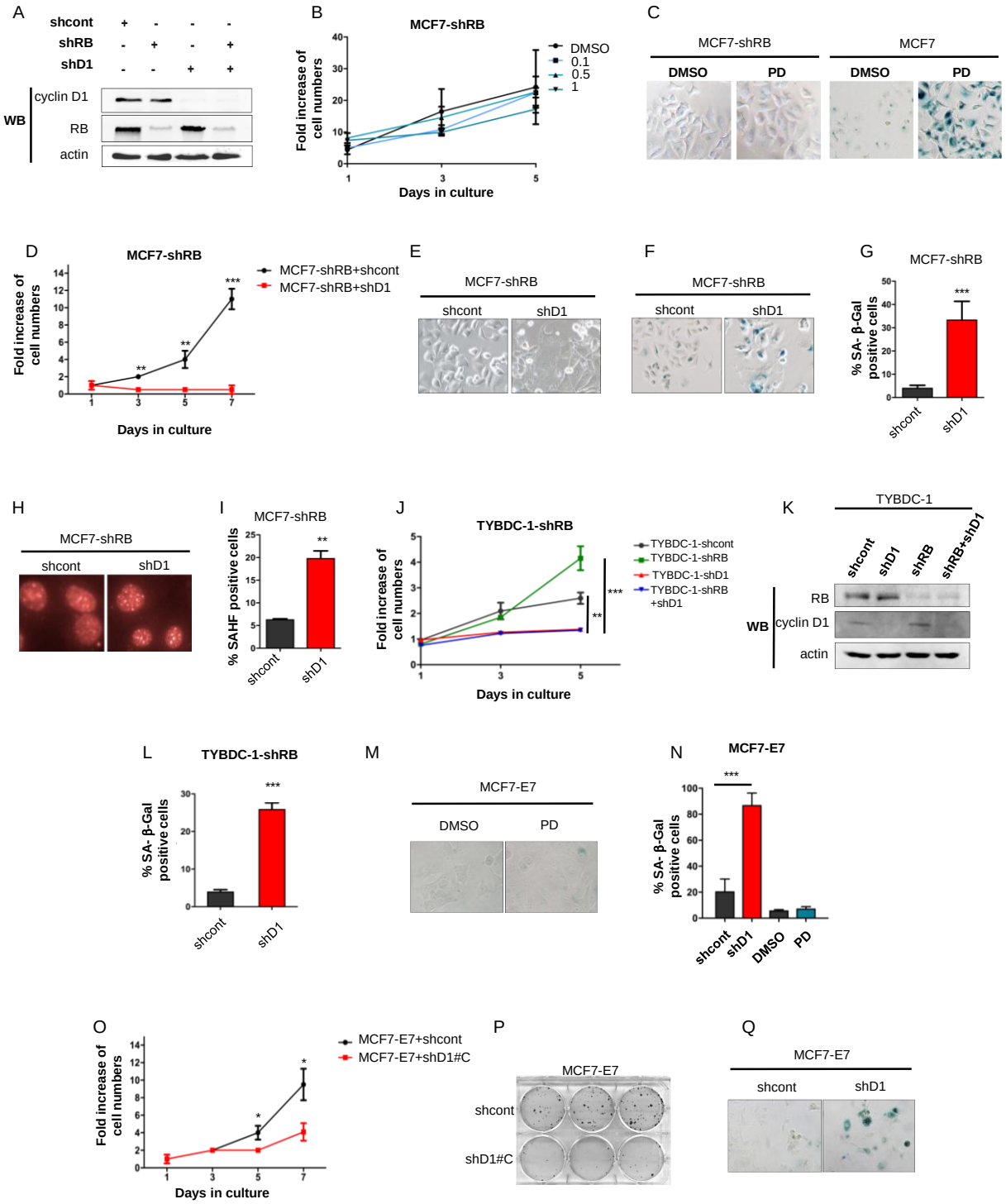


Figure 4 Reactive oxygen species (ROS) was upregulated in cyclin D1-depleted cancer cells and responsible for senescence.

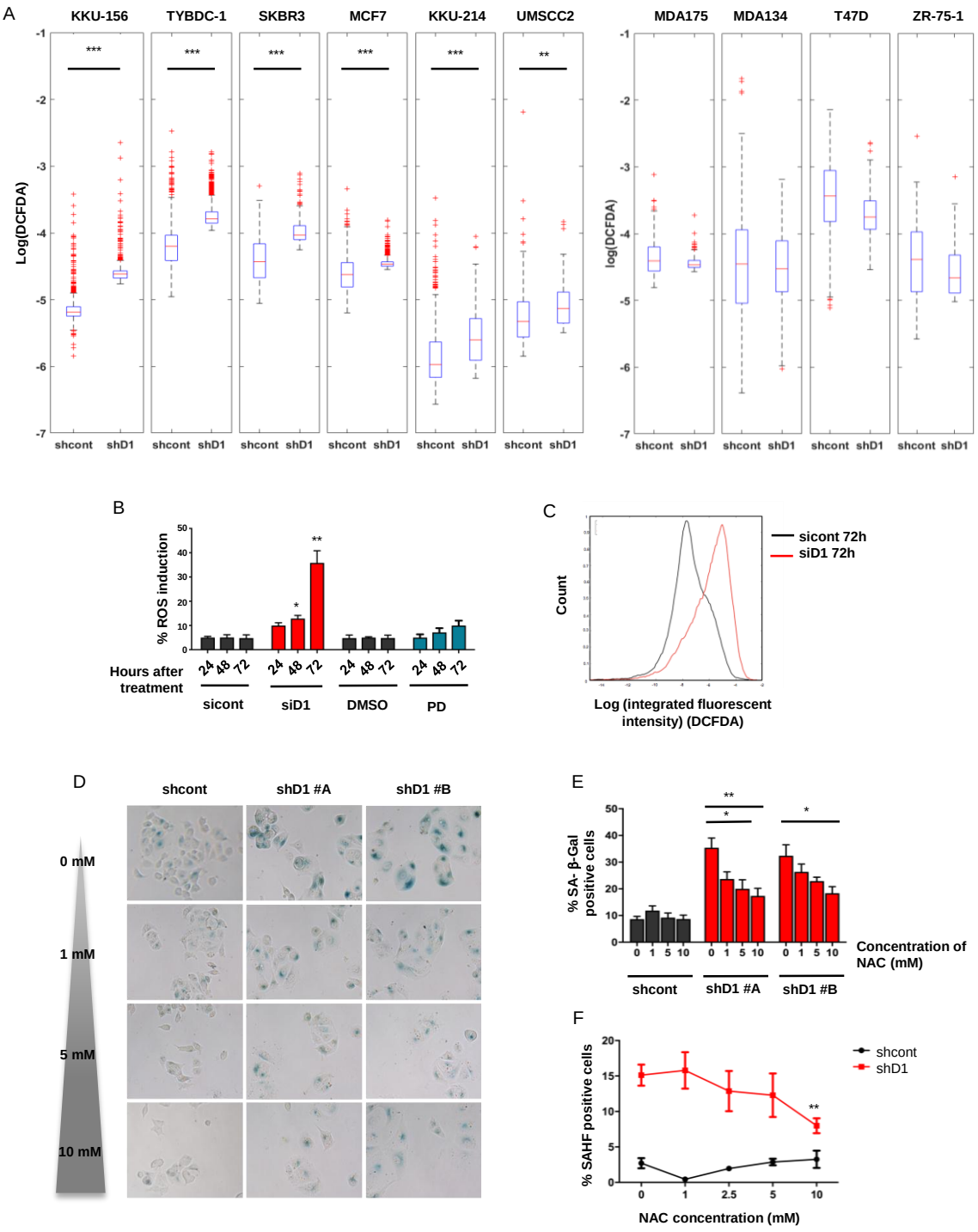


Figure 5 Upregulation of ROS in cyclin D1-depleted cells activated p38 and JNK pathways

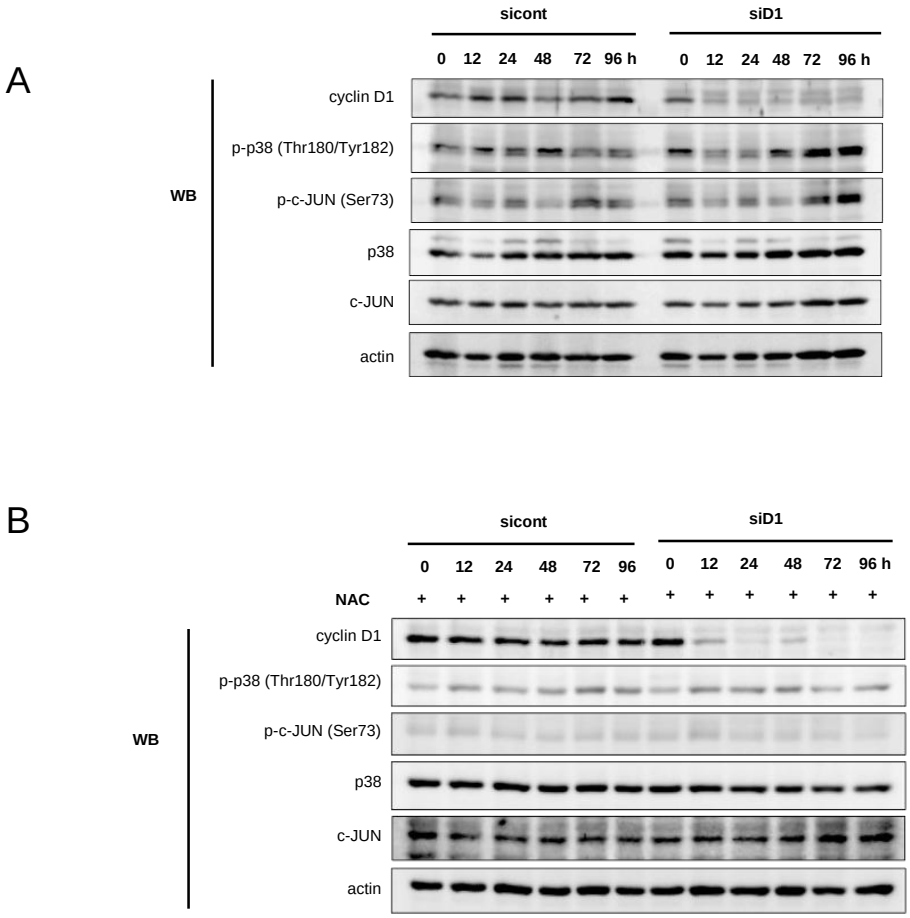


Figure 6 Elevated ROS induced hyper-functional FOXO3a.

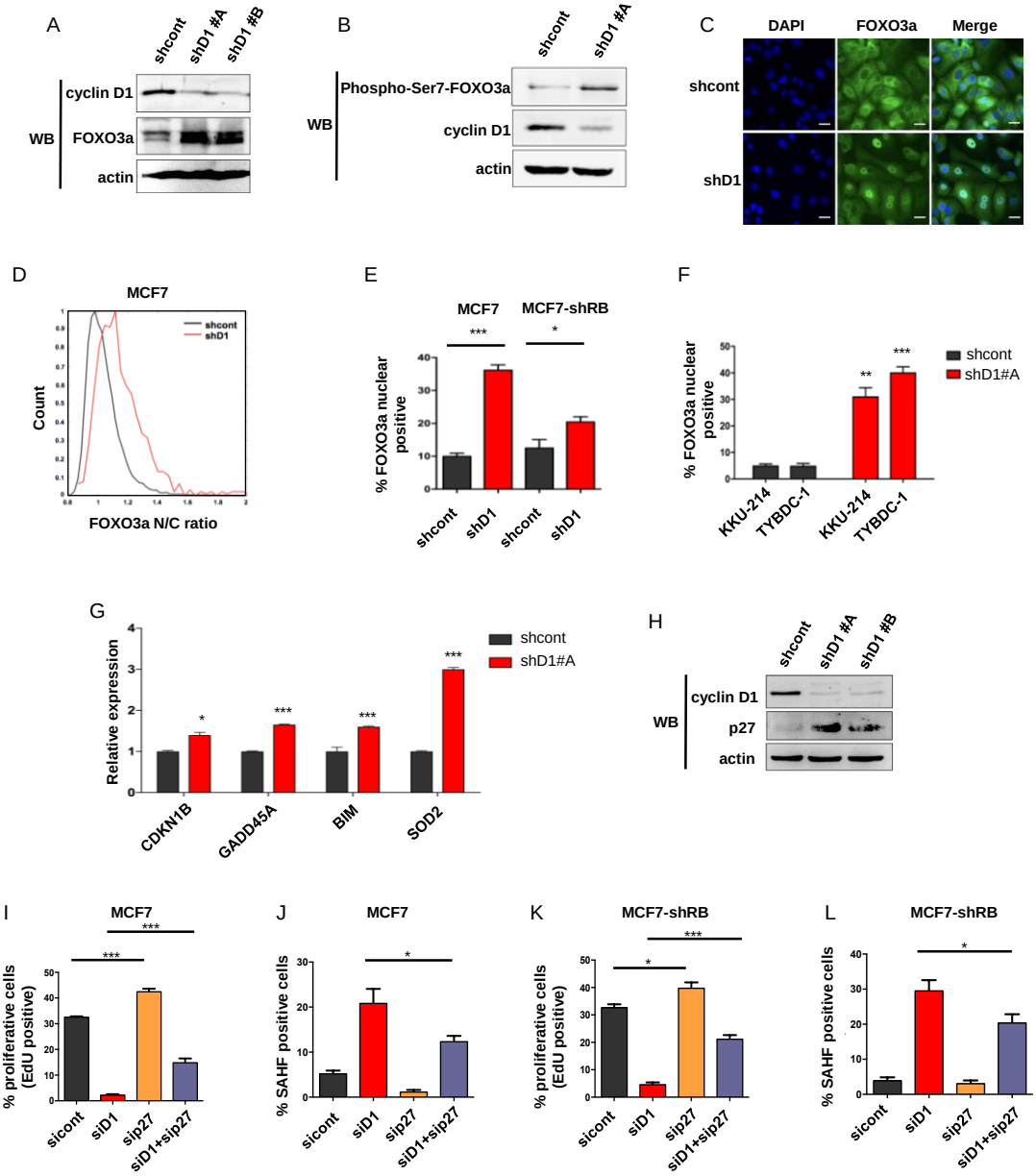
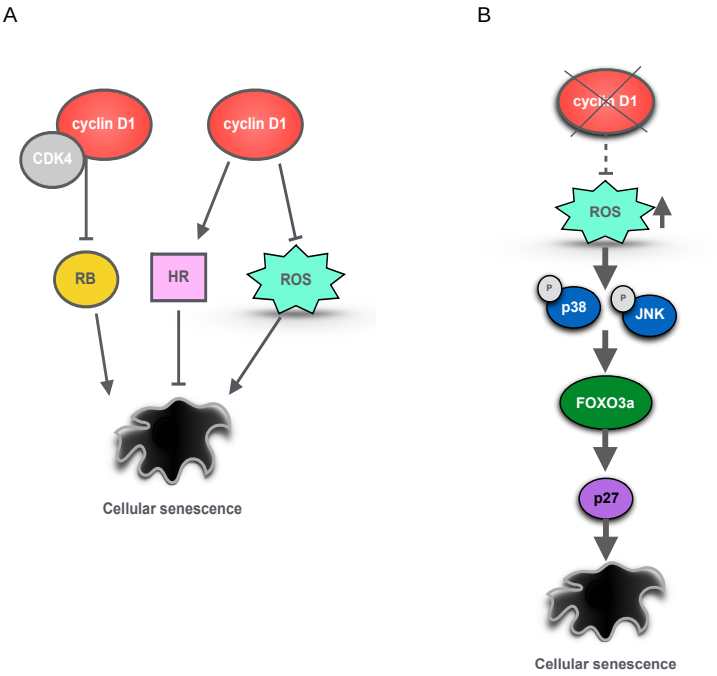
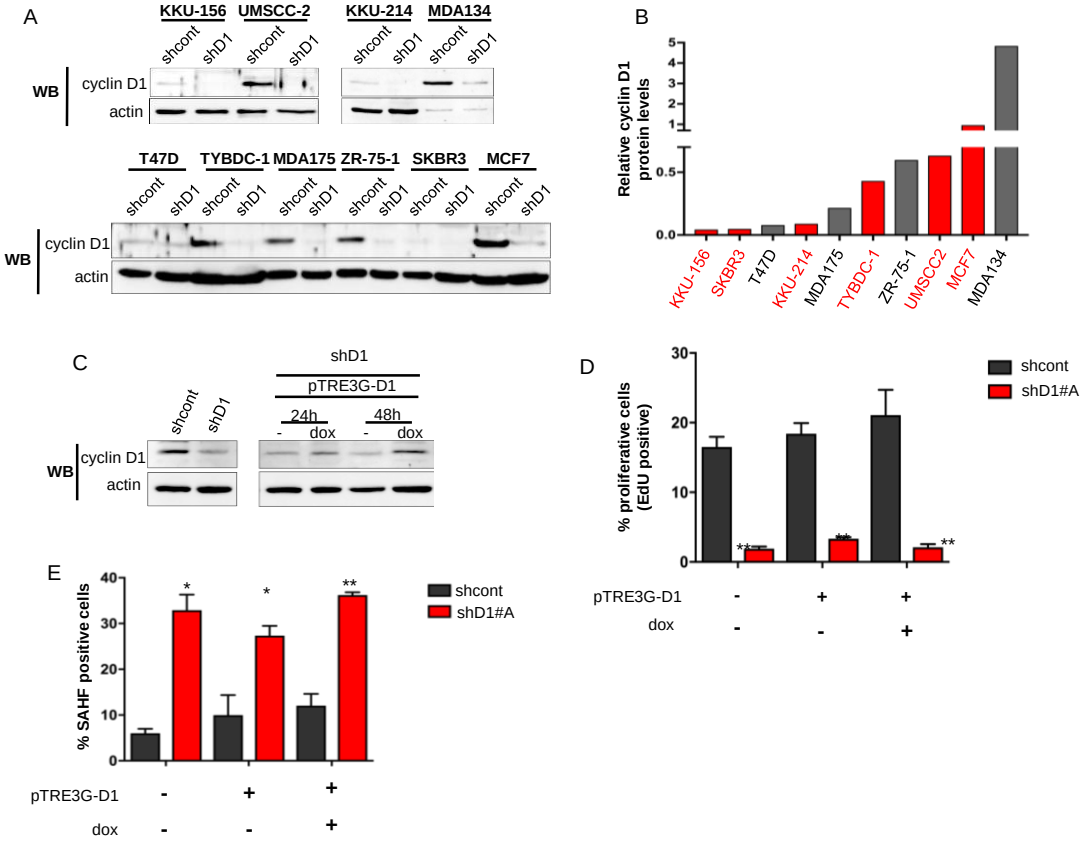


Figure 7 Anti-senescent role of cyclin D1 in cancer cells.



Supplementary Figure 1



A. Western blotting (WB) analysis of cyclin D1-knockdown efficiency in several types of cancer cell lines. Protein lysates were harvested 4 days after transduced with non-targeting shRNA (shcont) or cyclin D1-specific shRNA (shD1#A). Actin was used as a loading control.

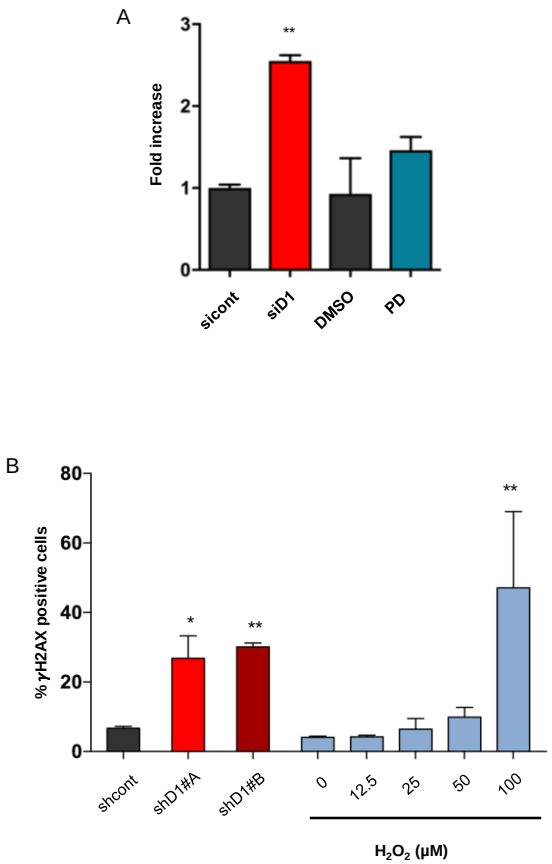
B. Relative protein levels of cyclin D1 in the cell lines from (a). Bars in grey are cell lines that did not undergo senescence and red bars are cell lines that underwent senescence upon cyclin D1 depletion.

C. Western blotting (WB) analysis of re-expressing cyclin D1-HA in the senescent cells. The inducible system (Takara Bio Inc., Mountain view, CA, USA), composed of pEF1α-Tet3G and pTRE3G-cyclin D1-HA plasmids was co-transfected into MCF7 cells, followed by cyclin D1 depletion using cyclin D1-specific shRNA (shD1#A). Expression of cyclin D1-HA was achieved by addition of 1 μM doxycycline. Re-expressions of cyclin D1-HA were examined at 24 and 48 hours after the induction. Actin was used as a loading control.

D. Re-expression of cyclin D1-HA did not increase cell proliferation in cyclin D1 depletion-induced senescent cells. Percentages of EdU-positive cells were analyzed at 48 hours after the induction. Bars represent averages of 3 experiments± s.d. (**$p \leq 0.01$)

E. Re-expression of cyclin D1-HA in the senescent cells did not reduced number of SAHF-positive cell. Analyses of SAHF-positive cell numbers were performed at the same time point as in (d). Doxycycline treatment; dox. Bars represent averages of 3 experiments ± s.d. (*$p \leq 0.05$ and **$p \leq 0.01$)
Statistical significance was determined by Student's t-test

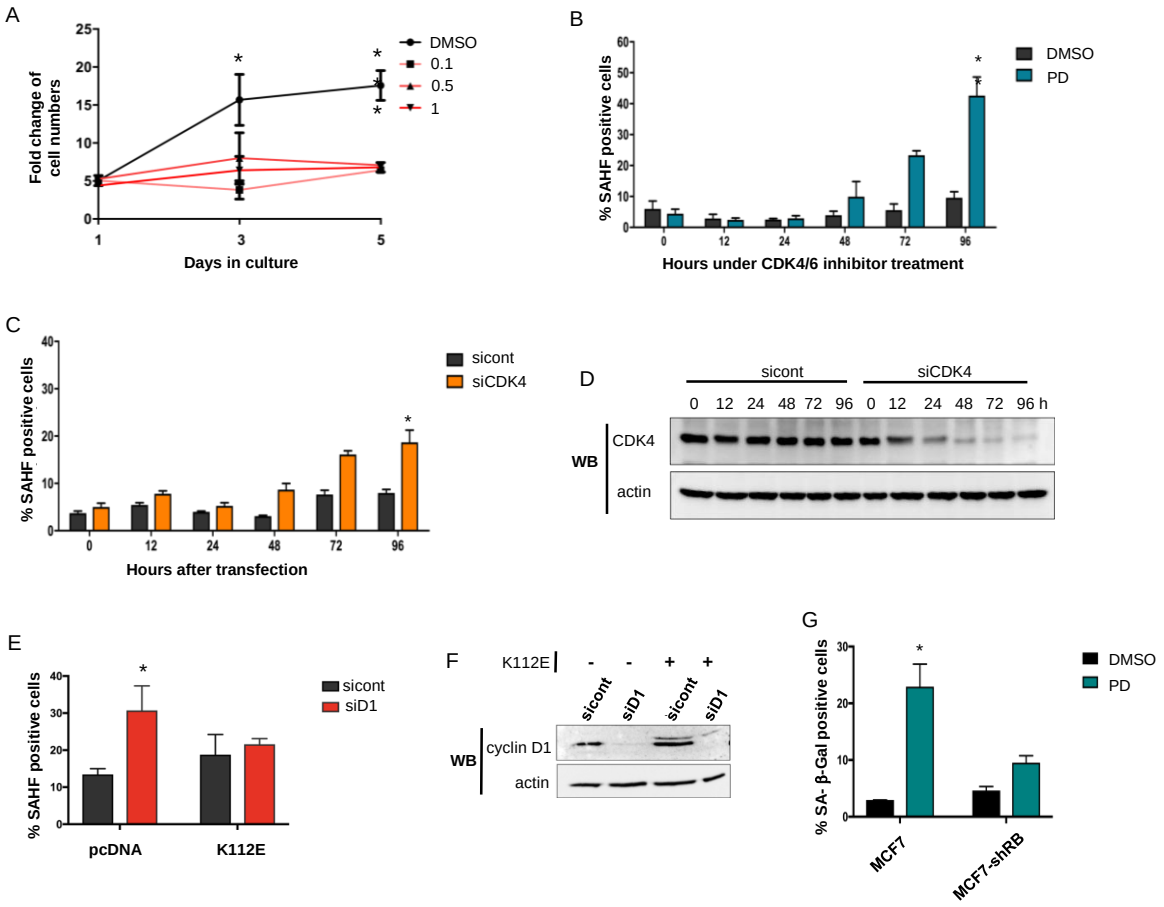
Supplementary Figure 2



A. Apoptosis detection of cyclin D1-specific siRNA (siD1) and CDK4/6 inhibitor treatment (PD), compared to control cells (sicont) and vehicle control (DMSO). Bars represent averages from 3 independent experiments ± s.d. Statistical significance was determined by Student's t-test (***p≤0.01*).

B. Cyclin D1-depleted cells (shD1#A, shD1#B) showed significant elevation of γH2AX foci-positive cells, compared to control cells (shcont). Percentages of γH2AX foci-positive MCF7 cells treated with increasing concentrations of H_2O_2 are shown as a reference (grey bars). At least 5,000 γH2AX-positive cells were analyzed. Bars represent averages from 3 independent experiments ± s.d. Statistical significance was determined by Student's t-test (**p≤0.05 and **p≤0.01*).

Supplementary Figure 3



A. Growth curves of MCF7 cells treated with CDK4/6 inhibitor (PD) at indicated concentration (0, 0.1, 0.5, and 1 μM).

B. Percentages of SAHF-positive MCF7 cells were analyzed at 0, 12, 24, 48, 72, and 96 hours after the cells were treated with 0.5 μM PD 0332991 (PD). DMSO was use as a vehicle control. Bars represent averages from 3 independent experiments ± s.d. *(**p≤0.01*).

C. Percentages of SAHF-positive MCF7 cells were analyzed at 0, 12, 24, 48, 72, and 96 hours after the cells were transfected with CDK4-specific siRNA (siCDK4), or non-target control siRNA (sicont). Bars represent averages from 3 independent experiments ± s.d. *(*p≤0.05*).

D. WB analysis of CDK4 depletion by the CDK4-specific siRNA at indicated time points as in (c). Actin was used as a loading control.

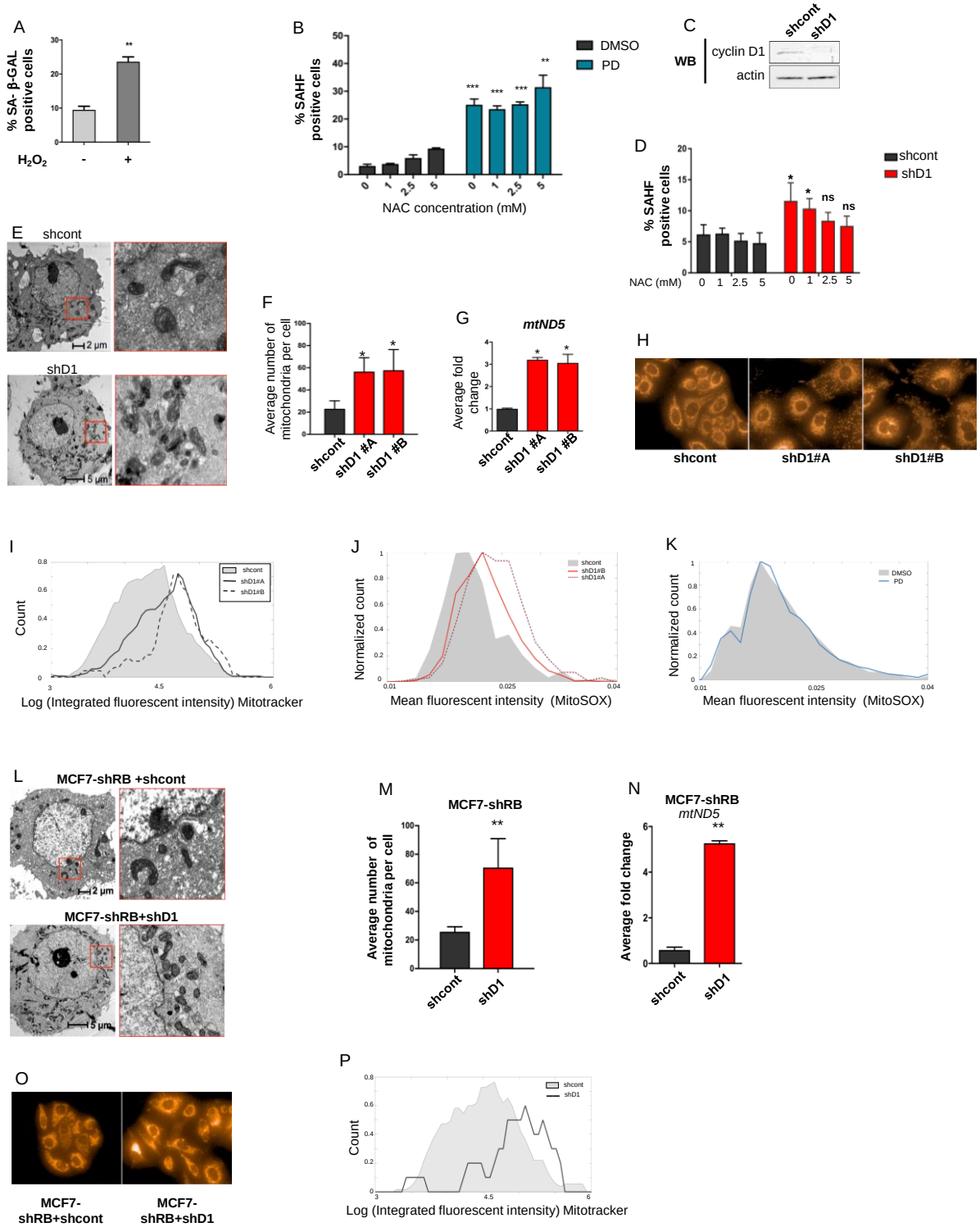
E. Cells were co-transfected control pladmid pcDNA or kinase dead cyclin D1 (K112E), with GFP expressing plasmid peGFP, followed by cyclin D1 depletion using cyclin D1-specific siRNA targeting 5'UTR of cyclin D1 mRNA. Percentages of SAHF-positive MCF7 cells were analyzed in GFP-positive cells after 72 hours of cyclin D1 depletion (**p≤0.05*).

F. WB analysis of MCF7 cells expressing kinase-dead cyclin D1 (K112E). Lysates were collected after 72 hours of siRNA transfection.

G. Percentages of SA-β-Gal-positive cells after 5 days of PD0332991 (PD) or vehicle (DMSO) treatment . Bars represent averages from 3 independent experiments ± s.d. (**p≤0.05*).

Statistical significance was determined by Student's t-test

Supplementary Figure 4



Supplementary Figure 4

A. MCF7 cells were treated with 1000 μM of H_2O_2 for 72 hours. Bars represent average percentages ± s.d. of SA-β-Gal-positive cells from 3 experiments *(**p≤0.01*)

B. PD0332991-induced senescence could not be prevented by N-acetyl cysteine (NAC). Percentages of SAHF-positive MCF7 cells were analyzed after 96 hours of 0.5 μM PD0332991 (PD) treatment. Indicated concentrations of NAC were added to the culture medium at the same time as PD treatment. DMSO was used as vehicle control. Bar represent average ± s.d *(**p≤0.01, ***p≤0.001*)

C. WB analysis of cyclin D1 in Capan-1 cells after transduced with non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1) for 4 days.

D. Percentages of SAHF-positive Capan-1 cells were analyzed after transfected with non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1) for 4 days . Indicated concentrations of NAC were added to the culture medium at the same time as cyclin D1 depletion. Bar represent average ± s.d *(*p≤0.05, ns; statistically not significant)*

E. MCF7 cells were transduced with non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1) for 4 days before analyzed by transmission electron microscope (TEM). Red boxes are enhanced to the right figures.

F. Average numbers of mitochondria per cell analyzed by TEM from (c) (shcont; n = 4 cells) (shD1 #A, and #B; n = 5 cells). Bars represent averages of 3 experiments ± s.d. (**p≤0.05*)

G. Mitochondrial DNA contents of *mtND5* gene in cyclin D1-depleted, and control cells. Bars represent averages of 3 experiments ± s.d. (**p≤0.05*)

H. Mitotracker staining of MCF7 cells expressing non-targeting control shRNA (shcont), or cyclin D1-specific shRNAs (shD1#A, and shD1#B).

I. Histograms show Log integrated fluorescent intensity of Mitotracker stained mitochondria from MCF7 cells expressing either non-targeting shRNA (shcont), or cyclin D1-specific shRNAs (shD1#A, and shD1#B), from (f).

J. Histograms show mean fluorescent intensities of MitoSox Red stained mitochondria in MCF7 cells expressing either non-targeting shRNA (shcont), or cyclin D1-specific shRNAs (shD1#A, and shD1#B).

K. Histograms show mean fluorescent intensities of MitoSox Red stained mitochondria in MCF7 cells treated either with vehicle (DMSO), or 0.5μM PD0332991 (PD)

L. TEM analyses of pRB-deficient MCF7 cells (upper panel) and pRB-deficient MCF7 cells expressing cyclin D1-specific shRNA (shD1# C) (lower panel). Red boxes are enhanced to the right figures.

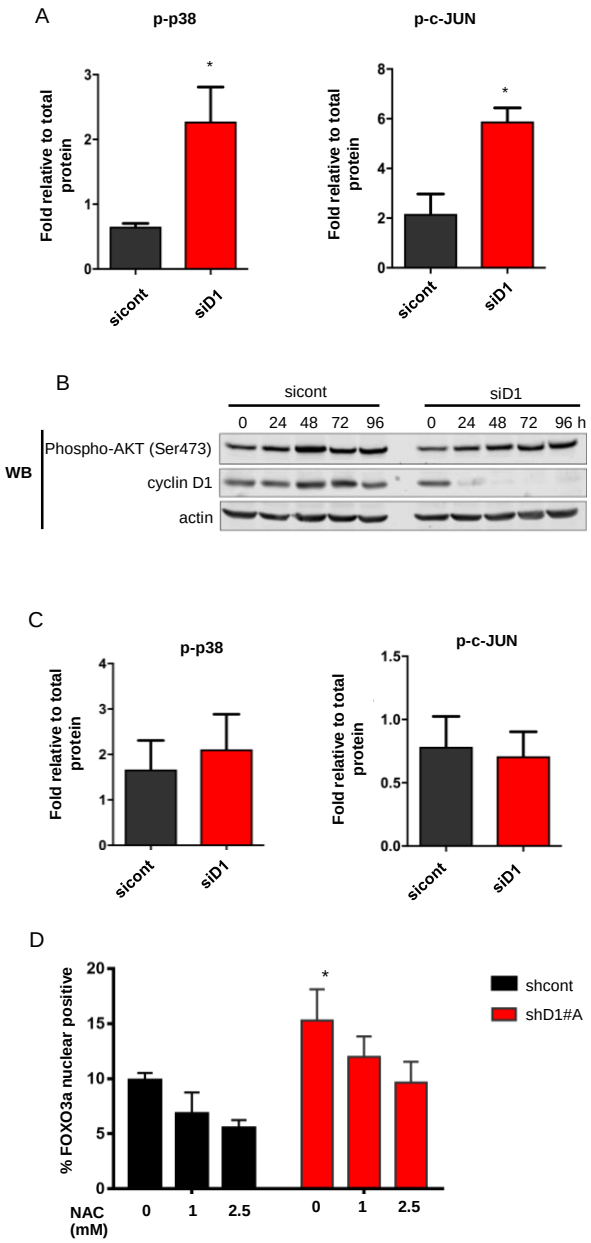
M. Average numbers of mitochondria per cell from (L). Bars represent averages ± s.d. (shcont, n = 5 cells; shD1, n = 5 cells) (**p≤0.01*)

N. Mitochondrial DNA content of *mtND5* gene in MCF7-shRB cells. Bars represent averages ± s.d. from 3 experiments (**p≤0.01*)

O. Mitotracker staining of MCF7-shRB cells expressing non-targeting shRNA (shcont), or a cyclin D1-specific shRNA (shD1#C).

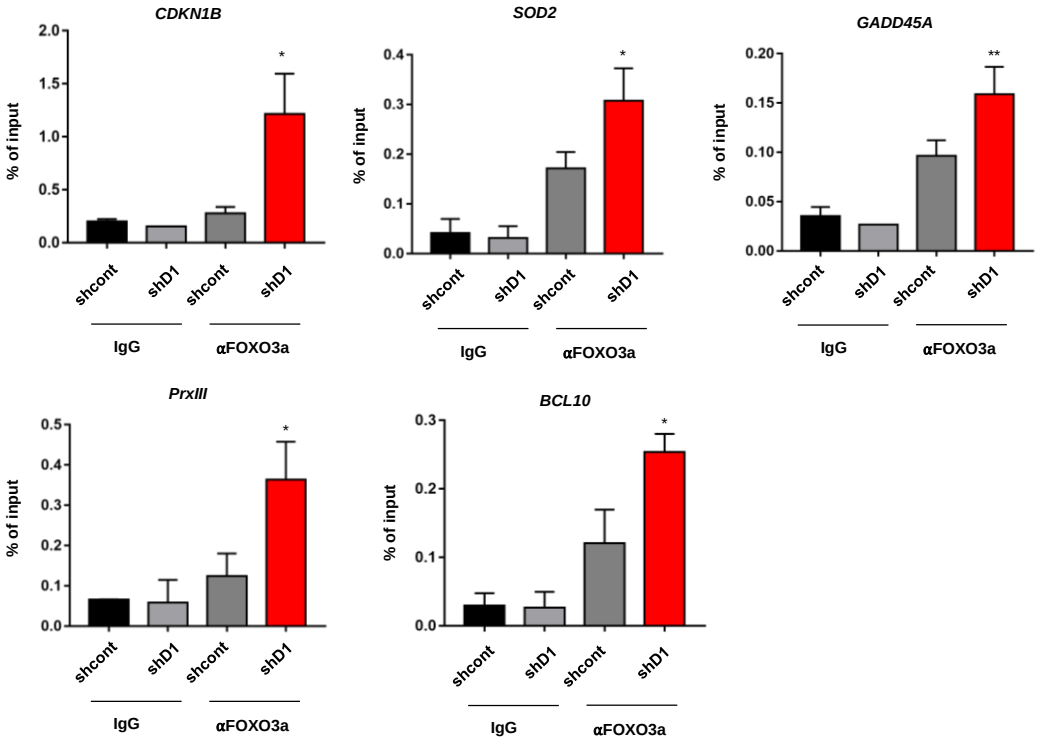
P. Histograms show log integrated fluorescence intensity of Mitotracker stained mitochondria from MCF7-shRB cells expressing either control shcont (shcont), or cyclin D1-specific shRNA (shD1), from (m).

Supplementary Figure 5



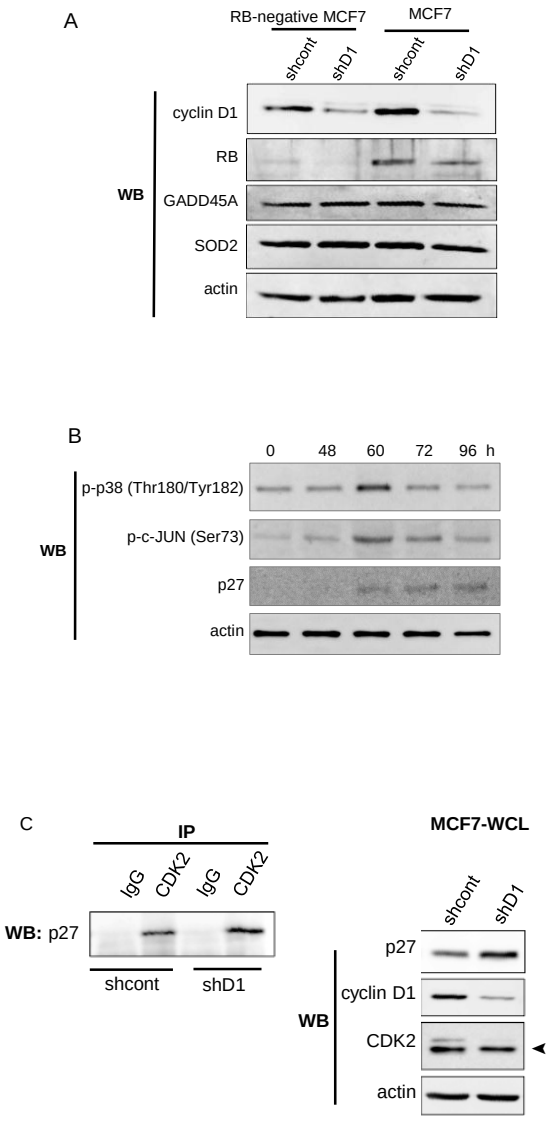
A. Cells were transfected with non-targeted siRNA (sicont) or cyclin D1 siRNA (siD1) transfection. Bars represent quantified signals of phospho-p38, and phospho-c-JUN normalized by total proteins at the 96-hour time point. (**p≤0.05*)
B. WB analysis of phospho-AKT Ser473, and cyclin D1 after transfected with with non-targeted siRNA (sicont) or cyclin D1 siRNA (siD1) at indicated time points. Actin was used as a loading control.
C. WB analysis was performed similarly as in **(A)**, except that freshly prepared NAC was added to the cells every other day from the 0 hour until the cells were harvested. Bars represent quantified signals of phospho-p38, and phospho-c-JUN normalized by total proteins at the 96-hour time point.
D. MCF7 cells were transduced with non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1) for 4 days under treatment of NAC at indicated concentration. At least 5,000 FOXO3a nuclear-positive cells were analyzed. Bars represent percentage of cell that have nuclear FOXO3a localization. *(*p≤0.05*).

Supplementary Figure 6



FOXO3a chromatin immunoprecipitation (ChIP) in cells expressing non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1). ChIP products were analyzed by semi-quantitative PCRs. IgG was used as a negative control for chromatin immunoprecipitation. Bars represent percent of input ± s.d. *(*p≤0.05, **p≤0.01*).

Supplementary Figure 7



A. MCF7 cells and MCF7-expressing shRB were transduced with non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1#C). Four days after transduction the cell lysates were collected and indicated proteins were analyzed by WB analyses. Actin was used as a loading control.

B. The upregulation of p27 coincided with p38 and c-JUN phosphorylations. WB analysis of p27, and p38/c-JUN specific phosphorylations. Protein lysates were prepared at 0, 48, 60, 72, and 96 hours after cyclin D1 siRNA (siD1) transfection.

C. (Left) WB analysis of p27 from immunoprecipitation by using IgG or CDK2 antibody in MCF7 cells expressing non-targeting shRNA (shcont) or cyclin D1 shRNA (shD1). (Right) WB analyses of whole cell lysate (WCL) from MCF7 cell with non-targeting shRNA (shcont), or cyclin D1 shRNA (shD1). Arrow indicated the CDK2 band.

ภาคผนวก 2

An Overview and Perspective: Non-Cell Cycle Functions of Cell Cycle Regulators

Phatthamon Laphanuwat[1], Siwanon Jirawatnotai[1 *]

[1]Siriraj Center of Research for Excellence (SiCORE) for Systems Pharmacology, Department of Pharmacology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, 10700, Thailand

Abstract

Core cell cycle regulators, including cyclin-dependent kinases (CDKs), cyclins, and cyclin-dependent kinase inhibitors (CKIs), are known for their well-characterized roles in facilitating normal cell division. However, recent studies have shed light on roles of these proteins that go beyond the cell cycle. Those roles range from the regulation of transcription as an integral part of various biological processes through several gene targets, the differentiation of cells, energy metabolism control, epigenetic and stem cell fate regulation, DNA damage repair, and oxidative stress. In addition, some of the functions contribute beyond the cell autonomous level, such as the emerging function in immune modulation. Here, we describe extensive examples of how cell cycle machineries regulate all of these crucial processes in cells. The additional roles of the cell cycle regulators add a new look to the cell cycle proteins as a group of proteins that not only facilitate the steps in cell division, but also holistically guard the cellular homeostasis of multicellular organisms.

Keywords: CDKs, cyclins, cancer, non-cell cycle functions, cell cycle

* **Correspondence:** Siwanon Jirawatnotai, Ph.D., Siriraj Center of Research for Excellence (SiCORE) for Systems Pharmacology, Department of Pharmacology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, 10700 Thailand. Email: siwanon.jir@mahidol.ac.th

Introduction

Our body has certain biological processes to maintain its cell numbers. Multicellular organisms develop and maintain the critical number of cells needed for tissue homeostasis through cell division. Cell division is governed by a group of core proteins called cyclin-dependent kinases (CDKs); cyclins; and its negative regulators, the cyclin-dependent kinase inhibitors (CKIs). The CDKs are Serine/Threonine kinase, which needs cooperation from a regulatory subunit, cyclin. A CDK protein activity can be regulated by the oscillation of a specific cyclin. However, in most metazoans, it can also bind to many cyclins to orchestrate a particular function. Mammalian cells contain 20 CDKs and 29 cyclins, which have been roughly divided into two groups based on their function: cell-cycle-related and transcription-related CDKs/cyclins (1). The canonical role of CDKs and cyclins in cell cycle control has been well-studied. In mammalian cells, upon growth factor stimulation, cyclin D (cyclin D1, D2, or D3) is upregulated and binds to its kinase partner CDK4 or CDK6. In turn, the binding activates CDK kinase activity and initiates phosphorylations on the pocket proteins pRB (p110), p107, and p130. The phosphorylated pocket proteins become inactive and release the E2F transcription factor, allowing S-phase gene synthesis to drive the cell cycle from the G1 to the S phase. In late G1, cyclin E (cyclin E1 and E2), which is regulated by E2F, activates its partner CDK2, causing further pocket protein phosphorylation. Consequently, various cell-cycle-related proteins are activated. The cyclin E-CDK2 complex also controls replication initiation by allowing the formation of a prereplication complex. During the S-phase, cyclin A partners with CDK2. As the cell passes from the G1 to the S phase, the cyclin A-CDK2 complex replaces the cyclin E-CDK2 complex. When the amount of cyclin A-CDK2 complex reaches a threshold level, it terminates the assembly of the prereplication complex made by cyclin E-CDK2. While the amount of cyclin A-CDK2 complex increases, the complex initiates DNA replication (2). As the cell cycle progresses through G2/M, the level of cyclin A declines. Eventually, the complex of cyclin B-CDK1 takes over as the G2-M cell cycle controller. During the onset of the G2 to the M phase, CDK1 associates with cyclin A or cyclin B; both types of the complex are essential for the timely M-phase progression of the cell (3). As such, the Cdk1 substrates are enriched by G2 and M phase-specific genes (4). Being a key regulator in crucial processes, the regulation of CDKs and cyclins is a tightly controlled process. The process is aided by gene transcription, phosphorylation programs, the turn-over rate and degradation of proteins (cyclins and substrates of CDKs), and the like. A major negative controlling mechanism is binding by one of the proteins belonging to 2 classes of the cyclin-dependent kinase inhibitors (CKIs). Classes of the CKIs are based on their structure and the target CDKs (5). The *Cip/Kip* family proteins, comprising $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, have a wide range of CDK targets. They negatively regulate the activity of cyclin E- and cyclin A-dependent kinases. In contrary they can also promote complex formation and the activity of the cyclin D-CDK4 complex. Another family, *INK4*, mainly inhibits the activities of CDK4 and CDK6. The *INK4* family consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. The cell cycle functions of CDKs, cyclins, and CKIs have been discovered, and the coordinating roles of these proteins in controlling a proper cell division have been well-described.

Apart from the roles of the cell cycle regulators, CDKs, cyclins, and CKIs, in cell division, emerging roles of these proteins in other biological processes have been reported. A growing body of evidence suggests that the cell cycle regulators are doing "part-time" jobs in various cellular processes. These unconventional roles support the steps involved in development and in tissue/whole body homeostasis, from the maintaining the cellular integrity of processes like metabolism, oxidative balance, energy control, DNA damage response; to keeping of a reservoir of stem cells and initiating and maintaining of the cellular differentiation. Importantly, many of these non-canonical functions, when deregulated, have been demonstrated to play roles in

diseases such as cancer.

In this review, we mainly focus on the non-canonical role of the cell cycle regulators, CDKs, cyclins, and CKIs, especially in mammalian cells. Rather than trying to describe the specific detail of every single discovery, we present evidence illustrating an overview of the non-cell cycle roles of the cell cycle proteins to try to understand this small aspect of the evolution of biological processes, and to reassess some of the proteins as a potential target of the pharmacological treatment of diseases such as cancer.

1. Transcriptional role of cell cycle regulators

The role of cell cycle regulators in transcription control during the G1-phase through the pRB-E2F axis has long been accepted. The phosphorylation of pocket proteins by CDK-cyclin complexes allows the liberation of the transcription factor E2F to initiate S-phase gene synthesis. The extended list of G1 cyclin-CDK complexes also shows that cell cycle regulators support cell cycle progression through phosphorylation of many transcription factor substrates beyond the pocket proteins. A list of known transcription factor substrates for CDKs is at Table 1. Phosphorylation of some of the non-pocket protein transcription factors either causes a deterioration of the growth inhibitory signal or creates a theoretically suitable environment for cell division. For example, the phosphorylation of Smad3 by CDK4 or CDK2 reduces the transcriptional activity and antiproliferative function of Smad3 (6-8). Phosphorylation of FOXM1 by CDK4 stabilizes the transcription factor, which is known to control G2/M phase-specific genes and antisenescence genes (9).

The non-cell cycle transcription roles of CDKs, cyclins, and especially G1 cell cycle proteins, emerged in the 1990s when a forced expression of cyclin D1 was found to inhibit muscle gene transcription. Cyclin D1 directly represses the myogenic bHLH regulator. This happened independently of the pRB inactivation role of cyclin D1, suggesting that the cell cycle regulator had a role in the negative control of cell differentiation (10). Since then, there has been a growing body of evidence supporting transcriptional regulation by cell cycle regulators (Table 1). Thus far, the cell cycle regulators have been found to regulate more than 30 transcription factors, involving several cellular processes, including those in the cell cycle control and non-cell cycle functions. The transcriptional roles of cell cycle proteins that are not related to the cell cycle exist in cell differentiation, the cytokine response, angiogenesis, and the like. Interestingly, when performing the transcription control, the role of these cell cycle regulators can be unconventional. In certain contexts, they can function independently of the CDK kinase activity.

Cyclin-CDK complexes can promote cell proliferation *per se* by phosphorylating substrates that play a role during the steps required for cell division. Cyclin B1-CDK1 phosphorylates and regulates the mRNA methyltransferase RNMT. This phosphorylation enhances the RNMT function and thereby promotes gene expression and cell proliferation (11). Cyclin A-CDK2 phosphorylates b-Myb and activates its transactivation activity (12-16). SMAD proteins are the key mediator for the TGF-β growth inhibitory effect. SMADs were found to be negatively regulated by CDK activity, blocking in turn the growth inhibitory signal from TGF-β (7, 8, 17).

The function of CDK inhibitors on transcription regulation has also been reported by several studies. p16 ^{INK4a} and p21^{CIP1} seem to be major players in regulating gene expression. p16 ^{INK4a} together with CDK4 physically form a complex with the transcription factor Sp1 and mediate the upregulation of microRNAs (miRNA-141 and miRNA-146b-5p) in response to UV-exposure, thereby inducing apoptosis in the UV-stressing cells (18). Breast cancer cell-induced

angiogenesis is negatively controlled by $p16^{INK4a}$. $p16^{INK4a}$ can form a complex with a master transcriptional regulator of the cellular response to hypoxia HIF-1α. The complex blocks transactivation of VEGF (19, 20). In addition to $p16^{INK4a}$ directly regulating transcription, it has been reported to enhance gene expression at the post-transcriptional level by inhibiting the mRNA decay-promoting AUF1 protein, thus promoting the stability of the cyclin D1 and E2F1 mRNAs (21). In response to DNA damage, downregulations of *myc* and *cdc25A* are induced by $p21^{Cip1}$, which binds and represses the promotors of the *myc* and *cdc25A* genes (22). Moreover, $p21^{Cip1}$ and $p27^{Kip1}$ play a sequential role in recruiting cyclin-CDK complexes on the promotor during the G1 phase, and they enable the gene repressing activities of p130 and E2F4 (23).

Among cyclins, the D-type cyclins seem to be a prominent family of cyclins with a direct transcriptional regulation role that is independent of its kinase activation function. Cyclin D1 potentiates estrogen hormone-dependent gene expression by directly interacting with the estrogen receptor (ER) *in vitro* and *in vivo* (24, 25). Cyclin D1 can not only bind directly, but it can also enhance the binding capacity of ER to the estrogen response element sequences; it does this by bridging the ER to a co-activator like SRC-1 (26) and the histone acetyl-transferase P/CAF (27, 28). Cyclin D3 also has a kinase-independent role in the positive control of vitamin D receptor (VDR) (29) and human activating transcription factor 5 (hATF5) transactivation (30). The direct transcription activity of cyclin D1 has been elaborated further when mice were genetically knocked-in the epitope-tags (FLAG and HA) into the endogenous cyclin D1 terminus, thus allowing the *in vivo* unbiased analyses of cyclin D1 interaction to DNA by immunoprecipitation, followed by ChIP-chip analysis. The results indicated that cyclin D1 physically binds to several promotor areas during mouse development, underscoring a prominent role of cyclin D1 in the control of gene expression. One of the cyclin D1 protein interactors was the CREB binding protein (CBP), which is recruited to the *Notch1* gene promotor in the retina and promotes the expansion of the retinal progenitor cells (31). The genetic ablation of cyclin D1 resulted in decreases in CBP recruitment and histone acetylation of the Notch1 promoter region as well as Notch1 production in the retina, leading to severe retinopathy (32, 33).

Despite the high level of homology found among CDK4 and CDK6, evidence indicates that CDK6 has distinct functions from those of CDK4. One of those unique functions has been discovered in the $p185^{BCR\text{-}ABL+}$ B-acute lymphoid leukemia mice model. CDK6 has been shown to be highly expressed in lymphoid malignancies. CDK6, independent of its kinase activity, has been demonstrated to be a part of a transcription regulatory complex that induces the expression of $p16^{INK4A}$ and the pro-angiogenic factor VEGF-A. In the context where $p16^{INK4A}$ is deleted, CDK6 promotes tumor growth while also ensuring the supply of oxygen and energy to the tumor (34). Apart from its direct transcriptional regulation, CDK6 contributes to erythroid cell structure by regulating the genes involved in cytoskeleton organization, such as *Gelsolin* and *Baiap2*, in a kinase-dependent manner (35). CDK6 also protects cancer cells from chemotherapy-induced death by phosphorylating the transcription factor FOXO3, which in turn induces transcription of the DNA damage checkpoint protein ATR (36). A list of evidence implicating the cell cycle regulators in transcription control is at Table 1.

It is an interesting notion that, although the well-known role of cell cycle proteins is indeed in the regulation of the E2F-mediated pro-proliferative transcriptional program, emerging evidence suggests that several of the G1 cyclins and CDKs (such as CDK6, CDK4, and cyclin Ds) are involved in other transcription programs in specific tissues and contexts, resulting in the homeostasis and maintenance of multicellular organisms. The existence of these G1 CDKs/cyclins may be a result of evolution in which specific kinase may be selected to mediate paracrine signaling and to couple cell division with tissue development.

2. Differentiation regulation by cell cycle regulators

In the process of organogenesis and development, the pluripotent cells organize their machineries to become more specialized in each lineage. This process is called terminal differentiation, which may be considered as a timer that is intrinsically set up to execute in an appropriate manner and at the right time. Generally, the occurrence of the process is inversely correlated with cell division. To complete terminal differentiation, cells must exit from the cell division cycle. The machineries that are used in support of proliferation have to be deteriorated. Therefore, cell cycle regulators such as cyclins and CDKs appear to be generally downregulated at this stage, whereas the cell cycle inhibitors CKIs appear to be generally upregulated.

In some of the tissues, certain regulators of the cell cycle have been shown to directly participate in differentiation. In differentiated myocytes, cyclin D1, cyclin A, and cyclin E have been reported to suppress expressions of muscle-specific genes. Cyclin D1, by pRb- and CDK-independent manners, directly interacts with a myogenic basic helix-loop-helix (bHLH) regulator, and it blocks expressions of MyoD and myogenin. Cyclin A and cyclin E can also suppress muscle-specific genes by an apparently different mechanism. Suppression of the myogenic genes by cyclin A and E depends on pRB hyperphosphorylation, suggesting that the suppression may be a consequence of unsuccessful cell cycle exit (10, 52). Cyclin-CDK complexes have also been shown to negatively control cellular differentiation *via* transcriptional regulation (Table 1). For example, cyclin D1-CDK4 phosphorylates RUNX2, which is a bone- and cartilage-specific transcription factor. RUNX2 Ser472 phosphorylation by cyclin D1-CDK4 results in a ubiquitin-dependent degradation of RUNX2 and prevents chondrocyte maturation (48, 49). Cyclin B/A-CDK1 and cyclin A-CDK2 negatively control neuronal differentiation by phosphorylating Sox2 at Ser39 and enhancing SOX2 activity in suppressing neuronal differentiation (50, 51). Cyclin D1 has been shown to be a positive regulator for astrocytes. Neural stem cell (NSC) differentiation into astrocytes requires the expression of cyclin D1. Cyclin D1 knockout mice could not differentiate to astrocytes; however, neuron differentiation appears to be normal (56). Since cyclin D1 knockout NSCs are arrested in G1, it is unclear whether the compromised astrocyte differentiation is a consequence of a disturbed NSC cell cycle.

Several reports have produced evidence in support of a prominent role for CDK6 in differentiation. Recent data have indicated that CDK6 is not essential for most mammalian cell cycles and may only be essential in some specialized cells (57). Mature erythroid cells require CDK6 transcriptional activity to control skeleton structure dynamics. Cdk6 is partly associated with the cytoskeleton of erythrocytes, and it directly regulates the genes involved in cytoskeleton organization (such as Gelsolin, Tubulin alpha-8, Baiap2, and Pip5k1b) in a kinase-dependent manner. The CDK6 knockout erythroblast showed several clear defects in the cytoskeleton architecture and functions (35). CDK6 has also been demonstrated to have a unique binding partner that does not overlap with CDK4. The EYA2 protein has been identified as a direct CDK6 binding partner by using a yeast 2-hybrid screen. This interaction with CDK6 leads to EYA2 protein degradation. The EYA2 protein is a transcriptional coactivator that is essential for multiple organ development, such as the developmental programs of the eyes, ears, kidneys, muscles, and nervous system. Its degradation abolishes the balance of differentiation (58, 59). Thus, these findings indicate that CDK6 may be a major transcriptional regulator and part of the conserved network of transcription factors regulating the differentiation of several tissues.

Mammary epithelial cell differentiation is a result of a collaboration between isoforms of the

C/EBPβ transcription factors, namely, LAP1, LAP2, and LIP. Cyclin D1 binds to LAP1, an event that causes activation of the transcriptional function of LAP1. Cyclin D1 binding relieves the LAP1 self-inhibited state. This cyclin D1-mediated activation of LAP1 has been shown to participate in mammary epithelial cell differentiation (60).

The expression of a positive regulator for the cell cycle may result in the halt of cell division and the facilitation of differentiation. Upon MYC overexpression, cyclin E is accumulated and induces a mitosis block. Consequently, keratinocytes could undergo differentiation. In this context, activations of MYC and cyclin E may trigger a tumor suppressor mechanism, in turn forcing aberrantly growing cells into differentiation (61).

The molecular composition of the postsynaptic protein composition is modified during synaptic plasticity, which forms the molecular basis of learning and memory. Changes in synaptic composition depends in part on the intricate regulation of phosphorylation of specific proteins *via* different protein kinases, including PKA, PKC, PKB, CaMKII, and Cdk5. A high level of cyclin E is known to express in the quiescent, post-mitotic cells in the nervous system, which has a very minimal proliferative potential. Recently, cyclin E has been demonstrated to form a complex with Cdk5 that restrains Cdk5 kinase activity in the cells of the nervous system. This process prevents CDK5-dependent phosphorylation on NR1, a subunit of N-methyl-D-aspartate (NMDA) receptors, consequently changing the surface localization of NR1. Cyclin E binding to CDK5 also reduces the neuronal CDK5 kinase activity toward Wave1 and Synapsin I, two known Cdk5 synaptic targets, allowing the two proteins to function. Wave1 promotes dendritic spine maturation and is essential for hippocampal-dependent learning and memory. Loss of cyclin E results in the reduction of neuron numbers and memory impairment (62, 63).

Exiting from the cell cycle seems to be a prerequisite for differentiation. Constraining hyperproliferating cells like cancer cells into becoming terminal differentiated cells is one of the possible approaches for cancer therapy. Persisting expressions of some cell cycle regulators are known to impede differentiation. In the leukemic murine erythroleukemia (MEL), which is a transformed erythroid precursor model, blocking of G1 CDKs, namely CDK2 and CDK6, triggered terminal differentiation *in vitro* and loss of tumorigenicity (64).

Likewise, the expression of some of the CKIs is considered a crucial step for differentiation. A lack of $p18^{Ink4c}$ and $p19^{Ink4d}$ results in infertility, specifically, in male mice with a normal level of luteinizing hormone. This finding argues for the essential role of the Ink4 proteins in male spermatogenesis (65). $p18^{Ink4c}$ was also found to work collaboratively with $p27^{Kip1}$ to control the timing of a neuron cell oligodendrocyte differentiation under thyroid hormone stimulation (66, 67). In this context, the activity of $p27^{Kip1}$ depends on its protein level. $p27^{Kip1}$ is progressively accumulated in the oligodendrocyte until reaching a certain level, when it triggered differentiation (68).

The differentiation control by $p27^{Kip1}$ appears to be cell-type specific. $p27^{Kip1}$ has a positive role in Schwann cells (69) and erythroid cell differentiation (70, 71), whereas the differentiation of neuroblastoma cells and memory CD4 T cells are found to be constrained by the $p27^{Kip1}$ protein level and activity (72).

Lastly, during osteoclastogenesis, the development of bone resorbing multinucleated cells, $p21^{Cip1}$ and $p27^{Kip1}$, spike upon treatment of the osteoclast differentiation factor and TNFα. Inhibitions of $p21^{Cip1}$ and $p27^{Kip1}$ by antisense oligonucleotide abolish the effect of the osteoclast stimulating factor (58). Although the mechanism underlying the differentiation-promoting role

of these CKIs is still unclear, the findings suggest that there is a crucial role of these two CKIs in osteoclasts.

A distinctive characteristic of cell cycle regulators in maintaining the terminally differentiated cells can be observed at the *in vivo* level in knockout animals. $p27^{Kip1}$-deficient mice have a significant enlargement of organs (such as the spleen; the thymus, pituitary, and adrenal glands; adipose tissue; and the gonadal organs), and they may develop pituitary tumors spontaneously (73-75). Mice lacking both $p27^{Kip1}$ and $p21^{Cip1}$ ($p21^{-/-}$, $p27^{-/-}$) have an extended proliferative life span of differentiating ovarian granulosa cells (76). Another CKI, $p18^{Ink4C}$, also plays a role in restricting the number of differentiated cells. $p18^{Ink4C}$-deficient mice undergo normal development well into their adulthood. However, $p18^{Ink4C}$-deficient mice display widespread organomegaly, specifically, in the spleen and in the thymus, adrenal, and pituitary glands (77). On the other hand, *CDK4*-deficient animals have a defect in the expansion in a set of differentiated cell types, such as somato/lactotrophs (78). Likewise, there have been reports that CDK4 and cyclins D2 are essential for pancreatic beta-cell expansion in adult mice (79, 80). Such evidence is exemplary of the unorthodox role of specific cell cycle proteins in the expansion of already differentiated cell populations for animals in response to development and the environment, and in controlling the proliferation-versus-differentiation balance.

All in all, cell cycle regulators are an integral part of tissue differentiation. They play both direct and indirect roles in controlling differentiation programs, and in creating a suitable cellular context for cells to undergo differentiation. In addition, they are an essential apparatus which is utilized to maintain the requisite numbers of the terminally differentiated cell populations of various tissues.

3. Energy controlling role of cell cycle regulators

Biological processes are driven by cellular fuels. Cells produce energy from nutrients, utilize it, and continually recycle the fuels *via* several pathways. Although the programs for the metabolic regulation may vary among each cell type in order to respond to the environment and maintain homeostasis, these processes are invariably tightly controlled. Generally, in highly proliferative cells, rapid energy consumption is observed. These cells usually utilize energy produced from glycolysis, which is in contrast to differentiated cells, which normally rely on oxidative phosphorylation to generate ATP (81). Therefore, regulation of cell fate is coordinated with the metabolic state. From this view, it is sensible that the energy production and cellular metabolism processes be coregulated by a set of cell cycle machineries.

The cellular metabolic control mostly relies on the activities of CDK1, CDK4, and CDK6 in both kinase-dependent and -independent manners. The connection between the two processes is evident even in organisms of low complexity. A study in yeast demonstrated that the cell cycle and mitochondrial regulations were connected by mitochondrial protein import. The yeast cyclin Clb3-activated Cdk1 phosphorylates and stimulates an assembly of the mitochondrial entry gate Tom6 protein. The activation leads to the formation of a protein import channel, which promotes the protein importation for mitochondria, especially during mitosis (82). In mammalian cells, a fraction of the cyclin B1-CDK1 complex is localized in the mitochondrial matrix, and it appears to play an essential role in energy control there. The phosphorylation of mitochondrial protein complex I, mediated by the cyclin B1-CDK1 complex, enhances the complex I activity and energy production (83). Particularly under conditions of genotoxic stress, when cells need more energy to drive DNA repair, cyclin B1-CDK1 enhances mitochondrial bioenergy production by mitochondrial complex I phosphorylation (84). In neurons, the mitochondrial activity of cyclin

B1-CDK1 is kept low to maintain the normal mitochondrial function. Excitotoxic damage triggers a buildup of mitochondrial cyclin B1 and promotes CDK1-mediated phosphorylation of the antiapoptotic protein Bcl-xL, leading to mitochondrial inner membrane depolarization and neuronal death (85, 86).

The cyclin D1-CDK4 complex has a remarkable role in energy and metabolism control. AMP-activated protein kinase (AMPK) is a master regulator of energy sensors. A subunit of AMPK, AMPKα2, is directly phosphorylated by the cyclin D1-CDK4 complex. The phosphorylation leads to AMPK inhibition, suppression of fatty acid oxidation, and a shift of the energy production toward anaerobic glycolysis (87). At the physiological level, CDK4 is also found to have a cell cycle-independent role in regulating insulin secretion and response. The CDK4-pRB-E2F1 axis cooperates to control the expression of Kir6.2, a key component of glucose-induced insulin secretion. Inhibition of CDK4 or the genetic inactivation of E2F1 results in a decreased expression of Kir6.2 and impaired insulin secretion, triggering glucose intolerance in mice (88). A repressive form of pRB-E2F is required to suppress oxidative metabolism. CDK4 kinase activity relieves the suppression, allowing cells to switch on the oxidative metabolism when adaptation to energy demand is required (89). CDK4 can also be activated in response to insulin and glucagon in adipocytes (90) and in hepatocytes (91), leading to an E2F-mediated activated gluconeogenic profile, higher Pck1 and G6pc gene expressions, and increased glucose production (92). These findings draw a picture of CDK4-pRB-E2F as a central coordinator that orchestrates energy utilization and cell cycles.

When CDK4 and E2F are active, cells require only a certain amount of energy. In stress conditions such as starvation, the cell cycle machineries respond to the stress by switching the cells to high energy production. Under such conditions, the activities of CDK4 and E2F are low, permitting a release of the oxidative pathway genes to overcome the lack of nutrients. Remarkably, the regulation of gluconeogenesis by the CDK4-pRB-E2F axis is able to elevate blood glucose, demonstrating the role of the cell cycle regulators in metabolic control beyond the cellular level. The role in metabolism at the physiological level may be assigned for the cell cycle regulators in multicellular organisms. Thus, in such contexts, the cell cycle regulators are able to exert their activity toward other cells.

CDK4 is also reported to couple with cyclin D3, playing a crucial role in inhibiting lipolysis and supporting lipogenesis in white adipose tissue, resulting in the prevention of insulin resistance. This outcome is mediated through the CDK4-mediated phosphorylation on insulin receptor substrate 2 (IRS2). This may explain why CDK4 null mice have a reduced body size and develop diabetes mellitus at an early stage of life (93). Likewise, the expression of the pancreatic-specific constitutively active CDK4 in obese mice (*db/db-CDK4*^{R24C}) reduces the progression of diabetes (90).

Interestingly, cyclin D1 is expressed in quiescent-stage liver tissue, suggesting the cell cycle-independent role of cyclin D1 in this tissue. The peroxisome-proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a key mediator that links the insulin response to glucose and lipid metabolism. It has been identified as a direct substrate of the cyclin D1-CDK4 kinase. CDK4-mediated PGC-1α phosphorylation controls its transcriptional activity. The cyclin D1-CDK4 complex suppresses the glucose-producing and oxidative phosphorylation-related genes *via* inhibition of PGC-1α (94). Not only does the cyclin D1-CDK4 complex control the gluconeogenesis by direct phosphorylation, the results of a cell-based high-throughput chemical screening revealed that the complex also indirectly controls PGC-1α by posttranslational modification. Phosphorylation of a histone acetyltransferase GCN5 by cyclin D1-CDK4 results

in increased GCN5 activity, thereby promoting GCN5-dependent PGC-1α acetylation and downregulation of PGC-1α activity, leading to suppressed gluconeogenesis in hepatic tissue (95). Thus, the overall actions of CDK4 are likely to increase insulin sensitivity and to conserve energy, simultaneously, by controlling the lipid and glucose-metabolism homeostasis. From this view, cyclin D1-CDK4 may have a function in energy preservation in post-mitotic cells.

Independently of its CDK4 counterpart, cyclin D1 has an additional function in controlling lipid metabolism and mitochondrial-related energy production. Deletion of cyclin D1 causes hepatic steatosis in mice. This is a consequence of its inhibitory effect on the transcription factor peroxisome proliferator-activated receptor γ (PPAR γ), which induces adipocyte differentiation and lipid oxidation. Cyclin D1 directly binds and sequestrates the DNA binding domain of PPAR γ using its helix-loop-helix region (96) (97). One study suggested that there is a CDK4-independent function of cyclin D1 on PPAR γ regulation. Cyclin D1 enhances the recruitment of histone deacetylase HDACs and histone methyltransferase SUV39H1 to regulate the PPAR response element, thereby repressing PPAR γ activity on those promoters (98).

Therefore, by its CDK4-dependent and -independent mechanisms, cyclin D1 creates a multi-leveled regulation of adipogenesis, which ensures the suppression of the adipocyte differentiation of fibroblasts and liver cells. The suppression also ensures the uncoupling of cell proliferation and fat differentiation.

Mitochondria are central players which regulate the homeostasis of cellular survival through energy production and control cell death by mitochondrial membrane-dependent death signals. A lack of cyclin D1 promotes an increase in mitochondrial numbers and functions *in vivo,* suggesting a connection of cyclin D1 to mitochondrial regulation (99). Cyclin D1 controls mitochondrial abundance and functions in both kinase-dependent and -independent ways. In the kinase-dependent manner, cyclin D1 couples with CDK4, which phosphorylates the nuclear respiratory factor 1 (NRF1), in turn restraining the nuclear-encoded mitochondrial genes (100). In the kinase-independent manner, cyclin D1 physically binds to the voltage-dependent anion channel and competes with hexokinase 2. The binding leads to a blockage of this channel and inhibits mitochondrial importation, thus reducing energy production (101). The activity of cyclin D1 in mitochondrial regulation is found in both normal and cancer cells. Several mechanisms leading to mitochondrial suppression by cyclin D1 highlight cyclin D1 as a major protein regulating energy production. This may be one of the explanations why cancer cells usually express an elevated level of cyclin D1 protein compared to normal cells: the overexpression of cyclin D1 may facilitate cancer formation by rewiring the energy balance in the cancer cells.

The list of cell cycle regulators controlling energy metabolism is not restricted to only cyclin D1 and CDK4. The metabolic regulation was recently shown to be driven by another G1 cyclin D-CDK set: cyclin D3 and CDK6. A study reported that in cancer cells containing high levels of cyclin D3-CDK6 activity, the cyclin D3-CDK6 complex phosphorylates 2 key enzymes in the glycolytic pathway, 6-phosphofructokinase and pyruvate kinase M2. The phosphorylation leads to an inhibition of enzyme activity, shifting the glycolytic intermediates into pentose phosphate (PPP) and serine, thereby allowing cyclin D3-CDK6-high cancer cells to survive oxidative stress (102).

4. Epigenetic and stem cell regulatory roles of cell cycle regulators

Stem cells are defined by their self-renewal ability and capacity to differentiate into several tissue types when needed. The control of the self-renewal and cell fate determination of stem cells is

closely related to the regulation of the cell cycle. When converting to differentiated cells, stem cells require extensive changes in their gene expression program as well as their epigenetic profile.

Cellular potency is characterized by the self-renewal and differentiation potential of stem cells. Embryonic stem cells (ESCs) are pluripotent stem cells which can potentially turn into any cell type. Adult stem cells are either multipotent or unipotent, and they can only differentiate into tissue-specific cell types. The potency is associated with the cell cycle regulation. Normally, pluripotent stem cells have a shortened G1 phase and lack a G1 checkpoint (103). Therefore, the control mechanism of embryonic stem cells is mostly driven by G2/M-phase machinery such as CDK1.

Results from knockout mouse studies have demonstrated that mouse development can take place without CDKs, except for CDK1. The targeted deletion of CDK4, 6, or 2 resulted in viable mice. However, CDK1-deficient zygotes died early at the 2-cell stage, suggesting a critical requirement for CDK1 in early mouse development and embryonic stem cell survival (104). As to the totipotent embryonic stem cells, in order to maintain their stemness, they require the expression and function of CDK1. The downregulation of CDK1 by siRNA induced mouse embryonic stem cell differentiation (105). A part of CDK1's function in stem cells is to preserve the stem cell DNA. CDK1 is found to contribute to the repair of double stranded breaks by a homologous recombination (HR). As ES cells are prone to DNA damage from fast proliferation, their DNA damage response, which comprises DNA-damage checkpoints and DNA repair, needs to be highly effective; it repairs the stem cell damaged DNA or those subject cells that are not fixable to apoptosis during the differentiation process (106). CDK1 depletion sensitizes the cells to severe DNA damage and G2 arrest upon irradiation (107). Similarly, treating ES cells with a CDK1 inhibitor induces DNA damage and the DNA damage response following chemically-induced DNA double stranded breaks, indicating that the DNA damage response function of CDK1 in stem cells is mediated by its kinase activity. CDK1 inhibition in stem cells promotes p53 activation and the downregulation of the antiapoptotic protein MCL1, leading to stem cell programmed cell death. This is specific to stem cells since it has not been observed in differentiated cells (108).

By reducing CDK1 activity to a level that has no effect on cell cycle progression, it has been demonstrated that CDK1 kinase activity is required for the pluripotency of hESCs. In addition, it has been shown that the activity of CDK1 maintains PDK1 phosphorylation and the full activity of the PDK1-PI3K/Akt kinase cascade, which is essential for stem cell pluripotency. Moreover, cyclin B1 stimulates a higher cellular level of LIN28A, a factor that can induce stem cell reprograming. This report also indicated that the stemness-promoting function of CDK1 is independent of its cell cycle function (109).

CDK1 itself can exert its effect on stem cell control in a kinase-independent manner. This has been demonstrated in the trophectoderm differentiation model. By directly binding with Oct4, the core transcription factor for embryogenesis, and enhancing the repression effect of Oct4 to the trophectoderm Cdx2 promotor, CDK1 prevents stem cells from undergoing trophectoderm differentiation. This function has been shown to be specific to only CDK1, not CDK2 or CDK4 (110).

Adult stem cells are the population of undifferentiated cells found among differentiated cells in the body. These cells still retain the ability to divide or self-renew when needed, for example, in the situation where cell dying occurs or there is tissue injury. However, the self-renewal ability

is limited to cell types of the organ from which the cells originated. To balance the tissue homeostasis, the regulation of adult stem cells requires tightly controlled mechanisms composed of transcription and epigenetic processes. Studies of mammalian adult stem cells are mostly performed with blood, skin, intestinal, and muscle stem cells. Hematopoietic stem cells (HSCs) have long been recognized as being able to remain in the resting (quiescent) state. HSCs can be divided into four subtypes, according to their ability to repopulate and retain a life-time blood production capacity. Thus, HSCs are generally in a heterogeneous pool. The ability of HSCs to exit from quiescence upon mitogen stimulation has been shown to be driven and fine-tuned by the expression level of CDK6. HSCs need a low level of CDK6 in order to maintain the quiescent state. Induction of the CDK6 level coupled with cyclin D1/D3 accelerates G0 exit (111). Another study reported that CDK6, independent of the kinase activity, acts as a transcriptional repressor that binds to the Egr1 promoter and represses Egr1 expression. This function of CDK6 is essential in Egr1 downregulation, a process necessary for HSCs or leukemic stem cells (LSCs) to exit quiescence and form colonies. Deletion of CDK6 in the stem cells reduces their repopulation ability. In the BCR-ABL^{p210+} leukemic stem cell model, *CDK6*$^{-/-}$ HSCs are unable to induce disease (112).

Even though the pluripotent stem cells are believed to only have a brief G1 phase, and the pRB-inactivating function of the G1 cyclins may appear dispensable, the expressions of G1 cyclins (including cyclin D1/D2/D3 and cyclin E1/E2) control the embryonic stem cell fate by coupling with their catalytic CDK partner to phosphorylate and stabilize the core stem cell transcription factors (Nanog, Sox2, and Oct4). G1 cyclin-deficient ES cells are predisposed to trophectodermal differentiation (113).

The reprogramming of somatic cells to induce pluripotent stem (iPS) cells is influenced by the expression of cyclin D1 and CDK4 activity. At the initial phase of reprogramming upon induction by Yamanaka factors, cyclin D1 is induced and physically binds to *Pax6* and *Nanog* promotors through NF-Y interaction. This results in suppressed *Nanog*, but activated *Pax6* expressions. Consequently, it leads to neural progenitor fate commitment and impaired pluripotency potential (114). Clues for the involvement of cyclin D1 in tissue-specific stem cells has been observed in knockout animals. Genetic deletion of cyclin D1 results in compromised organ development in the retina, and in the mammary and pituitary glands (32, 33). The retinal defect in cyclin D1-deficient mice was a result of prolonged retinal progenitor cell division and an early exit from the cell cycle. This cannot be rescued by the re-expression of cyclin D2, indicating that it is cyclin D1-specific (115).

In pluripotent human embryonic stem cells, cyclin D2 is prominently expressed, but cyclin D1 takes over from cyclin D2 during differentiation. Cyclin D2 expression facilitates p220(NPAT) phosphorylation, a step essential for stem cell renewal. Depletion of cyclin D2 or p220(NPAT) causes decreased cell cycle-dependent histone H4 expression and reduced S phase progression. Therefore, cyclin D2 is a principal cell cycle regulator that determines competency for self-renewal in pluripotent hES cells (116).

In breast cancer, the expression of the low molecular weight cyclin E (LMW-E) and the kinase activity of LMW-E-CDK2 have been shown to increase breast cancer stem-like cells (or CD44^{high}/CD24^{low} population) and induce epithelial-to-mesenchymal transition. This function is mediated by the phosphorylation of histone acetyltransferase (HAT) Hbo1 at Thr88 (117).

In order to exit the pluripotent state and complete differentiation, a large number of epigenetic changes are involved (118). Generally, differentiating cells are more reliant on gene silencing

than stem cells. This epigenetic landscape switching has been shown to be influenced by the activity of CDKs as well. CDK1 and CDK2 can phosphorylate the enhancer of zest homologue (EZH2), a protein essential for the histone H3 lysine 27 trimethylation (H3K27me3) and epigenetic gene silencing. The CDK1-mediated phosphorylation of EZH2 increases binding to the PRC2 recruiter and provides an epigenetic gene silencing profile suitable for the proliferation, migration, and blocking of differentiation (119, 120). CDK2 phosphorylates the histone methyltransferase Suv39H1, a heterochromatin regulator. The phosphorylation promotes Suv39H1 dissociation from chromatin and enhances occupancy of JMJD2A histone demethylase, resulting in altered inactive histone marks. Inhibition of the CDK2 phosphorylation on Suv39H1 leads to replication stress and reduced heterochromatin replication (121).

5. Roles of cell cycle regulators in balancing immune cell pool and functions

Several G1 cyclins and CDKs have been reported to play a role supporting the functions of immune cells and the immune system. The development and activation of cells in the immune system take place not only during embryonic development; rather, they persist dynamically throughout the life of multicellular organisms. Cell cycle regulators are involved in the complex development of cells in the immune system, partly as the machinery controlling the expansion of the immune cell population. However, the non-cell cycle functions of these proteins are also clearly implicated.

To shape a fully activated and functionally active lymphocyte to protect the body from pathogens, several steps need to be completed, ranging from antigen presenting processes to lymphocyte maturation. These processes require intense and repeated rounds of proliferation of the immune cells for a vigorous immune response (122). In some circumstances, the proliferation of immune cells also has to be restrained, such as upon infection. Thus, both positive and negative cell cycle regulators have a direct role in balancing the immune cell pool.

Macrophages are buried inside every tissue in our body, awaiting invading pathogens. They are an important mediator in the innate immune response to defend against pathogens such as bacteria. Several processes that macrophages contribute to in the defensive mechanism are phagocytic, and they have recently been found to be involved in innate immunological memory (123-125). Upon infection, the release of INFγ from activated T lymphocytes triggers macrophages to undergo full activation by modulating the proliferation of the macrophages. INFγ induces $p21^{Cip1}$ expression; consequently, macrophage cells undergo cell cycle arrest and escape from apoptosis. In a presence of another survival factor, the macrophage colony-stimulating factor (M-CSF), $p21^{Cip1}$ is also induced through the PI3K/Akt kinase pathway to protect macrophages from apoptosis (126, 127). The important of upregulating $p21^{Cip1}$ in controlling macrophage activity was also noticeable when mice that lack $p21^{Cip1}$ appeared to be more susceptible to LPS-induced septic shock with increasing levels of inflammatory factor IL-1β. The cytokine IL-1β is released from innate immune cells, such as macrophages, to further trigger self-stimulation and other immune cells, including neutrophils and monocytes. $p21^{Cip1}$ suppressed IL-1β at both the transcription and pro-protein levels, suggesting a role for $p21^{Cip1}$ in restraining excessive macrophage activation (128). Another CKI, $p16^{INK4a}$, was also found to play a macrophage inhibitory role by promoting LPS-triggered IL-1R-associated kinase 1 (IRAK1) degradation and impaired IL-6 production (129). The ability to infiltrate into tissue is one of the characteristics of macrophages. This ability is crucial as an initiation for the immune response as well as the inflammatory response. Two types of migration are employed by macrophages: amoeboid-like and mesenchymal. Amoeboid migration is used when migrating through loose tissues, whereas mesenchymal migration is used when migrating into a dense

matrix such as a tumor mass. A CKI, $p27^{Kip1}$, suppresses ROCK-mediated amoeboid migration and promotes mesenchymal migration, which is important for tumor infiltration, independent from its cell cycle inhibition (130) (Figure 1a).

The main function of B cells is to generate a specific immune response to pathogens by producing immunoglobulins (Igs). Several steps are required to acquire a fully activated B cell (plasma cell), including cell Ig class switching, clonal expansion, and clonal selection.

Cyclin D2 is important for B cell progenitor survival in bone marrow. Cyclin D2-deficient mice only have half the number of $Sca1^{+}B220^{+}$ progenitor cells (131). B cells can be activated by B cell receptor (BCR) ligation to promote proliferation and trigger apoptosis. The process needs cooperation between the cell cycle control and the antiapoptotic signal during the immune response. NF-κB/Rel signaling downstream of BCR activation promotes B cell development by regulating cyclin E-CDK2 activity, which in turn supports B cell survival by regulating the antiapoptotic protein Bcl-XL (132).

Following T cell antigens presenting in secondary lymphoid tissue, clonal expansion of B cells takes place by rapid proliferation to form a germinal center (GC). Within the GC, B cells undergo class switching, clonal expansion, and clonal selection in order to generate a high-affinity humoral immune Ig. Expression of the cyclin D3 is essential for the proliferation and Ig class switching of the activated B cells in the GC. Deletion of the cyclin D3 impairs GC development and class switching, leading to a compromised T cell-dependent antibody response (133, 134). A study by Minghui *et al.* revealed that the class switching process actually occurs in the late G1 to early S phase, and that it is mediated by the kinase activity of CDK2. CDK2 recruits the activation-induced cytidine deaminase (AID), a key regulator of class switch recombination, to a switch region DNA. Moreover, by replacing wild-type CDK2 with analog sensitive CDK2 (CDK2AS), which can be controlled by adenine analog, it has been demonstrated that CDK2 retains the nuclear level of AID (135). However, the precise mechanism of CDK2 in controlling AID is unclear. The terminal differentiation of the B cells is accompanied by a high level of antibody production and B-cell cell cycle arrest. A CKI, $p18^{INK4c}$, plays an essential role in the final maturation and Ig secretion of plasma B cells by inducing cell cycle arrest through CDK6 inhibition (136, 137). The role of CKI $p27^{Kip1}$ in mediating plasma B cell maturation has also been reported. The expression of $p27^{Kip1}$ seems to be a barrier for B cell proliferation and activation (138-140). Perhaps the cell cycle control of B cells is a dynamic and cooperative work between various CKIs, depending on the timing and context of activation during each stage of B cell development (Figure 1b).

When T lymphocytes become activated through the antigen receptor, this can give rise to two outcomes: cytokine responsive or unresponsive. The expression of CDK4 is required to respond to the IL-2 cytokine, which leads to T cell proliferation (141). T cell expansion is also controlled by CKIs. For example, $p18^{Ink4c}$ and $p27^{Kip1}$ act to restrict T cell proliferation. However, in some conditions like graft-versus-host disease, an inflammatory disease induced by alloreactive T helper cells, the expression of $p27^{Kip1}$ but the absence of $p18^{Ink4c}$ are required to abolish the immune response (142, 143). After clonal selection and expansion, some clones remain as a memory T cell. The CKI $p27^{Kip1}$ is upregulated to arrest cells in the G0/G1 phase along with the upregulation of Bcl-2 expression, believed to facilitate cells' resistance to apoptosis (144).

The peripheral tolerance of T cells is an important process to control the homeostasis in T cells. This process is driven mainly by the programmed cell death-1 (PD-1) receptor or CD279 to inhibit T cell proliferation. Although having a crucial role in restricting excess T cells, PD-1 is

found to be a major problem that compromises the T cell antitumor response. PD-1 signaling directly controls multiple cell cycle machineries to block the cell cycle at the G1 phase. PD-1 blocks cell cycle progression through suppression of SKP2 ubiquitin ligase, which leads to accumulation of $p27^{KIP1}$ and, consequently, an inhibition of retinoblastoma phosphorylation. PD-1 also triggers the upregulation of another CDK4/6 inhibitor, $p15^{INK4b}$, and suppresses the Cdk-activating phosphatase, Cdc25, *via* SMAD3 transcriptional activity (145, 146).

Deregulation of cell cycle regulators has also been shown to play a role in pathological immune cells. The normal B cell cycle is mostly driven by a G1 cyclin, cyclin D3. However, once the cell become cancerous, several transitions occur intrinsically that change the dependency of immune cells to rely on alternative molecules. The chromosome translocation of *CCND1* accounts for an overexpression of cyclin D1 in mantle cell lymphoma, while the normal counterpart B cells, naïve B cells, express cyclin D3 (147). Likewise, cyclin D1 is also found to be overexpressed in peripheral T-cell lymphoma by the chromosome translocation-independent mechanism (148). In chronic lymphocytic leukemia cells (B-CLL), cyclin D2 and cyclin D3 are both upregulated upon stimulation by cytokines along with the down regulation of $p27^{KIP1}$ to exert its rapid proliferation (149). These data suggest that the cyclins perhaps hold a driving role for the development of these immune cells, and that the deregulation of these proteins was enough to disrupt the normal differentiation/function of these immune cells.

Immune evading is one of the hallmarks of cancer. Cancer of the immune cells is likely to shift to utilize G1 machineries in order to proliferate and survive. As a result, arresting cells in the G1 phase by treatment of the small molecule CDK4/6 inhibitor is now an attractive strategy to treat cancer of the immune cells. For example, using the CDK4/6 inhibitor constrains the cell cycle entry and disease progression of T cell acute lymphoblastic leukemia (T-ALL). The effectiveness of the treatment may be a result of a dependency on cyclin D3 overexpression in these cells (150, 151). Recently, several independent reports have demonstrated a critical requirement of CDK4/6 activity in tumor immunosurveillance by using small molecule CDK4/6 inhibitors. PD-L1 is a surface antigen that expresses on the tumor cell membrane. This ligand is believed to mediate suppression of immune cells by inducing T cell exhaustion. Protein abundance of PD-L1 is an indicator for predicting the response to PD-L1 inhibition treatment. The higher the PD-L1 expression, the better the cancer response to the checkpoint inhibitors. The ligand abundance is found to be regulated *via* cyclin D-CDK4 kinase activity on the proteasome degradation-related protein SPOP. Therefore, treatment by the small molecule CDK4/6 inhibitor increases the PD-L1 protein and enhances the response to the anti PD-1 immunotherapy (152). Inhibition of the CDK4/6 activity also stimulates cancer cell production of endogenous retroviral elements, leading to enhanced tumor antigen presentation (153).

The CDK4/6 inhibitor not only acts on tumor cells, but also acts on the immune cells that are responsible for antitumor immunity. The CDK4/6 inhibitor suppresses proliferation of the regulatory T cell (Treg), an immunosuppressive T cell, and relieves the Treg inhibition on T cells (153). Inhibition of CDK4/6 can result in the relief of the NFAT family proteins and their target genes, critical regulators of the T cell function, which in turn increases tumor infiltration and activation of effector T cells. Therefore, the blocking of CDK4/6 activity on T cells facilitates the cytotoxic activity of T cells to kill tumor cells (Figure 1c) (154).

6. DNA damage response role of cell cycle regulators

Unfixed DNA damage can lead to various outcomes, ranging from the silencing of mutations to misfunctioning proteins or fault messages, which can be passed to daughter cells during the cell

division process. Therefore, it is crucial for every cell to have an effective DNA damage response (DDR) that can effectively prevent the passing on of faulty messages to daughter cells. DDR consists of a sensory mechanism which detects DNA lesions and conveys distress signals to the DNA repair proteins and the cell cycle machineries. Two processes need to coordinate to ensure that the damaged DNA has been repaired before the cell division continues. Once the repair has been completed, the DDR is turned off, and the cells resume the cell cycle. Thus, DNA repair and cell cycle regulation must have a close relationship and work well collaboratively in order to keep the DNA information intact. Many types of DNA damage repair are employed, depending on whether the DNA lesions are a single- or double-stranded break (DSBs), and in what phase of the cell cycle the DNA-stranded break has occurred. Normally, the type of DNA double-stranded break responses is more dependent on the cell cycle phase. The two major types of repair are the non-homologous end-joining (NHEJ) repair, which directly joins the DNA ends where it is broken, and the homologous recombination mediated repair (HR), which utilizes the sister chromatid as a template for the process, thereby giving a more precise repair (155).

Early discoveries of the role of cell cycle regulators in DDR were demonstrated in yeast. Upon DNA damage, a group of proteins that sense DNA lesions are activated and decide whether the cell will use either an NHEJ or HR repair. The decision-making involves DNA resection, which can modify the broken DNA ends to be more favorable toward HR while at the same time be unfavorable for NHEJ repair. DNA resection is performed by the Mre11-Rad50-Xrs2 (MRX) protein complex. The resection activity of this complex is driven by the Cdc28/Cdk1-mediated phosphorylation of Xrs2 in the cell cycle-dependent manner, thereby enabling the highest activity of the MRX resection complex during the G2/M phase of the yeast cell cycle (156). As a result, the resected DNA ends are preferably repaired by HR during G2/M.

In mammalians, the homolog of the yeast MRX complex, the MRE11-RAD50-NBS1 (MRN) complex, plays a role in DSBs by resecting the broken ends and facilitating recruitment of a checkpoint protein, ATM kinase, and its targets. Similar to the Xrs2 protein in yeast, NBS1 is regulated by cyclin B-CDK1. Phosphorylation on NBS1 Ser432 by CDK1 triggers the transformation of the DNA structure at the breaks into one that is favorable for HR repair. In addition, the NBS1 phosphorylation allows resumption of DNA replication after replication-fork stalling (157). During the DNA ends resection, the CtIP protein is recruited to MRE11 and NBS1 by CDK1/2 phosphorylation. CtIP phosphorylation by the CDKs is a prerequisite for CtIP phosphorylation by ATM, which in turn will activate a second step for ends resection, the long-range resection (158, 159). Moreover, CDK1/2 also phosphorylates nuclease EXO1, an enzyme functioning during the long-range resection. Inhibition of CDK1/2-specific phosphorylation sites on EXO1 blunts the resection and shifts the repair pathway to NHEJ repair (160). Therefore, these findings emphasize the major contribution of CDK1/2 in the choosing of a DNA repair pathway.

Several molecular cascades are involved to ensure that the repair is well-regulated and occurs at the appropriate time. A DNA repair protein breast cancer type 2 susceptibility protein (BRCA2) is one of the targets of CDK1/2. BRCA2 and a recombinase RAD51 co-localize at the DNA lesions where the HR repair occurs. CDK1/2 plays a role to control this interaction by phosphorylating at the carboxy-terminal Ser3291 of BRCA2 to inhibit physical interaction between the BRCA2 C-terminus and RAD51. This inhibitory phosphorylation is needed to prevent unauthorized HR. Phosphorylation of S3291 takes place when the HR repair is not required, and it is maintained during a normal uninterrupted cell cycle. However, when DNA damage occurs, the phosphorylation is decreased, thereby allowing RAD51 to bind to the C-terminus of BRCA2 and activate HR (161).

Not only do the S/G2 cell cycle machineries play roles in this step, but the G1 machinery is also crucial for HR. The G1 cyclin, cyclin D1, was formerly believed to be degraded once the DNA damage response was activated (162). The degradation of cyclin D1 is required to block proliferation and support the proliferating cell nuclear antigen (PCNA) to relocate and repair DNA using HR (163). The degradation of cyclin D1 upon DNA damage has been shown to be mediated by the F-box protein, FBXO31, *via* the proteasome directed pathway (164). Interestingly, DDR-induced cyclin D1 degradation may not be totally true since analyses of cyclin D1-interacting proteins have revealed that cyclin D1 physically binds to RAD51 upon DNA damage, and it is recruited to DNA damage lesions in a BRCA2-dependent manner upon radiation. This function of cyclin D1 has been shown to be CDK4-independent. It has been demonstrated that, at a low dose of ionizing radiation (up to 5 Gy), the cyclin D1 protein was not degraded. However, it switched function from supporting the cell cycle to facilitating HR by recruiting RAD51 to the C-terminus of BRCA2 (165). To do that, cyclin D1 interacts with the C-terminus of BRCA2 using its 20-19 amino acids and precludes the CDK1/2-dependent HR inhibitory Ser3291 phosphorylation. As a result, the binding of the cyclin D1 stabilizes the interaction of the RAD51/BRCA2 complex and promotes effective HR (166). Cyclin D1 has also been found to be a mediator for the hormone-dependent DNA damage response; for example, cyclin D1 is required for dihydrotestosterone-mediated DNA repair, which targets cyclin D1 to DNA lesions and promotes radioresistance in prostate cancer (167). In agreement with the positive role of cyclin D1 in HR, several reports have revealed that cancer cells with cyclin D1 overexpression are more resistant to DNA-damaging agents or radiation therapy, and that depletion of cyclin D1 from the cancer cells sensitized the cells to DNA damage (168-171). The opposite has been found in estradiol (E2)-mediated DNA repair when E2 treatment delays DDR. Cyclin D1 needs to be excluded from the nucleus in order to induce DDR by estradiol upon a single-stranded break caused by UV irradiation (172). Perhaps different levels of DNA damage stress lead to different signaling cascades and protein behavior.

Cell cycle arrest is required for cells to conduct a complete DNA damage response and fix all DNA injuries. Therefore, CKIs play crucial roles in cell cycle arrest upon damage. In order to arrest the cell cycle, DNA damaged cells require the transcription activity of p53, which is activated by the DNA damage checkpoint, to induce the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (173). $p21^{Cip1}$ also directly inhibits the kinase activity of the G1/S cell cycle machineries, cyclin D-CDK4/ and cyclin E-CDK2, and subsequently arrests cells in G1 (174). $p21^{Cip1}$ has been reported to induce G2 arrest by degrading cyclin B1 in response to DNA damage (175). $p21^{Cip1}$ not only blocks the cell cycle during DNA damage but inhibits the DNA replication process by inhibiting the PCNA function without affecting the PCNA-dependent nucleotide-excision repair (176). $p16^{INK4a}$ is activated as part of the DDR as well, and it can cause G1 arrest upon DNA damage (177). Apart from its role in the G1/S checkpoint, $p21^{Cip1}$ has been shown to have a distinct function in promoting HR in an observation that $p21^{Cip1}$-deficient cells could not perform HR, as detected by the GFP reporter assay. In addition, the deletion of $p21^{Cip1}$ disturbed the assembly of the MRN complex by enhancing the CDK1/2-dependent BRCA2 Ser3291 phosphorylation (178). In contrast, another study suggested a role of $p21^{Cip1}$ in augmenting the NHEJ repair. The domain that $p21^{Cip1}$ uses to bind with PCNA is important for the recruitment of the protein and colocalization with Ku80, a protein specializing in NHEJ repair. This suggests an additional role of $p21^{Cip1}$ in initiating NHEJ-mediated DNA repair when cells are stopped in the G1 phase (179) (Figure 2).

Although $p21^{Cip1}$ can potentially exert its functions in a few pathways in the DDR, the physiological role of $p21^{Cip1}$ in DNA damage-mediated cancer formation is still unsettled.

$p21^{Cip1-/-}$ mice are not prone to radiation-induced carcinogenesis, compared to wildtype control (180). Other CKIs that may be involved in DNA repair are $p19^{INK4d}$ and $p57^{Kip2}$. Upon UV irradiation, $p19^{INK4d}$ relocates into the nucleus of neuroblastoma cells, and it protects cells from undergo apoptosis. In addition, its expression enhances DNA repair and decreases chromosomal aberrations. These roles are independent from its CDK4 interaction (181, 182). Recently, $p57^{Kip2}$ has been shown to be an effector of DDR in cancer cells. Upregulation of $p57^{Kip2}$ is found upon exposure to genotoxic stress in a p53-independent manner. The upregulated $p57^{Kip2}$ causes cell cycle arrest, but it protects the cells from apoptosis upon DNA damage. Depletion of $p57^{Kip2}$ sensitizes cancer cells to doxorubicin-induced cell death (183).

Collectively, the roles of the cell cycle regulators in DDR are regarded as necessities that support DNA repair and cell survival during genotoxic stress. Members of the cell cycle protein families mediate signals for the DNA damage checkpoint as well as directly facilitate DNA repair.

DNA repair and the cell cycle processes are both evolutionary conserved. Before the advent of radiation and chemotherapy, DNA replication during the S-phase of the cell cycle was the major source of endogenous DNA damage. Therefore, proteins controlling the cell cycle may need to also oversee DNA integrity. Thus, the role of cell cycle regulators in DNA repair may not be just an odd-job for the cell cycle regulators. However, the physiological relevance of the DNA repair role and its contribution to human diseases will require further evidence and evaluation.

7. Oxidative stress control role of cell cycle regulators

Reactive oxygen species (ROS) are signaling molecules that can influence the vast majority of nuclear and cytosolic processes, leading to cell fate outcomes such as growth, arrest, or death. Naturally, cell cycle regulators are effectors of these signals. The activity of cell cycle regulators is modulated in response to external and internal stimuli during the steps of gene expression, protein production, and posttranslational modifications. ROS or redox signals have been reported as influential in all of these processes (184). Conversely, cell cycle regulators have been shown to control ROS homeostasis. Oxidative stress, a condition in which ROS homeostasis is disturbed, is caused by an imbalance between ROS production and ROS scavenging. To support growth, several cell cycle regulators have been found to play an extra role in controlling the oxidative balance to ensure that the oxidative status of the cells remains in a healthy state.

In Drosophila, the overexpression of cycD-cdk4 (cyclin D and CDK4 in mammalian) promotes the production and activity of mitochondria; however, cycD-cdk4 activity seems to sensitize the flies to the low oxygen condition and reduce their life span. This might result from the excessive mitochondrial oxidative phosphorylation and ROS production that has been found upon overexpression of the proteins (185). The increased mitochondrial oxygen species upon cycD/cdk4 expression were also confirmed in the neuron and shown to cause neurodegeneration (186).

In mammalian cells, as opposed to Drosophila, the cyclin D1-CDK4 complex performs an important role in negatively regulating mitochondrial biogenesis (99, 100). In response to oxidative stress, the cyclin D1-CDK4 complex relocates to mitochondria and exerts redox control by regulating the mitochondrial reactive oxygen species through direct phosphorylation of manganese superoxide dismutase (MnSOD), a major ROS-detoxifying enzyme in mitochondria, thus protecting the cells from oxidative stress. Phosphorylation of MnSOD at Ser106 enhances its antioxidant activity (187). Following IR-radiation treatment, the DNA damage checkpoint is activated and the cell cycle is arrested. Cyclin B1 is redistributed to the cytoplasm and

mitochondria, and it induces changes in the mitochondria, such as a decrease in the MnSOD function, a decrease acronitase function, an increase in complex II activity, and a decrease in membrane potential. Interestingly, this response is reversible. The result demonstrates cross-talk between the DNA damage checkpoint, cell cycle control, and the mitochondrial response to oxidative stress induced by IR, all mediated by a single cell cycle protein cyclin B (188) (Figure 3a).

How cells react to oxidative stress seems to vary from cell type to cell type. For example, skin cells are likely to be more resistant to oxidative stress than other cell types, and cancer cells can tolerate a high level of ROS better than normal counterparts. Within the same cell type, the cellular response can vary depending on the level of the ROS trigger. Some of the underlying reasons for the distinctions may lay in the cell cycle control and the activity of the cell cycle proteins.

Cell fate decisions upon oxidative stress inducer treatment have been reported to be controlled by CDK2. Normal cells respond to H_2O_2 by undergoing senescence; this is in contrast to cancer cells, which show an enhanced cell proliferation. It has been shown that cancer cells can escape from the H_2O_2-induced senescence by elevating the activity of CDK2 (189).

Depletion of cyclin D1 in cancer cells also triggers cellular senescence. The undergoing of senescence by cancer cells is associated with an elevated ROS. Suppression of the ROS in cyclin D1-depleted cells can prevent cancer cell senescence. These results suggest a pivotal role of cyclin D1 in suppressing excessive oxidative stress for cancer by maintaining the ROS at a level that does not cause cell damage. This ROS suppression function of cyclin D1 has been found to be independent of its role in activating CDK4 and inhibiting pRB. Cyclin D1-depleted cells activate the p38-FOXO3a-p27 pathway to promote cancer senescence. This holds true for both pRB-active and -inactive cells (190) (Figure 3a). Cyclin E is another G1/S cyclin that can regulate intracellular ROS. During the terminal differentiation of erythroid cells, cyclin E inhibits mitochondrial ROS accumulation, thereby restraining oxidative stress (191). The CDK-independent role of the cell cycle inhibitor $p16^{INK4A}$ protein in preventing oxidative stress has been investigated. The loss of $p16^{INK4A}$ leads to several phenomena that trigger oxidative stress (for example, the upregulation of ROS formation (192), and increased mitochondrial biogenesis and functions *via* activation of the mitochondrial biogenesis transcription factors PRC and TFAM). It could also facilitate cancer migration (192, 193). These cellular adaptions after $p16^{INK4A}$ loss are indeed an integral part of the hallmarks of cancer transformation (Figure 3a). According to this view, the role of $p16^{INK4A}$ as a gatekeeper for ROS balance may be just as important as its role in restraining cell proliferation because each role has a tumor-suppressing effect. This supports the notion that the loss of $p16^{INK4A}$ is a prerequisite for cancer formation, and it explains why $p16^{INK4A}$ is the most frequently lost tumor suppressor across all types of human cancer. A high level of ROS interferes with several biological processes in the cells because of its ability to react with other molecules; DNA damage and protein dysfunction result from highly reactive ROS. The p38 stress-activated protein kinase (SAPK) is one of the ROS sensor proteins. Once activated by ROS, p38 conveys a message to other target molecules in order that they will respond to oxidative stress by ROS. p38 is found to phosphorylate the cell cycle inhibitor $p57^{KIP2}$ and to enhance its CDK2 inhibition activity, resulting in a delayed cell cycle progression (194) (Figure 3a).

The hypoxia-inducible factor 1 (HIF-1α) is a central protein which has a crucial role in response to the low oxygen condition. HIF-1α supports cellular/systemic survival. HIF-1α is regulated *via* the cellular oxygen amount. A low level of oxygen stabilizes HIF-1α, while a high level of

oxygen triggers proteasome degradation of this protein. A recent report shed light on a novel link between CDKs and HIF-1α. CDK2 and CDK1 can regulate HIF-1α degradation through physical interaction. It is interesting that both cell cycle CDKs show opposite HIF-1α controlling outcomes in that CDK1 inhibits lysosome degradation whereas CDK2 promotes the process. Differential regulation of HIF-1α by CDK2 and CDK1 may implicate a differential role of HIF-1α during DNA replication in the S-phase (where CDK2 activity is dominant) and during hypoxia in the G2/M phase (where CDK1 is dominant; (195) (Figure 3b). However, further investigation is required to understand the physiological context of the control.

Some functions of the cell cycle regulators in controlling oxidative balance are still controversial and need to be further explored. In various contexts, ROS is also necessary for cells to drive many biological processes that are important for cell survival. There is a fine, hypothetical line separating useful and harmful levels of ROS, which also depends on the cellular context. Likewise, equally complicated means of ROS regulation by cell cycle regulators are anticipated.

Conclusions and perspective

The list of novel functions of cell cycle regulators is still being extended as research in this field is very active. Of note, the emerging, non-canonical functions of the proteins have been linked to human diseases, making this new research area relevant and important for the development of a new therapeutic approach for diseases, such as cancer.

Some of the non-canonical functions of the cell cycle regulators overlap with their traditional cell cycle roles. Some of the functions are in cell cycle-connected processes, such as the DNA damage response, energy control, oxidative balance, transcription, and epigenetics; thus, the cell cycle regulators are obliged to be part of the processes to ensure that cell division takes place smoothly. These functions may be critical for multicellular organisms that have relatively long lifespans, during which new cells and parts of tissue have to be constructed, when needed, with accuracy and minimum cellular stress or damage (Figure 4).

In yeast, a relatively simple cell cycle control is steered by a single CDK and a few yeast cyclins. During the evolution from lower to higher organisms, several important CDKs and cyclins developed, in particular, the G1 CDKs and cyclins. They seem to be specific for, and to accompany, the extended G1 phase found in multicellular organisms. Each G1 cyclin and CDK may serve as an essential mediator for specific growth-signaling pathways that differentially regulate each type of cell/tissue in the body, with different outcomes. Hence, cell cycle proteins may have to function not only to keep the differentiated cells safe from apoptosis and to permit differentiated cell expansion, but also to maintain the differentiation status of the cells. Thus, it may be assumed that the spinoff of the G1 cell cycle proteins from the primitive mitotic CDK/cyclins is to serve more meticulous functions in adult multicellular organisms. An argument in favor of this hypothesis is the fact that cell division during early embryo development does not require any G1 cell cycle proteins.

It is interesting that there are several non-canonical functions of the cell cycle regulators that transcend the single-cell level. These functions maintain the homeostasis of the whole organism. Functions related to stem cell maintenance, insulin secretion, and T cell development need interactions between many cell types. It is perplexing how multicellular organisms happened to rely on a set of cell cycle regulators to serve these purposes. The search for the underlying reasons may possibly lead to a better understanding of our evolution and the mechanism of deregulated tissue homeostasis.

Deregulation of the cell cycle regulators is often detected in diseases such as cancer. Evidence indicates that the non-canonical roles of the cell cycle regulators also contribute to the severity of diseases. For example, our recent work, along with others, has shown that the overexpression of cyclin D1 in cancer cells facilitates resistance to DNA-damaging treatments (165). We have also demonstrated that cyclin D1 protects cancer cells from succumbing to cancer oxidative stress (190). These functions of cyclin D1 are independent of its traditional CDK4-pRB-regulating role. These findings highlight that cyclin D1 is a potential target for cancer therapy. In addition, the non-cell cycle function of cyclin D3-CDK6 in cancer metabolism has been proposed as a target for the treatment of cyclin D3-CDK6-high cancers. Inhibition of the CDK6 activity in these cancer types not only blocks the cancer cell cycle but also sensitizes the cancer to oxidative stress-induced apoptosis (102). Recently, the immunomodulatory functions of CDK4/6 have been recognized. The inhibition of the CDK4 activity by small molecule inhibitors has been shown to promote the efficiency of the immune checkpoint inhibitors (152-154, 196).

To date, several specific CDK inhibitors have been developed. Some of these have shown promise for the treatment of many human cancers. It is more difficult to develop specific inhibitors for cyclins which lack enzymatic activity. However, considering the number of non-canonical roles of cyclins, which is very substantial compared to that of CDKs, it may also be worth reconsidering the approach to inhibit these cyclins.

Author Contributions

SJ planed and wrote the manuscript, and PL wrote manuscript. Both authors approved the final manuscript**.**

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Conflict of Interest Statement

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure legends

Fig 1 Roles of cell cycle regulators in immune response

a. Innate and adaptive immune responses need to work collaboratively to achieve a full immune response.

Pathogen-activated T cell stimulates macrophages *via* a cytokine INFγ release to trigger macrophage maturation. The cell cycle inhibitor $p21^{CIP1}$ is upregulated upon stimulation; in turn, it suppresses the macrophage cell cycle and protects the macrophages from apoptosis. $p16^{INK4a}$ inhibits pro-inflammatory cytokine IL-6 production through IRTK suppression. The macrophages' choice of movement is controlled by $p27^{KIP1}$. $p27^{KIP1}$ promotes the tumor-infiltrating mesenchymal migration by blocking the amoeboid-like migration through RACK suppression.

b. Cyclin D2 plays an important role in the survival of the B cell progenitor. NF-κB/Rel signaling downstream of B cell receptor (BCR) activation promotes B cell proliferation by positively regulating cyclin E/CDK2 activity, and it supports survival by regulating the antiapoptotic protein Bcl-XL. Following the antigen presenting process, CDK2 helps to recruit activation-induced cytidine deaminase (AID) to initiate class switching in the germinal center. Cyclin D3 also plays a class switching-promoting role. After clonal selection, B cells become plasma cells and start producing a specific antibody (with help from $p18^{INK4c}$ activity, which produces Ig) and to block the cell cycle.

c. The cell cycle inhibitor $p27^{KIP1}$ promotes memory T cell survival by inhibiting cell cycle progression. T cell exhaustion is controlled by PD-1. Upon activation by its ligand PD-L1, the signals are transduced to promote cell cycle inhibition *via* inhibition of SKP2 activity, which in turn promote $p27^{KIP1}$ and directly enhances $p15^{INK4b}$ activity. The cytokine responsiveness of T cells is also controlled by CDK4/6 activity upon IL-2 stimulation. Moreover, to prevent overactivation of T cells, CKIs $p18^{INK4c}$ plays an important role in suppressing T cell proliferation. The kinase activity of CDK4/6-cyclin D is important for regulatory T (Treg) cell proliferation. On the other hand, CDK4/6-cyclin D also acts on tumor cells by inhibiting the expression of PD-L1 and inhibiting type III interferon production, which in turn activates the antigen presentation process.

Fig 2 Roles of cell cycle regulators in DNA damage response

Upon DNA breakage, cells undergo cell cycle arrest in order to allow enough time for the DNA repair process to complete. Alterations of several cell cycle proteins occur during this process, such as upregulation of the cell cycle inhibitors $p21^{CIP1}$ and $p16^{INK4a}$ along with downregulation of cyclin A and D1. However, residual cyclin D1 remains to repair the damaged DNA.

There are two major pathways following DNA damage: HR and NHEJ. The MRE11-RAD50-NBS1 (MRN) complex initiates HR and disfavors NHEJ by resecting the broken DNA ends. NBS1 activity is positively regulated by CDK1/cyclin B1 kinase activity. CDK1/2 phosphorylates and recruits CtIP and nuclease EXO1 to the repair site. Following DNA resection, BRCA2 and a recombinase RAD51 co-localize at the DNA lesions, where the HR repair occurs.

When HR-mediated DNA repair is not needed, this interaction is negatively regulated by CDK1/2 kinase activity. Upon DNA damage, cyclin D1 binds to the BRCA2 C-terminus, precluding CDK1/2-mediated BRCA2 phosphorylation and facilitating RAD51 recruitment to the BRCA2-bound repair site.

Expression of $p21^{CIP1}$ also inhibits CDK1/2-mediated BRCA2 phosphorylation. In NHEJ, $p21^{CIP1}$ binds to PCNA and facilitates recruitment of NHEJ proteins such as Ku80. This suggests a role of $p21^{CIP1}$ in initiating NHEJ-mediated DNA repair when cells are in the G1 phase.

Fig 3 Roles of cell cycle regulators in cellular oxidative status

a. Various pathways are coordinated in the response to oxidative stress. Cyclin B1 is a mediator that coordinates the cell cycle and mitochondria. Redistribution of cyclin B1 as a result of genomic stress induces changes in the mitochondria, such as decreased MnSOD function, increased complex II activity, and decreased acronitase activity. MnSOD, a major ROS-detoxifying enzyme in mitochondria, is also positively regulated by CDK4/6-cyclin D1 activity. The balance of the ROS in the mitochondria are tightly regulated by cyclin E, cyclin D1, and $p16^{INK4a}$. Moreover, $p16^{INK4a}$ constrains mitochondrial biogenesis by inhibiting the TFAM and PRC transcription factors. Imbalance of ROS causes oxidative stress and triggers cell cycle arrest *via* the p38/JNK-FOXO3a-p27/p57 pathway.

b. HIF1α is directly controlled by cell cycle regulators. In normoxic conditions, CDK2 inhibits the HIF1α activity. However, when cells are in a hypoxic condition, CDK1 and CDK2 coordinate to enhance the HIF1α activity.

Fig 4 Summary of various functions of the cell cycle regulators

a. The cell cycle control of cell cycle regulators is well-established. However, there is a growing body of evidence on cell cycle-independent functions. Some of these are cell autonomous functions, such as transcriptional regulation, DNA damage repair, energy metabolism, and oxidative balance control. The roles of the cell cycle regulators in those pathways are fundamental for the survival of the cells. The functions have been conserved from yeast to mammals.

b. Regulation of the stem cell pool and differentiation to various cell and tissue lineages are important for multicellular organisms. Cell cycle regulators play these roles through transcription, epigenetic control, and apoptosis control, utilizing various CDK and cyclin family members.

c. Cell cycle regulators also participate in the expansion of several, terminally differentiated cell types as well as in many steps of the development of immune cells and their functions.

Figure 1

a. Macrophage

pathogens

T cell

INFγ

p21

p16

p27

IRTK

IL-6

apoptosis

RACK

Macrophage

Ameboid migration

Mesenchymal migration

loose tissue infiltration

Tumor infiltration

b. B cell

cyclin D2

Progenitor cell

bone marrow

BCR

NF-kB/Rel

cyclin E

CDK2

Bcl-xL

B cell survival

T cell

B cell

cyclin D3

CDK2

AID

class switching

class switching

class switching

clonal selection

p18

plasma cell

Ig production

c. T cell

Memory T cell

p27

Memory T cell survival

CDK4/6

cyclin D

Treg proliferation

Treg

T cell exhaustion

T cell proliferation

p18

CDK4/6

p27⬆

p15⬆

IL-2

SKP2

PD-1

PD-L1

CDK4/6

cyclin D

typeIII INFs production

tumor cell

Figure 2

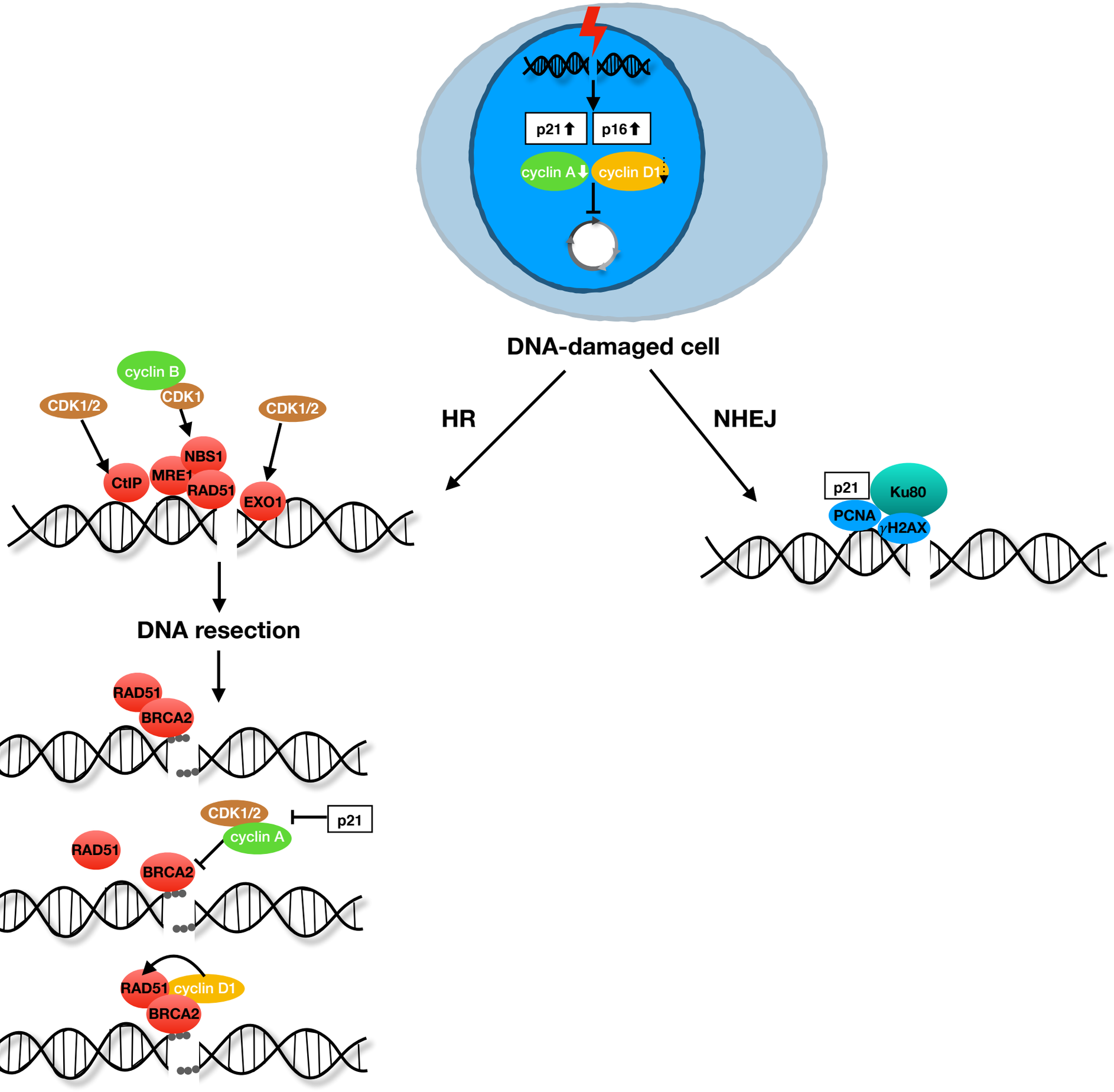
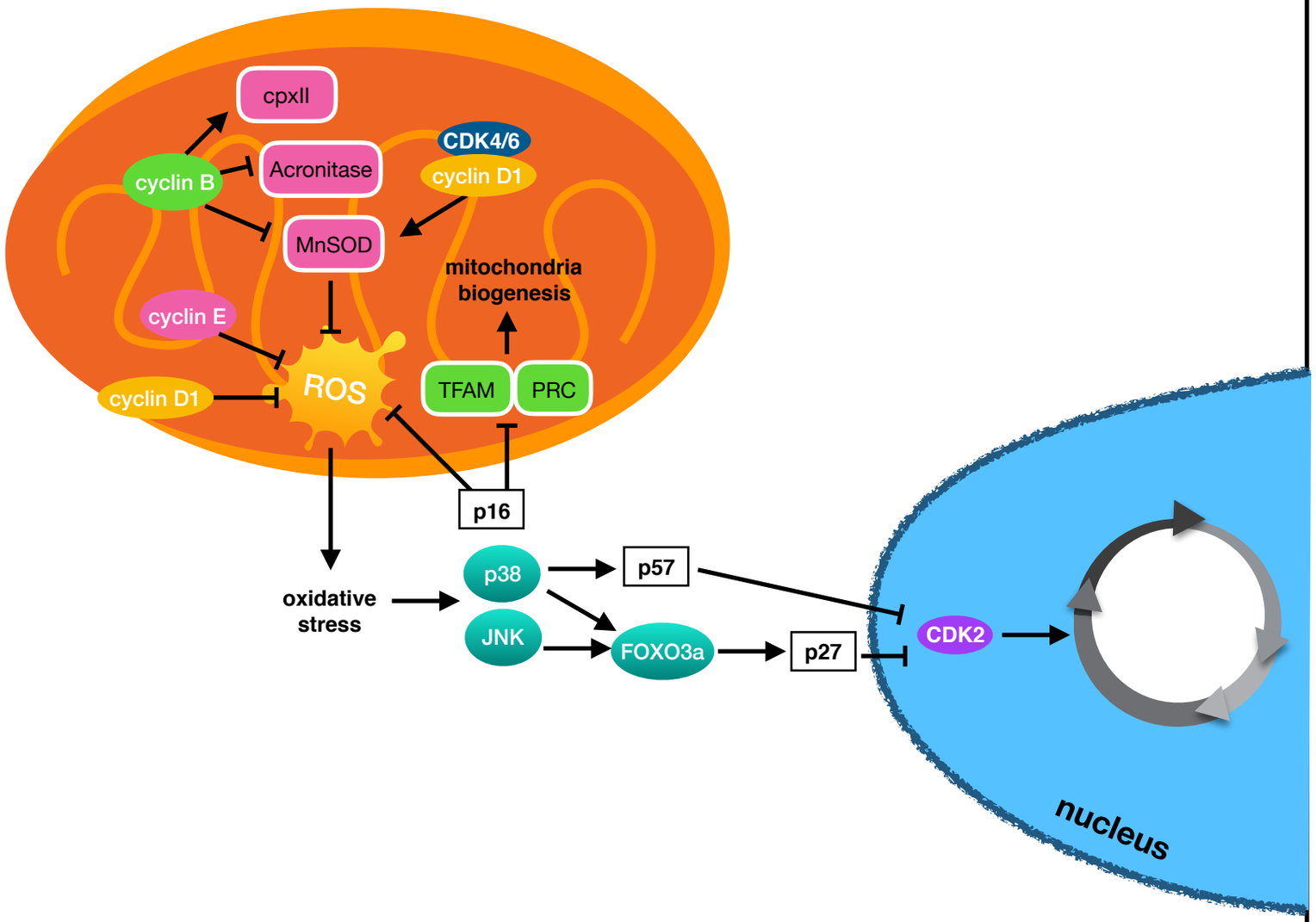
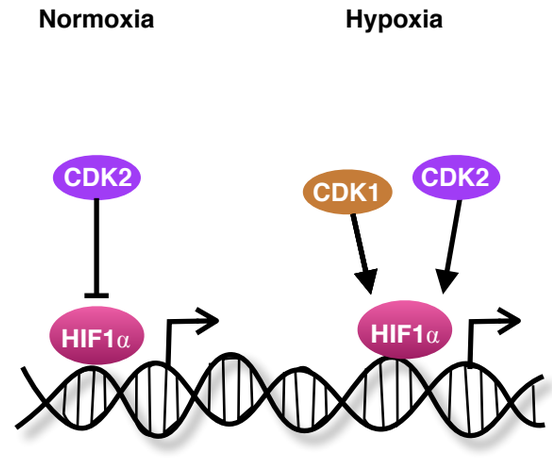

p21
p16
cyclin A
cyclin D1
DNA-damaged cell
HR
NHEJ
cyclin B
CDK1
CDK1/2
CDK1/2
NBS1
CtIP
MRE1
RAD51
EXO1
p21
Ku80
PCNA
γH2AX
DNA resection
RAD51
BRCA2
CDK1/2
p21
cyclin A
RAD51
BRCA2
RAD51
cyclin D1
BRCA2

Figure 3



a. Mitochondrial-related functions and oxidative stress response



b. hypoxic respones

Figure 4

Cell Autonomous functions

a.

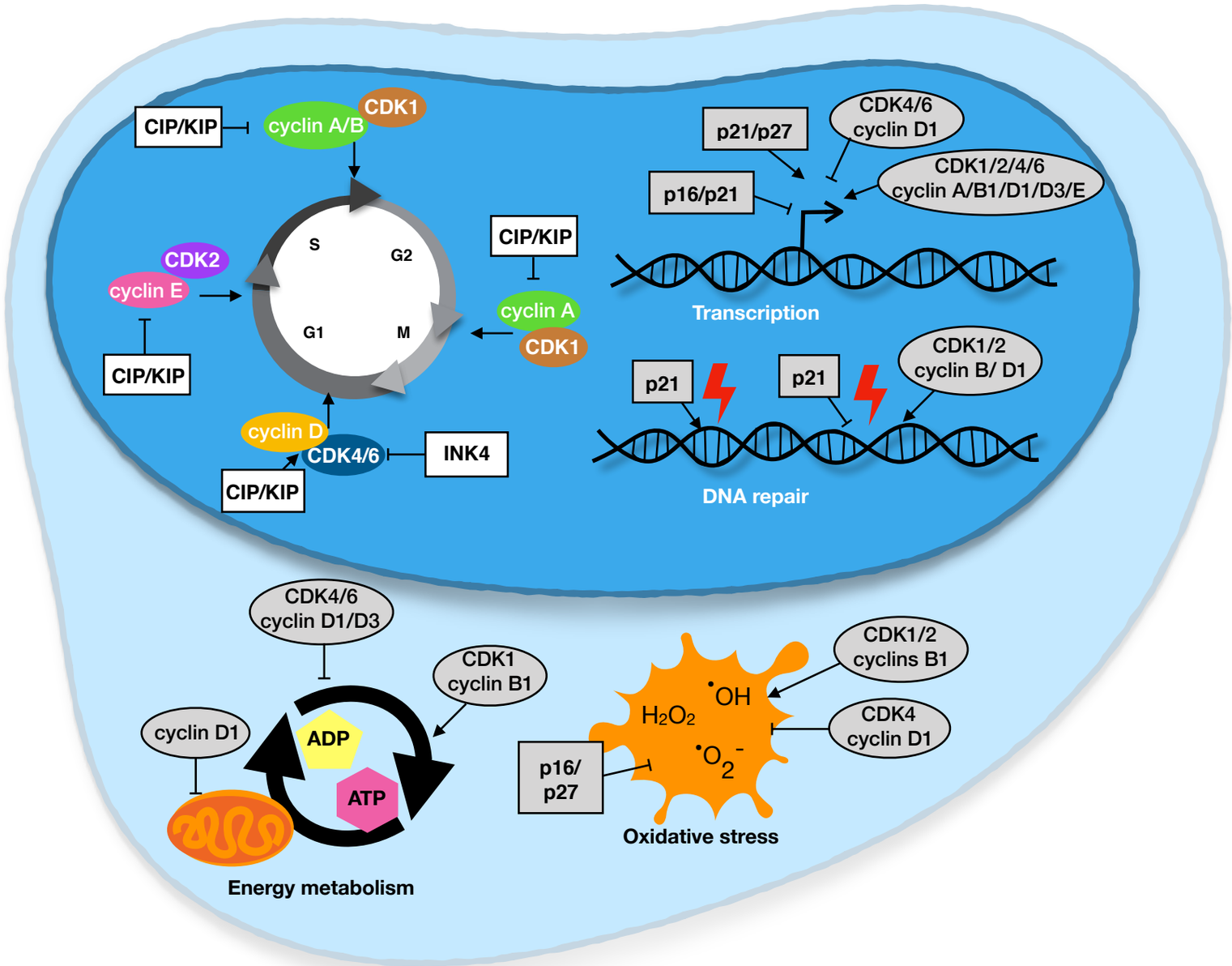
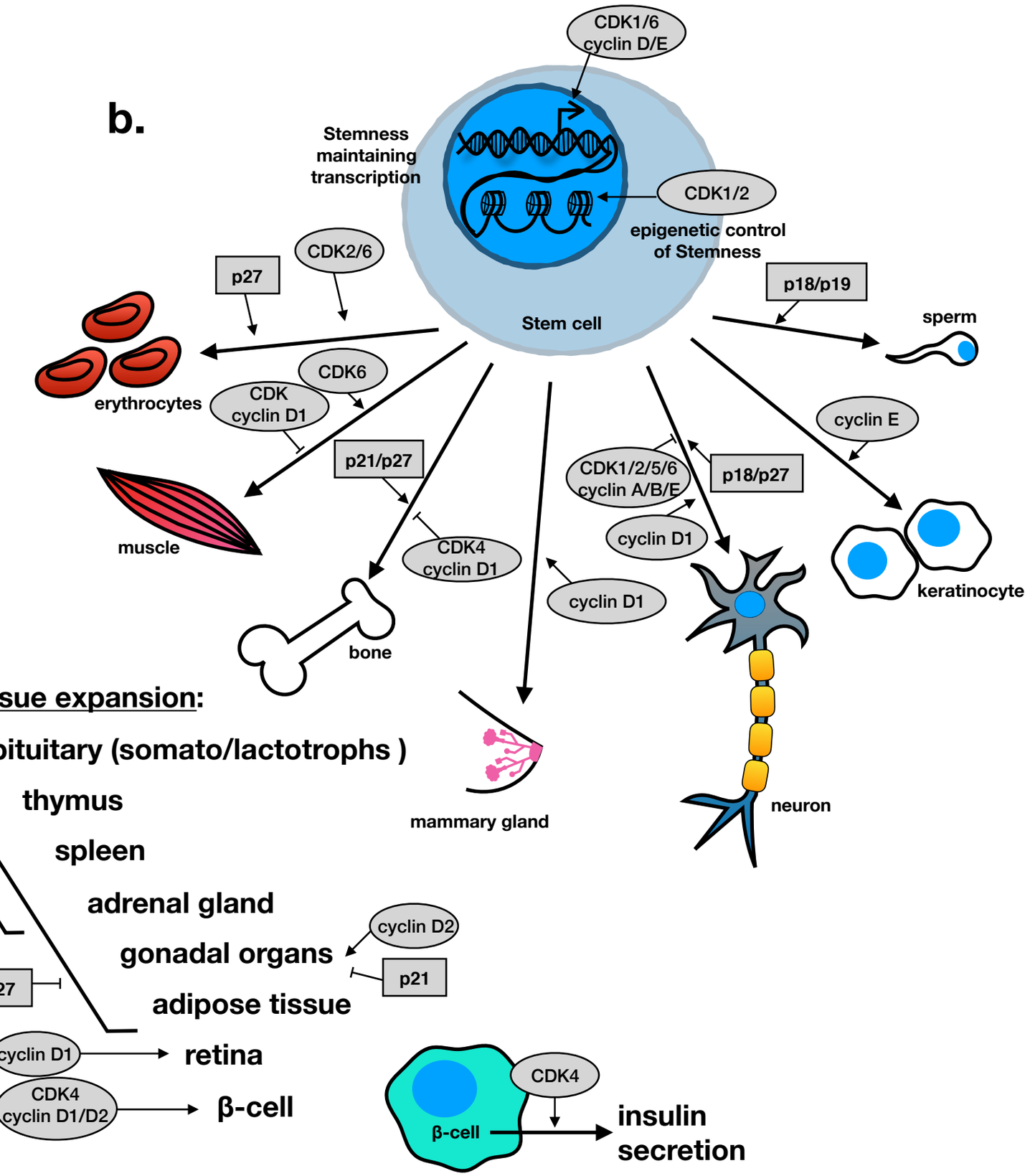


Figure 4

non-cell autonomous functions

b.



c.