Published in final edited form as:

Leuk Lymphoma. 2017 March ; 58(3): 676–688. doi:10.1080/10428194.2016.1198958.

Cyclin D1 Depletion Induces DNA Damage in Mantle Cell Lymphoma Lines

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Abstract

Elevated cyclin D1 (CCND1) expression levels in mantle cell lymphoma (MCL) are associated with aggressive clinical manifestations related to chemoresistance, but little is known about how this important proto-oncogene contributes to the resistance of MCL. Here, we showed that RNA interference-mediated depletion of CCND1 increased caspase-3 activities and induced apoptosis in the human MCL lines UPN-1 and JEKO-1. *In vitro* and xenotransplant studies revealed that the toxic effect of CCND1 depletion in MCL cells was likely due to increase in histone H2AX phosphorylation, a DNA damage marker. DNA fiber analysis suggested deregulated replication initiation after CCND1 depletion as a potential cause of DNA damage. Finally, in contrast to depletion or inhibition of cyclin-dependent kinase 4, CCND1 depletion increased chemosensitivity of MCL cells to replication inhibitors hydroxyurea and cytarabine. Our findings have an important implication for CCND1 as a potential therapeutic target in MCL patients who are refractory to standard chemotherapy.

Keywords

Cyclin D1; DNA damage; replication stress; chemoresistance; mantle cell lymphoma

Introduction

Mantle cell lymphoma (MCL) is an incurable B-cell malignancy that accounts for 6-8% of all non-Hodgkin lymphomas. Most MCL patients respond initially to conventional chemotherapy, but then quickly develop resistance, leading to a median survival of 3-5 years

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Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed by all authors.

[1, 2]. MCL is characterized by the recurrent t(11;14) chromosomal translocation [3], resulting in CCND1 overexpression. CCND1 binds and activates cyclin-dependent kinase (CDK) 4 or CDK6 to mediate phosphorylation-dependent degradation of the major G1 checkpoint protein retinoblastoma protein (RB) and promote S-phase entry [4-6]. Other D-type cyclins, cyclin D2 (CCND2) and cyclin D3 (CCND3), which also associate with CDK4 and CDK6 [7], are less common in MCL, and their expression are more associated with CCND1 negative cases [8, 9]. Salaverria et al. has reported 55% of MCL cases that lacked CCND1 expression had chromosomal rearrangement at the *CCND2* locus [9]. Translocation at the *CCND3* locus has been reported only in one CCND1 negative case [8] and this translocation appear more common in other B-cell lymphomas [10]. While the CDK4/RB-dependent function of CCND1 is important for MCL development, targeting this pathway by inactivating CDK4/6 kinase causes G1 arrest with little cytotoxicity in the lymphoma cells [11-15].

Overexpression of CCND1 has been shown to correlate with increased chemoresistance and poor survival in many cancers [16-19]. In MCL, Rosenwald *et al.* demonstrated that elevated CCND1 expression levels are associated with high tumor cell proliferation and aggressive clinical manifestations, likely related to chemoresistance [20]. In another study by Tiemann *et al.*, the authors showed that depletion of CCND1 using siRNA increased the sensitivity of MCL cell lines to etoposide or doxorubicin [21]. These reports suggest an important role of CCND1 in protecting MCL cells against conventional chemotherapeutic agents, but the mechanisms underlying this function are largely unknown. In this study, we address this question by hypothesizing that CCND1 is essential for genomic stability in MCL tumors. We tested the hypothesis by determining the effects of CCND1 depletion on DNA stability and cellular response to the DNA damaging agents hydroxyurea (HU) or cytarabine in MCL lines and primary MCL cells in vitro and in a xenotransplant model.

Materials and methods

Cell lines and culture conditions

Human MCL lines UPN-1 and JEKO-1 were kindly provided by Dr. Louis Staudt. The UPN-1 cell line was confirmed to carry the cyclinD1/IGH fusion gene or t(11;14) translocation and other chromosomal rearrangements, as previously described [22], by metaphase fluorescence in situ hybridization (FISH) analysis (Supplementary Figure S1). JEKO-1 cells were previously reported to carry the t(11;14) translocation [23]. Cells were cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin/streptomycin and maintained in a humidified, 5% CO₂ incubator at 37°C.

Primary MCL preparation

Viably cryo-preserved MCL cells were obtained from the tumor bank of the Pathology Department of City of Hope as de-identified samples after approval by the Institutional Review Board. Cells were briefly thawed in 37°C water bath, washed in RPMI-1640 medium and subsequently cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum.

Antibodies and chemicals

The following antibodies were used: pRB-S807/811 (Cell Signaling Technology, Danvers, MA), pRBL2-S672, RBL2 (Abgent, Inc., San Diego, CA), CCND1, CDK4, CDK6, GAPDH, β -ACTIN (Santa Cruz, Dallas, TX), RB (BD Biosciences, San Jose, CA), and γ H2AX (Millipore, Billerica, MA). The following chemicals were used: PD-0332991 (Selleck Chemicals, Houston, TX), hydroxyurea, cytarabine, and aphidicolin (Sigma, St. Louis, MO).

RNA interference reagents and siRNA transfection

All shRNA constructs and sequences were obtained from a previously generated shRNA library [24] and listed in Supplementary Table S1. SMARTpool On-Targetplus siRNA targeting CCND1 or CDK4 was purchased together with scramble control siRNA from Dharmacon (Dharmacon, Lafayette, CO). Two rounds of siRNA transfection were performed on day zero and day two as recommended by the manufacturer.

Western blotting

Cells were lysed in the presence of protease inhibitors (Roche) and phosphatase inhibitors (Sigma, St. Louis, MO) for 30 minutes. Lysates were cleared by centrifugation and protein concentrations were determined by BCA protein assay (Life Technologies, Grand Island, NY). Thirty to 80 μ g of lysates were separated by 4-15% SDS-PAGE and immobilized on the nitrocellulose membranes for immunoblotting.

Viability and apoptosis measurements

Cell viability was performed either by using the TC20 Automated Cell Counter (Biorad, Hercules, CA), based on the trypan blue exclusion method, or by a Celltiter-Glo assay (Promega, Madison, WI) following instructions by the manufacturer. To measure apoptosis, cells were stained with anti-active Caspase-3 and anti-cleaved PARP antibodies (BD Biosciences, San Jose, CA) or analyzed by Annexin V-based methods using Annexin V Apoptosis Detection Kit (eBioscience, Inc., San Diego, CA).

Retroviral transduction

A retroviral vector and a mixture of helper plasmids for viral envelope and *gag/pol* were transfected into HEK293T cells using Lipofectamine2000 (Life Technologies, Grand Island, NY). Retroviral supernatants were harvested 48 hours after transfection and were used to transduce ecotropic receptor-expressing target cells by centrifugation at 1360 g for one hour in 4 μ g/ml polybrene (Sigma, St. Louis, MO).

Rescue of shRNA toxicity by cDNA complementation

Complementation studies were performed in the UPN-1 cell line. Briefly, cells were transduced with retroviral vectors that express a 3' UTR-directed CCND1 shRNA. CCND1 shRNA-transduced cells were subsequently infected with retroviruses co-expressing a wild-type CCND1 cDNA and hygromycin resistant gene. Hygromycin-selected cells were induced with doxycycline to induce shRNA expression and apoptosis was monitored over time by active caspase-3/cleaved PARP staining.

Immunofluorescence

Cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton-X-100, followed by blocking with 2% BSA and 0.05% Tween-20 in phosphate buffered saline. Cells were stained with primary antibodies and then with fluorescently labeled secondary antibodies, followed by nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Grand Island, NY).

Xenograft study

Mouse care and experimental procedures were performed in accordance with established institutional guidelines and approved protocols from the Institutional Animal Care and Use Committee of the City of Hope. Five millions of CCND1 shRNA-transduced UPN-1 cells were subcutaneously injected into dorsal skin area of 7–8 weeks old immunodeficient NOD/SCID/IL2R-Gamma (NSG) mice (Jackson Laboratories, Bar Harbor, ME). After tumors reached average size of approximately 5 mm, sucrose or doxycycline (Sigma, St. Louis, MO) was added to mouse drinking water and tumor growth was monitored every other day by caliper measurements.

DNA combing assay

Exponentially growing cells were pulse-labeled with 50 μ M iododeoxyuridine (IdU) followed by 100 μ M chlorodeoxyuridine (CIdU) for 20 and 40 minutes each. Labeled cells were harvested and DNA fiber spreads were prepared processed as described [25].

Cell cycle analysis

Cells were cultured with bromodeoxyuridine (BrdU) for 30 minutes, washed in PBS, and fixed overnight in 70% ethanol. Labeled cells were permeabilized in Perm/Wash buffer (BD-Biosciences) and treated with 4N hydrochloric acid and 1% Triton X-100. Acid was neutralized by sodium tetraborate (pH 8.5). Cells were incubated with an anti-BrdU antibody (BD-Biosciences) followed by washing and resuspending in RNase/PI-staining solution (Life Technologies). Flow cytometric analysis was performed on a FACSCalibur (BD-Biosciences).

Statistical analyses

A two-tailed Student t-test was used for normally distributed values. For non-normally distributed values, a two-tailed Mann-Whitney test was used. A Chi-square test or a two-sided Fisher's exact test was used for categorical data. All tests were analyzed using Prism Version 6.0b (GraphPad Software, Inc.). $P < 0.05$ was considered significant.

Complete methods are available in Supplementary Methods.

Results

CCND1 is required for the survival of MCL cells

To examine CCND1 function in the proliferation and survival of MCL, we depleted this protein in the human MCL lines UPN-1 and JEKO-1 using short-interfering (si) RNA. To

distinguish other roles of CCND1 from those of its well-characterized pathway through CDK4 or CDK6, we also depleted CDK4 in parallel experiments, as the cell lines we chose do not express CDK6 (Figure 1A). Protein knockdown were confirmed by immunoblot analysis (Figure 1B). Depletion of CCND1 or CDK4 after 7 days decreased cell proliferation in both cell lines (Figure 1C). To determine whether the growth inhibition observed in these cells was due to cytotoxic effects of gene silencing, the apoptotic marker active caspase 3 was evaluated by flow cytometry. Compared to controls, CCND1 depletion induced apoptosis in UPN-1 and JEKO-1 cells more than CDK4 depletion (Figure 1D, E). As an alternative method to evaluate cell death, Annexin V and propidium iodide (PI) positive cells were analyzed by flow cytometry on day 5 after siRNA transfection. Consistent with the results shown in Figure 1D, E, depletion of CCND1 but not CDK4 significantly increased Annexin V and PI staining in UPN-1 and JEKO-1 cells (See DMSO-treated samples in Figure 5A, B).

To determine that little toxicity in UPN-1 and JEKO-1 cells was not due to partial CDK4 knockdown, cells were treated with the CDK4/6-specific inhibitor PD-0332991 [13]. The effectiveness of this drug was verified by its ability to block the phosphorylation on RB, a known target of CDK4/6. Since UPN-1 cells do not express RB [26], the alternative CDK4/6 target protein RB-like 2 (RBL2) was evaluated. In a dose-dependent manner, PD-0332991 effectively blocked the phosphorylation of RB or RBL2, leading to decreased cell proliferation in both cell lines (Figure 1F, G). However, compared to DMSO-treated controls, PD-0332991 only modestly induced apoptosis in UPN-1 and JEKO-1 cells (Figure 1H, I). These results were similar to the effects of siRNA-mediated CDK4 depletion shown in Figure 1, D and E.

We next used shRNA as an alternative gene knockdown method to confirm the effects of CCND1 depletion. UPN-1 and JEKO-1 cells were transduced with retroviral vectors that co-expressed control or CCND1 shRNA and enhanced green fluorescence protein (eGFP). The proliferation and/or survival of transduced cells were then determined by monitoring the fractions of eGFP positive cells over time using flow cytometry. Compared to controls, CCND1 shRNA significantly reduced the viability of both UPN-1 and JEKO-1 cells in a time-dependent fashion (Figure 1J). Because CCND1 depletion is toxic in the MCL lines used in our study, we generated a stable UPN-1 cell line that conditionally expresses CCND1 shRNA, using a doxycycline (Dox)-inducible retroviral expression system [24]. To validate that the toxicity of the CCND1 shRNA was not due to an off-target effect, we demonstrated that a cDNA vector encoding wild-type CCND1 was able to rescue apoptosis induced by a 3'UTR-directed CCND1 shRNA (Figure 1K). We next compared the effects of CCND1 and CDK4 shRNA-mediated knockdown on proliferation and survival using stable UPN-1 cell lines that conditionally express CCND1 or CDK4 shRNA. To ensure similar CCND1 and CDK4 knockdown efficiencies, cells with similar expression levels of the co-expressed eGFP marker were sorted by flow cytometry to normalize shRNA expression (Supplementary Figure S2). As expected, shRNA-mediated depletion of CCND1 in UPN-1 cells led to more growth inhibition and apoptosis than depletion of CDK4 in multiple time points starting on day 4 after Dox induction (Figure 1L, M). Altogether, we conclude that survival of UPN-1 and JEKO-1 cells is more dependent on CCND1 than on CDK4.

CCND1 is required for DNA stability in vitro and in a xenograft model of MCL

To determine whether apoptosis induced by CCND1 depletion was due to DNA damage, we evaluated DNA double-strand break (DSB) formation by detecting histone H2AX phosphorylation on Ser139 (gamma-H2AX), an established DSB marker [27], in UPN-1 and JEKO-1 cells transfected with CCND1 siRNA. Compared to controls, CCND1-depleted cells had increased gamma-H2AX expression on day 3 and 4 after siRNA (Figure 2A). In contrast, gamma-H2AX induction was not detected in these MCL lines treated with PD-0332991 (Figure 2B) or depleted of CDK4 (Supplementary Figure S3) for up to four days. Increased gamma-H2AX signals were also observed in primary MCL cells four days after transduction with CCND1 shRNA-expressing lentivirus (Figure 2A). The tumor content in this primary MCL sample was confirmed to have greater than 95% of CD19+CD5+ lymphoma cells (Supplementary Figure 4). As an alternative method of detecting DSBs, we used immunofluorescence to identify cells with nuclear gamma-H2AX foci. Using inducible knockdown UPN-1 cell lines generated above, we demonstrated that depletion of CCND1, but not CDK4, significantly increased the number of gamma-H2AX⁺ foci (Figure 2C), which were more frequent in the S and G2 phases of the cell cycle (Supplementary Figure S5). These data indicate that CCND1, but not CDK4, plays an important role in preventing DNA damage in MCL cells.

DNA damage after CCND1 depletion in MCL cells prompted us to evaluate genomic stability in these cells. Using a cytokinesis-block micronucleus assay [28] to detect nuclear damage during mitotic segregation, we observed that depletion of CCND1, but not CDK4, significantly increased micronucleus formation in UPN-1 cells (Figure 2D). Further cytogenetic analysis of chromosomal aberrations by metaphase karyotyping following CCND1 silencing in UPN-1 cells revealed chromosomal breaks, chromatid breaks and recurrent chromosomal gaps or constrictions that are associated with fragile sites (Supplementary Figure S6, Supplementary Table S2).

We next evaluated the *in vivo* relevance of CCND1 inactivation by engrafting CCND1 shRNA-expressing UPN-1 cells into immunodeficient NOD/SCID/IL2R-Gamma (NSG) mice. CCND1 depletion resulted in reduced growth (Figure 3A-C) and increased apoptosis, as detected by active caspase 3 immunofluorescence (Figure 3E), in the transplanted tumors. Increased accumulation of gamma-H2AX foci was also observed in CCND1-depleted, but not control, xenografts (Figure 3D). These findings are consistent with the *in vitro* effects of CCND1 depletion in UPN-1 cells on cell growth, apoptosis and DNA stability described above. Together, these data indicate that CCND1 is essential for maintaining DNA stability in MCL cells and specifically in the UPN-1 cells both in *in vitro* and *in vivo* conditions.

CCND1 depletion leads to defective DNA replication in UPN-1 cells

In the absence of induced DNA damage, DNA breakage often occurs during replication stress, a process when DNA synthesis becomes inefficient [29]. To determine whether DNA damage in CCND1-depleted UPN-1 cells were related to defects in DNA replication, the DNA combing assay [25] was used to assess replication initiation. In this assay, CCND1- or CDK4-depleted UPN-1 cells were pulse-labeled with the thymidine substitutes iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) for 20 and 40 minutes each. DNA

fibers were extracted from cell lysates and spread on glass slides for immunofluorescence analysis using antibodies specific for IdU or CldU. Compared to uninduced or CDK4-depleted cells, CCND1-depleted cells showed a markedly reduced distance between replication origins, indicating increased frequency of origin firing (Figure 4). Thus, CCND1 may have an important role in DNA replication initiation and reduced CCND1 expression levels may result in unscheduled origin firing and replication stress-related DNA breakage.

CCND1 depletion sensitizes MCL cells to DNA replication inhibitors

We next determined whether CCND1 plays a role in the chemoresistance of MCL cells to the replication inhibitors hydroxyurea (HU) or cytarabine. To do so, we depleted CCND1 by siRNA in UPN-1 and JEKO-1 cell lines for three days. Equal numbers of siRNA-transfected cells were re-plated and treated with increasing concentrations of HU or cytarabine for two days followed by evaluation of apoptosis using Annexin V-based flow cytometry.

Knockdown of target proteins in these experiments was confirmed by immunoblot analysis (Figure 5A, B insets). CCND1 depletion increased sensitivity of UPN-1 and JEKO-1 cells to HU (Figure 5A, B) or cytarabine (Figure 5C, D) dose-dependently. Using the inducible CCND1 knockdown UPN-1 cell line, we confirmed that induced CCND1 shRNA sensitized UPN-1 cells to HU or to another replication inhibitor aphidicolin (Figure 5E). Enhanced chemosensitivity of UPN-1 and JEKO-1 cells after CCND1 depletion was not due to CDK4 inactivation, as CDK4 depletion produced results similar to control knockdown cells (Figure 5A-D). In addition, pharmacologic inhibition of CDK4 using PD-0332991 increased resistance of these cells to HU (Figure 5F, G). These results indicate that CCND1, but not CDK4, is required for resistance of MCL cells to DNA replication inhibitors.

Effects of CCND1 depletion on the cell cycle of MCL cells

Because CCND1 primarily regulates cell cycle progression through CDK4, increased chemosensitization in MCL cells after CCND1 depletion led us to examine the effect of loss of CCND1 on the cell cycle. Control or CCND1-depleted UPN-1 and JEKO-1 cells were labeled with bromodeoxyuridine (BrdU) and propidium iodide (PI), and the cell cycle was analyzed by flow cytometry. The CDK4 inhibitor PD-0332991 was also used in parallel experiments to compare with the effects of CCND1 depletion. Silencing of CCND1 in both UPN-1 and JEKO-1 cells was confirmed by qPCR (Figure 6A). While depletion of CCND1 or inhibition of CDK4 induced cell cycle arrest in both cell lines, more G1 arrest was observed in CDK4-inhibited cells (60-80%) than in CCND1-depleted cells (~50%) (Figure 6B, C). A strong induction of G1 arrest by CDK4 inhibition was consistent with the chemoprotective effect of PD-0332991, which prevented cells from entering the S phase where they were more sensitive to HU (see Figure 5F, G). In contrast, weaker G1 arrest induced by CCND1 depletion sensitized cells to HU or cytarabine (see Figure 5A-E). These data indicate a functional difference between CCND1 and CDK4 signaling with respect to chemosensitivity and suggest a CDK4-independent role of CCND1, which becomes essential when cells encounter replication inhibitors.

Discussion

The present study demonstrated that CCND1 is essential for the maintenance of established MCL tumor cell lines and uncovered a new role for CCND1 in preserving genomic stability during DNA replication in MCL cells. There are conflicting reports on the role of CCND1 in the survival of MCL cells. Klier *et al.* showed that silencing of CCND1 in the MCL lines GRANTA-519, JEKO-1, and Z-138 for up to seven days caused growth arrest but not cell death [30]. In contrast, Weinstein *et al.* detected apoptosis in the GRANTA-519 and JEKO-1 lines eight days after CCND1 siRNA transfection [31]. In agreement with the study of Weinstein *et al.* [31], our data indicate that CCND1 is essential in JEKO-1 (and UPN-1) cells. Apoptosis in JEKO-1 cells after CCND1 depletion was detected both in the Weinstein *et al.* [31] and the present study but not in the study by Klier *et al.* [30]. This discrepancy is probably due to the use of more sensitive apoptotic markers like Annexin V by Weinstein *et al.* [31] or active caspase 3/cleaved PARP 1 in our study, whereas Klier *et al.* [30] assessed apoptosis by quantifying the sub-diploid populations.

We found that CCND1 depletion resulted in increased origin firing, leading to replication stress and DNA DSB. However, it is unclear how CCND1 plays a protective role in preventing DNA damage during replication in MCL cells. In a comprehensive proteomics study of CCND1-overexpressing human cancers, including one MCL line (Granta-519), Jirawatnotai *et al.* demonstrated direct binding between CCND1 and the DNA repair protein RAD51 in the context of radiation-induced DNA damage [32]. As RAD51 can also be recruited to the stalled replication forks independently of DNA breakage [33, 34], it would be of future interest to investigate whether CCND1 and RAD51 interaction is required to maintain genome stability during unperturbed replication in MCL.

Because CCND1 depletion is toxic in the MCL lines used in the present study, detailed studies of its function require a conditional knockdown system. By generating Dox-inducible knockdown cell lines in UPN-1 cells, we uncovered a previously unrecognized role for CCND1 in preserving genomic stability during DNA replication. This genome protective function of CCND1 is thus relevant to the resistance of MCL to chemotherapeutic agents. These data were, however, limited to the inducible knockdown system generated in UPN-1 cells. Multiple attempts to generate inducible CCND1 shRNA in JEKO-1 cells have been proven technically challenging, likely due to the inherent leakiness of the tetracycline inducible system in this cell line. Nonetheless, the roles of CCND1 in promoting survival and chemoresistance in JEKO-1 cells reported in this study are consistent with previous studies for this cell line [21, 31].

Aberrant CCND1 signaling is considered central to MCL pathogenesis and major efforts to target this pathway have been directed to its catalytic partner CDK4 using the highly specific inhibitor palbociclib (PD-0332991). Despite being a potent inhibitor of CDK4-RB signaling that can efficiently suppress tumor growth both in vitro and in vivo [13-15], palbociclib induces little or very modest cell death in MCL, as demonstrated by others [15] and the present study. Our data indicate that the survival and chemoresistance of established human MCL lines are more dependent on CCND1 than on CDK4, suggesting CCND1 as a valid target in MCL. However, these cell line results were confirmed by only limited data on

primary MCL cells (see Figure 3A) due to technical challenges in genetic manipulation of primary cells. Currently, there are no small-molecule inhibitors of CCND1 under clinical development, but pharmacologic agents that indirectly reduce CCND1 levels have been reported (see review in [35]). These agents will be helpful to confirm our cell line observations in primary MCL cells. Of interest, recent MCL treatment advances using ibrutinib, a Bruton's tyrosine kinase inhibitor, have produced durable responses in MCL patients [36]. Further studies by Ma *et al.* to characterize MCL cells with different sensitivity to ibrutinib showed that this drug can down-regulate CCND1 levels in certain MCL cell lines [37]. Our findings on the essential role of CCND1 in the survival and maintaining genomic stability of MCL cells are therefore highly relevant to future efforts to improve the efficacy of this emerging therapy.

In summary, this study has demonstrated a role for CCND1 in the survival and chemoresistance of MCL cells. CCND1 plays these essential roles likely through involvement in DNA replication to minimize DNA damage. These findings, thus, have important implications for understanding and treating the chemoresistance of aggressive MCL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Louis M. Staudt and Yandan Yang for MCL cell lines and the Tet-repressor expressing UPN-1 cells, and Dr. Jeremy Stark for feedback and technical advice. We also thank Dr. Ravi Bhatia for helpful discussions. This work was supported in part by the National Cancer Institute of the National Institutes of Health under grant number P30CA033572. A.S. is a Tim Nesvig Fellow. D.D.W is supported by NIH/NCI grant 5U01CA62505-19. V.N.N. is supported by the Tim Nesvig Lymphoma Fellowship, the Stop Cancer Foundation and the American Hematology Society.

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Figure 1. CCND1 is required for survival of MCL cells

A. Immunoblot shows UPN-1 and JEKO-1 cells express little CDK6 with U2-OS cells as a positive control for CDK6 expression. **B.** Immunoblot shows protein knockdown in indicated MCL cell lines on day four after transfection with scramble control (sc), CCND1 (D1), or CDK4 (K4) siRNA. Numbers below the bands are relative densitometric values between target proteins and GAPDH loading control, followed by normalization to the control siRNA for each protein. **C.** Effects of siRNA knockdown on cell proliferation. Shown are the numbers of viable cells as determined by trypan blue exclusion on day seven after transfection with indicated siRNA. **D.** Detection of apoptosis in indicated cell lines seven days after transfection with indicated siRNA. Shown are representative histograms of live cells stained with the active caspase-3 antibody followed by fluorescence-activated cell sorting (FACS) analysis. **E.** Quantification of active caspase 3-positive cell fractions described in (**D**). **F.** Immunoblot analysis of indicated cell lines 48 hours after treatment with indicated doses of PD-0332991. Inhibition of CDK4/6 activity by PD-0332991 was shown by dephosphorylation of the CDK4/6 target protein RB or RBL2. RB was absent in UPN-1 cells and, therefore, RBL2 was analyzed. Relative densitometric values were calculated as described in (**B**). **G.** Effects of PD-0332991 on cell proliferation. Shown are percentages of viable cells compared to DMSO-treated cells as determined by the Celltiter-Glo assay seven days after treatment with indicated doses of PD-0332991. **H.** Detection of apoptosis in indicated cell lines after seven-day treatment with PD-0332991 as described in (**D**). **I.** Quantification of active caspase 3-positive cell fractions described in (**H**). **J.** Toxicity of CCND1 shRNA in MCL cell lines. Shown are the normalized fractions (to day 0 values) of GFP⁺, shRNA-expressing cells relative to GFP⁻, shRNA-negative fractions at indicated times. **K.** Rescue of CCND1 shRNA toxicity by cDNA complementation. UPN-1 cells were

transduced with a doxycycline (dox)-inducible 3' UTR-directed CCND1 shRNA (shCCND1-2) and a rescue vector constitutively expressing HA-tagged, wild-type (WT) CCND1 coding regions or an empty vector. Apoptotic cells were assessed as described in (D) at indicated times after shRNA induction and shown as percentages of active caspase 3/cleaved PARP-1-positive cells. Inset, immunoblot verifies CCND1 overexpression or knockdown two days after shRNA induction. # non-specific bands. **L.** Effects of CCND1 or CDK4 depletion on cell growth. Shown are the means of live cell numbers of control or shRNA-induced cells as determined by trypan blue exclusion on indicated days. **M.** Apoptosis in UPN-1 cells transduced with indicated shRNA was detected at indicated times after shRNA induction by staining with active caspase-3 and cleaved PARP-1 antibodies. Inset, immunoblot confirms protein knockdown two days after shRNA induction. Values in line graphs or bar graphs are the means of three independent experiments. Error bars, SD. **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$ by a two-tailed student's t-test.

Figure 2. CCND1 is required for DNA stability in MCL cells

A. CCND1 depletion induces gamma-H2AX expression. Immunoblot analysis of indicated MCL cell lines on day three or four after transfection with control (con) or CCND1 (D1) siRNA. Primary MCL cells (MCL #1) with more than 95% of tumor content (see Supplementary Figure S4) were transduced with control or CCND1 shRNA-expressing lentivirus and analyzed by immunoblot on day 4 after transduction. Relative densitometric values were calculated as described in Figure 1B. **B.** Effect of CDK4 inhibition on gamma-H2AX expression. UPN-1 and Jeko-1 cells were treated with 250 nM of PD-0332991 for indicated times and analyzed by immunoblot. Relative densitometric values were calculated as described in Figure 1B. **C.** CCND1 depletion induces DNA DSB foci. CCND1 or CDK4 shRNA-transduced UPN-1 cells were induced with Dox for four days. Shown are representative confocal immunofluorescence images of uninduced and induced cells stained with Alexa-Fluor 555-conjugated phospho-H2AX (S139) antibody (red) followed by nuclear staining with DAPI (blue). Scale bars, 20 μ m. Bar graphs show the mean values of cell fractions that are positive for phospho-H2AX (γ H2AX) foci from two independent experiments. Error bars, SEM. Foci of at least 1mm in diameter were considered positive for γ H2AX. On average, 500 cells from each group were counted. **** $P < 0.0001$ by a Chi-square test; ns, not significant. More γ H2AX foci in uninduced CCND1 shRNA-transduced

cells than in CDK4 shRNA-transduced cells indicate “leakiness” of the Dox inducible system in UPN-1 cells. **D.** CCND1 depletion induces genomic instability. Representative bright-field images of uninduced or day-four induced UPN-1 cells treated with cytochalasin B followed by Giemsa nuclear staining. Scale bars, 20 μ m. Bar graphs show the percentages of binucleated (BN) cells with micronucleus (MN) formation from two separate experiments. Error bars, SEM; **** $P < 0.0001$ by a two-sided Fisher's exact test. Approximately 200 binucleated (BN) cells from each group were counted.

Figure 3. Effects of CCND1 depletion in a xenograft model of MCL

Five millions of CCND1 shRNA-transduced UPN-1 cells were injected subcutaneously into NSG mice and shRNA expression was induced by adding Dox to mouse drinking water. **A.** Shown are the means of tumor volumes ($n = 7$). Error bars, SEM. **B.** Shown are the means of tumor weights. Error bars, SD. **C.** Lysates of MCL tumors harvested from control or Dox-induced mice on day 21 were immunoblotted with indicated antibodies. **D, E.** Representative immunofluorescence images of tumor sections from control and Dox-induced groups were stained with indicated antibodies followed by fluorescently labeled secondary antibody and nuclear staining with DAPI. Scale bars, 10 μ m. Bar graphs show the percentages of γ H2AX+ or active caspase 3+ cells from control ($n=3$) or Dox-induced ($n=3$) xenografts. Foci with at least 1mm in diameter were considered positive for γ H2AX. At least 500 DAPI-positive cells were counted in non-overlapping fields of immunofluorescence images. Error bars, SD; *** $P < 0.001$; * $P < 0.05$ by a two-tailed Student's t-test.

Figure 4. CCND1 depletion increases replication initiation frequency in UPN-1 cells
 CCND1 or CDK4 shRNA-transduced UPN-1 cells were induced with Dox for four days and sequentially pulse-labeled with IdU and CldU, as illustrated in the diagram (A). B. DNA fibers from labeled cells were spread on glass slides and incorporated IdU and CldU were detected by immunofluorescence. Shown are representative fluorescence images of labeled DNA fibers. C. Inter-origin distances as illustrated in (A) and shown in (B) were measured in micrometers and quantified by boxplots, with bars contained in boxplots representing median values. Scale bars, 10 μ m; **** $P < 0.0001$. ns, not significant. P values are from the two-tailed Mann-Whitney test for 100-200 scorable tracks on average per sample.

Figure 5. CCND1 depletion sensitizes MCL cells to DNA replication inhibitors

A-D. MCL lines were transfected with siRNA against CCND1 (D1) or CDK4 (K4) or a control siRNA on day zero and again on day two (see Materials and Methods). On day three, 1.5×10^5 live cells were counted and re-plated, followed by addition of HU (A, B) or cytarabine (C, D) at indicated doses. Apoptosis was assessed by Annexin V/PI staining on day five. Shown are percentages of Annexin V+/PI+ cells. Inset, confirmation of protein knockdown three days after siRNA transfection by immunoblotting with indicated antibodies. **E.** CCND1 shRNA sensitizes UPN-1 cells to DNA replication inhibitors. CCND1 shRNA-transduced UPN-1 cells were induced with Dox for four days followed by treatment with HU (0.25 mM) or aphidicolin (APH, 1000 nM) for two days and analyzed for apoptosis by Annexin V/PI staining. **F, G.** CDK4 inhibition increases chemoresistance in MCL cells to HU. UPN-1 or JEKO-1 cells were treated with indicated doses of HU in combination with DMSO or 250 nM of PD-0332991 for two days and analyzed for apoptosis by Annexin V/PI staining. Bar graphs show means of three independent experiments. Error bars, SD. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ by a two-tailed student's t-test.

Figure 6. Effects of CCND1 depletion on the cell cycle

A. Verification of CCND1 knockdown. Control or CCND1-depleted UPN-1 or JEKO-1 cells were analyzed for relative CCND1 mRNA expression by qPCR after 4 days of shRNA induction. Shown are the means of 3 independent experiments. Error bars, SD. **B.** Cell cycle profiles of MCL cells after CCND1 depletion. Four days after CCND1 depletion, cells were BrdU labeled for 30 minutes followed by FACS analysis. Shown are representative FACS plots of control or CCND1 depleted UPN-1 or JEKO-1 cells. Stacked column graphs show percentages of cells in each cell cycle phase, as defined by the drawn gates. Values are the means from 3 independent experiments. Error bars, SD. **C.** Cell cycle profiles of MCL cells after CDK4 inhibition. Indicated cells were treated with PD0332991 (250 nM) for four days and were labeled with BrdU as described in (B). Shown are representative FACS plots of control or treated UPN-1 or JEKO-1 cells. Stacked column graphs were generated as described in (B). Values are the means from 3 independent experiments. Error bars, SD.