Cyclin D1oncogenic overexpression induces global transcriptional downregulation in malignant lymphoid cells

Robert Albero[1,7], Anna Enjuanes[2,4,7], Santiago Demajo[1], Giancarlo Castellano[3], Magda Pinyol[2,4], Noelia García[1], Cristina Capdevila[1], Guillem Clot[1], Helena Suárez-Cisneros[2], Mariko Shimada[5,6], Ken Karube[5,6], Mónica López-Guerra[1,4,6], Dolors Colomer[1,4,6], Sílvia Beà[1,4], José Ignacio Martin-Subero[1,4], Elías Campo[1,4,6] and Pedro Jares[1,4,6*]

[1]*Lymphoid Neoplasm Program, August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain.*

[2]*Genomics Unit, August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain.*

[3]*Molecular Biology Core, Hospital Clinic of Barcelona, Barcelona, Spain*

[4] *Centro de Investigación Biomédica en Red de Cáncer (CIBERONC)*

[5]*Hematopathology Unit and Cell Biology, Graduate School of Medicine and Faculty of Medicine, University of the Ryukyus, Nishihara, Japan*

[6] *Haematopathology Unit, Department of Anatomic Pathology, Hospital Clınic, University of Barcelona, Barcelona, Spain*

[7]*These authors contributed equally to this work.*

Conflict of interest statement: The authors have declared that no conflict of interest exists

***Corresponding author:** Pedro Jares, Anatomic Pathology Department, Hospital Clinic de Barcelona, Villarroel 170, Barcelona, Spain, phone: 34-932275400 ext 2184, fax +34-932275572; e-mail: pjares@clinic.ub.es

ABSTRACT

Cyclin D1 is an oncogene frequently overexpressed in human cancers that plays a dual function as cell cycle and transcriptional regulator, although the latter is widely unexplored. Here, we investigated the transcriptional role of cyclin D1 in lymphoid tumor cells with cyclin D1 oncogenic overexpression. Cyclin D1 showed widespread binding to the promoters of most actively transcribed genes and the promoter occupancy positively correlated with the transcriptional output of targeted genes. Despite this association, the overexpression of cyclin D1 in lymphoid cells led to a global transcriptional downmodulation that was proportional to cyclin D1 levels. This cyclin D1 dependent global transcriptional downregulation was associated with a reduced nascent transcription and an accumulation of promoter-proximal paused RNA Polymerase II (Pol II) that colocalized with cyclin D1. Concordantly, cyclin D1 overexpresion promoted an increment of the Poll II pausing index. This transcriptional impairment seems to be mediated by the interaction of cyclin D1 with the transcription machinery. In addition, cyclin D1 overexpression sensitized cells to transcription inhibitors revealing a synthetic lethality interaction that it was also observed in primary MCL cases. This global transcriptional dysregulation expands the oncogenic cyclin D1 functions and places the transcriptional machinery as a potential therapeutic target in cyclin D1 overexpressing tumors.

Keywords: Cyclin D1, transcription, RNA Pol II; CDK9; malignant lymphoid cells, transcription inhibitors

INTRODUCTION

Cyclin D1 plays a central role in cell cycle regulation and it is frequently upregulated in cancer by different genomic alterations including amplifications in breast tumors and respiratory airway (1-4) and chromosomal translocations in mantle cell lymphoma (MCL) and multiple myeloma (MM) (5, 6). Moreover, point mutations disrupting the nuclear export process have been described in esophageal and endometrial carcinomas (7, 8). All these genetic alterations lead to an oncogenic overexpression of cyclin D1. Remarkably, *CCND1*, which encodes cyclin D1, is the most significantly amplified gene among the twelve tumor types recently analyzed in The Cancer Genome Atlas Pan-Cancer analysis project (9).

The classical tumorigenesis model considers that cyclin D1 mediates its oncogenic effect through its binding to CDK4 followed by pRB phosphorylation, E2F release and subsequent promotion of the G1/S phase transition (10). However, during the last decade a growing body of evidence has established that cyclin D1 has additional roles besides its canonical cell cycle function (11-13). The description of cyclin D1 interactions with transcription factors, chromatin-remodeling and histone-modifying enzymes has revealed a potential role of cyclin D1 as a transcriptional regulator in different cell models (14-17). However, whether the oncogenic overexpression of cyclin D1 is responsible for a transcriptional dysregulation in cancer cells remains unknown.

Mantle cell lymphoma is an aggressive lymphoid neoplasm that represents the paradigm of a neoplasia with cyclin D1 dependent oncogenesis. The t(11;14)(q13;q32) translocation that leads to the constitutive overexpression of cyclin D1 is the initial oncogenic event in this lymphoma (18). The relevance of cyclin D1 dysregulation in MCL pathogenesis is stressed by the recognition that MCL cells

adopt different mechanisms to increase the levels of cyclin D1 such as the amplification of the translocated allele or secondary rearrangements and mutations involving the 3' untranslated region that generate more stable *CCND1* transcripts (19-21). The expression of these abnormal transcripts correlates with the presence of higher protein levels and increased aggressiveness of the tumors(22). Recently, mutations at the cyclin D1 N-terminal region have been identified in MCL that also lead to an increased stability of the protein (23, 24).

In this study, we have investigated the role of cyclin D1 overexpression as a transcriptional regulator in malignant lymphoid cells. The integration of chromatin-immunoprecipitation sequencing (ChIP-seq) data of cyclin D1 with that of histone modifications and the transcriptional output of MCL cell lines revealed that cyclin D1 binds to the promoters of most actively transcribed genes and its overexpression led to a global downmodulation of the transcriptome program. This effect was associated with an accumulation of proximal-promoter paused RNA Polymerase II (Pol II) that overlapped with cyclin D1 bound regions. In concordance with the presence of higher levels of paused Pol II, the overexpression of cyclin D1 promoted an increased Pol II pausing index. This transcriptional dysregulation seems to be mediated by the physical interaction of cyclin with the transcription machinery. Finally, cyclin D1 overexpressing cells showed higher sensitivity to transcription inhibitors, a phenotype also observed in primary MCL cases, suggesting a synthetic lethality interaction that may open new therapeutic opportunities in cyclin D1 overexpressing tumors.

RESULTS

Cyclin D1 shows an extensive genome-wide chromatin binding in MCL cells

In order to characterize the genome-wide chromatin binding pattern of cyclin D1 we performed ChIP-seq of endogenous cyclin D1 in four MCL cell lines (Z-138, GRANTA-519, Jeko-1, and UPN-1). All these cell lines carry the t(11;14) translocation and display variable levels of cyclin D1 protein overexpression (Supplemental Figure 1A). Noteworthy, we found an outstanding number of cyclin D1 DNA binding regions with 19860 peaks common to all four MCL cell lines (Figure 1A). Interestingly, the number of identified peaks displayed a strong positive correlation with the amount of cyclin D1 protein (r=0.87) (Supplemental Figure 1B). The annotation of the peaks as promoter, gene body (exon or intron) or intergenic revealed enrichment in promoters (Supplemental Table 1). Peaks at promoters showed higher tag density and, concordantly, when a tag density filter was applied more than 50% of the peaks were classified as promoters (Figure 1B and Supplemental Table 2). In total, an average of 11583 coding genes displayed cyclin D1 binding to their proximal promoters and more than 74% of them were common among the four cell lines (n=8638) (Figure 1C). The actual distribution of cyclin D1 binding sites showed that these interactions tend to occur close and centered around the transcription start sites (TSS) of the genes (Figure 1D). Functional pathway analysis of genes showing cyclin D1 occupancy at promoters revealed that these genes are related to processes such as translation, RNA processing, cell cycle and DNA damage and repair among others (Figure 1E and Supplemental Table 3).

To validate the cyclin D1 promoter binding we performed ChIP-qPCR of eight genes with cyclin D1 peaks close to their TSS (Figure 1F and Supplemental Figure 1C). All the tested genes showed a significant cyclin D1 binding enrichment when

compared to a negative region (Figure 1G). The interaction of cyclin D1 with promoters is consistent with a transcriptional function of the cyclin in MCL cells, and the extensive binding observed across the genome is compatible with a global transcriptional role.

Cyclin D1 binds to open chromatin regions enriched in active histone marks

To further characterize the global binding of cyclin D1 and its potential transcriptional role, we investigated the features of the cyclin D1 associated chromatin. To do so, we compared the cyclin D1 binding pattern to the profiles of several histone marks and DNase I hypersensitive sites obtained for Z-138 cell line in the context of the Blueprint Epigenome Consortium (25). We observed that promoters occupied by cyclin D1 were enriched in active histone marks (H3K4me3 and H3K27ac) and DNAse I hypersensitivity sites (Figure 2A, B). Conversely, cyclin D1 was not present at promoters lacking active histone marks and DNAse I hypersensitivity sites (Figure 2A). Although cyclin D1 localized preferentially to active promoters, characterized by the presence of H3K27ac and H3K4me3 histone modifications, a fraction of cyclin D1 peaks also occurred at active enhancers, characterized by H3K27ac and H3K4me1 histone modifications (Figure 2C). The cyclin D1 binding density at active promoters was significantly higher than the observed in enhancers (Figure 2D; $P<2.2E-16$). Taken together, these results indicate that cyclin D1 binds to regions of open chromatin enriched in active histone marks, which include promoters and enhancers.

Cyclin D1 occupies promoters of highly expressed genes

To study the relationship between the binding of cyclin D1 to promoters and the transcriptional output we performed RNA-sequencing (RNA-seq) of the MCL cell lines. Interestingly, when all coding genes were stratified according to the mRNA expression levels, the groups with higher gene expression values displayed larger fractions of cyclin D1-bound genes (Figure 3A). Furthermore, cyclin D1 promoter occupancy showed a strong positive correlation with gene expression levels (rho= 0.98, P<2.2E-16) (Figure 3B) and the average binding density around the TSS was proportional to the transcription output of the genes (Figure 3C and Supplemental Fig 2A). These results were consistent with the high degree of overlap observed between cyclin D1 binding sites and active chromatin marks. Moreover, when we combined the expression of primary MCL cases (n=122) with the cyclin D1 peak-density found in MCL cell lines we observed a highly significant correlation between the cyclin D1 promoter binding density in MCL cell lines and the expression levels of the genes in primary MCL cases (Figure 3D, rho= 0.97, P<2.2E-16). This result shows that genes actively transcribed in MCL cell lines with cyclin D1 promoter binding are also highly expressed in primary MCL cases. Together, our results demonstrate that cyclin D1 binding occurs predominantly around the TSS of abundantly transcribed genes suggesting that cyclin D1 might regulate transcription in MCL cells.

Genome-wide recruitment of exogenous cyclin D1 to active gene promoters

We wanted to investigate whether exogenous cyclin D1 would be recruited to promoters and preferentially to the actively transcribed, in a similar way than the endogenous cyclin D1. To that end, we transduced a cyclin D1-negative lymphoblastoid leukemic cell line (JVM13) with a constitutively nuclear active form

of cyclin D1 carrying the T286A mutation (JVM13-cD1^{T286A}). This mutation prevents the physiological nuclear export and subsequent degradation of the cyclin allowing to reach cyclin D1 protein levels more similar to those observed in MCL cell lines (Supplemental Figure 2B). The cyclin D1 DNA binding profile observed in JVM13-cD1^{T286A} cells was comparable to the pattern identified in the MCL cell lines (Figure 3E), despite a lower number of cyclin D1 peaks was observed. The reduced number of peaks could be due to the lower amount of cyclin D1 protein constitutively expressed by JVM13-cD1^{T286A} compared with the MCL cell lines. In fact, the strong correlation observed between the number of identified peaks and the amount of cyclin D1 protein in MCL cell lines was strengthened when JVM13-cD1^{T286A} data were included (r=0.98; Supplemental Figure 2C). This result suggests that the degree of cyclin D1 chromatin binding is proportional to the amount of cyclin D1 protein.

To determine the possible association between the chromatin binding of exogenous cyclin D1 and the transcriptome output we performed RNA-seq of JVM13-cD1^{T286A} cells. The integration of ChIP-seq and RNA-seq data sets showed a strong positive correlation between the gene expression levels and exogenous cyclin D1 promoter density (rho= 0.97, P<2.2E-16; Figure 3F). Moreover, the average binding density around the TSS was proportional to the transcription levels of the genes, similar to what was observed for the endogenous cyclin D1 (Supplemental Figure 2D). In addition, the combination of the basal gene expression profile of JVM13 cells with the cyclin D1 chromatin binding showed that genes bound by exogenous cyclin D1 were already actively transcribed prior to the presence of cyclin D1 and the genome-wide recruitment around the TSS correlated with the previous mRNA levels of the bound genes (Supplemental Figure 2E). Altogether, these results suggest that cyclin D1 may be recruited to promoters that are pre-configured in an

active state, and the extent of recruitment would be dependent on the transcriptional levels of the targeted genes.

Cyclin D1 overexpression leads to a global RNA synthesis reduction in lymphoid cells

The cyclin D1 chromatin binding pattern identified in MCL cell lines and JVM13 cells overexpressing cyclin D1 evidenced important similarities to those recently reported for MYC (26-28). Both proteins display an extensive genome-wide binding to open chromatin, preferentially centered around the TSS of highly expressed genes, together with an unimodal distribution in a peak-density histogram (Supplemental Figure 3A) (26-28). Indeed, when we compared the chromatin binding of cyclin D1 with the MYC peaks previously described in a normal B-cell (GM12878) we observed that more than 23% of promoters bound by cyclin D1 also had been reported to be occupied by MYC in normal B cells (29). Moreover, both proteins showed a similar chromatin binding profile (Supplemental Figure 3B). In addition, the cyclin D1 peaks that colocalized with MYC showed significant higher tag density (P<2.2E-16; Supplemental Figure 3C). Interestingly, the particular chromatin binding pattern of MYC was associated with an increase in total RNA and mRNA which led to define MYC as a global transcriptional amplifier (26-28). To analyze whether the genome wide chromatin binding pattern of cyclin D1 is related to a potential role of the cyclin as a global transcriptional regulator we explored the relationship between cyclin D1 expression and the total cellular RNA content. We overexpressed in JVM13 cells either the highly stable nuclear form of cyclin D1 (JVM13-D1^{T286A}) or the wild-type form of the protein (JVM13-D1). As expected, inducible JVM13-D1^{T286A} cells showed higher cyclin D1 protein levels than JVM13-

D1 cells (Figure 4A). Following cyclin D1 induction we performed a spectrophotometric analysis of the absolute levels of total RNA obtained from a fixed number of cells (Figure 4B). Unexpectedly, cyclin D1 overexpressing cells showed significant lower amounts of total RNA per cell than JVM13 control cells (Figure 4B). In order to exclude that the reduction in the amount of total RNA could be related to the cell cycle distribution of the cells we measured the amount of total RNA by flow cytometry using pyronin Y (RNA) and Hoechst (DNA) co-staining. This approach confirmed that the significant decrease in the absolute levels of RNA content due to cyclin D1 overexpression was independent of the cell cycle phase, since it was observed in both G1 and G2/M cells (Figure 4C and Supplemental Figure 4A). Remarkably, the reduction was superior in JVM13-D1^{T286A} cells that express higher levels of cyclin D1 protein. In addition, we transduced the lymphoblastoid JVM2 cell line, which expresses low levels of endogenous cyclin D1 despite harbouring the t(11;14), with the stable nuclear (JVM2-D1^{T286A}) or the wild-type form of cyclin D1 (JVM2-D1) (Supplemental Figure 4E). The RNA quantification showed that JVM2-D1^{T286A} cells displayed a significant diminution in the amount of total RNA per cell when compared to JVM2 control cells. The reduction was not observed in JVM2-D1 cells that displayed low cyclin D1 levels (Supplemental Figure 4B-C and 4E).

To further confirm these results, we knocked-down cyclin D1 in the MCL cell line GRANTA-519 using two independent shRNA constructs (Figure 4D). In agreement with the overexpression experiments, cyclin D1 depletion led to a significant increase in the total RNA content per cell (Figure 4E-F). Once again, the cyclin D1 effect was dose-related, since the cells with the most knock-down efficiency showed the highest increase in RNA amount, and it was cell cycle

independent (Supplemental Figure 4D). Then, we investigated whether the different MCL cell lines showed the predicted association between cyclin D1 levels and the amount of total RNA. The analysis identified a highly significant inverse correlation between the cyclin D1 protein levels and the total RNA amount measured by flow cytometry (Figure 4G and Supplemental Figure 2E). This association remained significant when exogenous cyclin D1 overexpressing and cyclin D1 depleted cells were included (P<4.77E-4; Figure 4G and Supplemental Figure 4E). We next investigated whether this effect was also observed in multiple myeloma (MM), a lymphoid neoplasm showing cyclin D1 upregulation due to the t(11;14) translocation or gene amplification in a substantial fraction of the cases. The analysis of MM cell lines with or without the t(11;14) translocation confirmed in a different cancer cell model the negative correlation between cyclin D1 protein levels and the total RNA cell content (Figure 4H and Supplemental Figure 4F). Finally, the RNA quantification in primary MCL cases (n=11) also evidenced a negative correlation between the levels of cyclin D1 and the amount of total RNA although it did not reach statistical significance. (r=-0.4, P=0.2; Supplemental Figure 4G and 7C).

In order to test whether this reduction on the steady-state RNA levels reflects a decrease in RNA synthesis we performed nascent RNA-labeling experiments. We measured the incorporation of 5-ethylnyl-uridine (EU) in JVM13, JVM13-D1 and JVM13-D1T286A cells to quantify the rate of nascent transcription at single cell level. Interestingly, cyclin D1 overexpression led to a significant reduction in transcription (Figure 4I and Supplemental Figure 5A). Accordingly, cyclin D1 depletion in GRANTA-519 cell line revealed a significant increase in RNA synthesis (Figure 4J and Supplemental Figure 5B). Altogether, these results suggest that cyclin

D1 oncogenic overexpression induce a reduction in the steady-state RNA levels in a dose-dependent manner by decreasing total transcription.

Cyclin D1 produces a global downmodulation of mRNAs in lymphoid cells

We investigated whether the reduced transcription promoted by cyclin D1 overexpression also determines a global messenger RNAs (mRNAs) downregulation. The decrease in the quantity of total RNA per cell following cyclin D1 overexpression compromises the use of genome-wide approaches such as microarrays or RNA-seq to explore the presence of a global mRNA downmodulation, since these strategies usually compare the same amount of RNA between conditions (30). To overcome this limitation, we took advantage of the NanoString's nCounter technology, able to perform a direct digital quantification of cell extracts. We hybridized a commercial pre-defined panel containing 48 genes derived from the Microarray Quality Control Study (MAQC) (31) with cell extracts from three different amounts of cells corresponding to the cyclin D1 overexpression model (JVM13 and JVM13-cD1^{T286A}). The high correlation observed between mRNA counts and the number of cells confirmed the suitability of this approach to test the global effect of cyclin D1 (JVM13-Ctrl, r=0.969±0.016; JVM13-cD1^{T286A}, r=0.987±0.007; Figure 5A). In accordance with the total RNA quantification data, the cells overexpressing cyclin D1 showed lower gene expression levels than control cells at the three different amounts of cells (P<2.2E-16; Figure 5A). Interestingly, all 36 genes expressed in control cells were downregulated in cyclin D1 overexpressing cells. To further confirm the global impact of cyclin D1 in mRNA levels we analyzed the expression of a commercial pre-designed panel of 236 cancer related genes in JVM13-D1^{T286A} cell line following cyclin D1 induction. Control cells expressed 154 of these 236 genes. Consistently

with the RNA-seq, we observed an association between the cyclin D1 tag density and the gene expression levels in JVM13-D1^{T286A} cells (Figure 5B, *P*=1.7e-06). Remarkably, the average expression of these genes was significantly lower in JVM13-D1^{T286A} (Figure 5C). In this line, 94% (n=145) of the genes expressed in control cells showed lower mRNA levels in JVM13-D1^{T286A} (Figure 5D). To explore whether this mRNA downmodulation would include tumor suppressor genes (TSG) we compiled a set of 583 genes defined as TSG (32). We found that 30 out of 33 (91%) expressed TSG were downregulated in JVM13-D1^{T286A} cells. Interestingly, the TSG analyzed in the cancer panel showed more frequently cyclin D1 peaks at their promoters than the rest of genes (81% vs 61%, *P*=0.038, Supplemental Figure 6A). Then we explored the binding of cyclin D1 to the promoters of the 583 TSG in the MCL cell lines. We observed that a significant higher fraction of TSG showed cyclin D1 peaks in MCL cell lines (48% vs 36%, *P*<3.3E-9, Supplemental Figure 6B). Concordantly, the analysis of TSG in primary MCL cases evidenced that this set of genes tends to show high expression levels in MCL cases (Supplemental Figure 6C). Altogether, these results strongly suggest that the binding of cyclin D1 to gene promoters leads to a global transcription downmodulation of expressed mRNAs involving also TSG.

Cyclin D1 physically interacts with the transcription machinery and increase promoter-proximal Pol II pausing

To explore whether the cyclin D1 dependent transcriptome downmodulation was associated with changes in the RNA polymerase II chromatin binding profile we performed Pol II ChIP-seq experiments in control (JVM13) and cyclin D1 overexpressing cells (JVM13-D1^{T286A}) (Supplemental Figure 7A). Concordantly with the association between cyclin D1 chromatin binding and the transcriptional levels,

we observed a strong correlation between the tag density of cyclin D1 and Pol II together with a colocalization of both binding profiles around the TSS (Figure 6A-B). Interestingly, JVM13-D1^{T286A} cells showed higher Pol II loading at promoters and this difference was more evident in highly expressed genes (Figure 6B, Supplemental Figure 7B). These results suggest that the observed transcriptome downmodulation was not due to a reduced Pol II recruitment to promoters but it was related to an increased promoter-proximal Pol II pausing. Consistent with this scenario, cyclin D1 overexpressing cells displayed similar levels of Pol II protein than control cells (Figure 6C; Pol II, N-20) indicating that the increased promoter occupancy by Pol II was not due to the presence of higher Pol II levels. Interestingly, the phosphorylation status of the C-terminal domain (CTD) of Pol II was different in cyclin D1 expressing cells as compared to the control cells. Cyclin D1 overexpressing cells showed lower levels of Ser-2 phosphorylation, a CTD modification associated with active elongation (Figure 6C). Concordantly, the 8WG16 antibody that binds to unphosphorylated CTD and more weakly to Ser-5 phosphorylated CTD, showed higher signal in cyclin D1 overexpressing cells (Figure 6C) (33). These Pol II forms are associated with paused polymerase or early elongation complexes. This Pol II phosphorylation pattern supports the view that cyclin D1 overexpression increase promoter-proximal Pol II pausing. To confirm whether the Pol II pause release was affected by the overexpression of cyclin D1 we established the pausing index by performing the ratio between the Pol II occupancy at promoters and gene body regions (34). This analysis showed that cyclin D1 overexpression led to an augmented Pol II pausing index (Figure 6D). Altogether, these results strongly support that the reduction of total nascent transcription following cyclin D1 overexpression observed in lymphoid cells was due to an increased promoter-proximal Pol II pausing.

To investigate whether the phosphorylation pattern of Pol II could be dysregulated in primary MCL cases we analyze the expression of Pol II in a series of cases (n=14). All the samples showed expression of the CDT hyperphosphorylated form of Pol II (IIo) whereas the expression of the hypophosphorylated CTD form (IIa) was heterogeneous among primary MCL cases (Supplemental Figure 7C). We established a ratio between the levels of the IIa and IIo forms and the amount of total Pol II. When we classified the primary MCL cases in two groups based on their low or high cyclin D1 protein levels (Supplemental Figure 7C), we observed that the proportion of hyperphosphorylated Pol II (IIo) form was significantly higher in MCL cases with low cyclin D1 levels (Figure 6E), whereas the MCL cases with high cyclin D1 displayed significantly higher proportion of the hypophosphorylated form (IIa) (Figure 6E). This association between the hypophosphorylated form of the Pol II and the levels of cyclin D1 was confirmed using the 8WG16 antibody (Figure 6F, Supplemental Figure 7C). Overall, these results suggest that the overexpression of cyclin D1 may impact in the transcription process by dysregulating the RNA polymerase II CDT phosphorylation.

We next wanted to explore whether the transcription dysregulation promoted by cyclin D1 overexpression could be mediated by an interaction of the cyclin with the transcription machinery. Cyclin D1 co-immunoprecipitation analysis in MCL cell lines showed that indeed cyclin D1 interacts with Pol II (Figure 6G and Supplemental Figure 8A). Furthermore, the association found between the amount of cyclin D1 and the hypophosphorylation pattern compatible with Pol II paused led us to investigate whether this phenotype could be mediated by an off-target effect of the overexpressed cyclin D1 onto the cyclin-dependent kinase CDK9. This CDK is the kinase subunit of the positive elongation factor b (P-TEFb) required for release of paused Pol II and

responsible of Ser-2 phosphorylation during active elongation (35). In order to test whether cyclin D1 interacts with CDK9 we transduced 293T cells with cyclin D1 ($D1^{T286A}$) and CDK9 (CDK9-Flag) and performed co-immunoprecipitation experiments. Interestingly, we observed an interaction between CDK9 and cyclin D1 proteins (Figure 6H). Moreover, endogenous co-immunoprecipitation experiments in MCL cell lines confirmed this cyclin D1-CDK9 interaction (Supplemental Figure 8B-C). These results suggest that cyclin D1 overexpression may dysregulate the release of paused Pol II through its binding to CDK9. Altogether, these results indicate that cyclin D1 overexpression determines a global transcriptome downmodulation by interacting with the transcription machinery and interfering with the release of promoter-proximal paused Pol II.

Cyclin D1 overexpression sensitizes cells to transcription inhibitors

The global transcriptional downmodulation caused by cyclin D1 overexpression led us to hypothesize that cyclin D1 overexpression could render cells more sensitive to drugs targeting the transcription machinery. To explore whether cyclin D1 overexpression sensitizes cells to transcription inhibitors we treated control (JVM13) and cyclin D1 overexpressing (JVM13-$D1^{T286A}$) cells with DRB (5,6-Dichloro-1-beta-Ribo-furanosyl Benzimidazole), a transcription inhibitor that causes premature chain termination, being CDK9 its major target. We used DRB at two different concentrations (20μM and 40μM) below the levels reported to fully inhibit transcription (100μM) (36). This experiment demonstrated significantly increased sensitivity to DRB in cyclin D1 overexpressing cells compared to control cells (Figure 7A). To explore whether the sensitivity to transcription inhibitors was related to the cyclin D1 levels we analyzed the response of MCL cell lines to DRB. The MCL cell

lines showed a significant increase of apoptosis following DRB treatment (Figure 7B). Interestingly, the MCL cell lines with higher amount of cyclin D1 and lower RNA content per cell showed higher sensitivity to DRB (Z-138 and UPN-1).

To further investigate the synthetic lethality between cyclin D1 overexpression and transcription inhibition we treated MCL and MM cells lines with Triptolide, a transcription inhibitor that has been used for cancer treatment with promising results (37). This inhibitor promotes the degradation of RPB1, the largest subunit or RNA pol II (38). Again, UPN1 and Z-138 showed the highest sensitivity to Triptolide (Figure 7C). Interestingly, the cyclin D1 positive MM cell lines that showed lower amount of total RNA also displayed a significant higher sensitivity to Triptolide than cyclin D1 negative MM cell lines (Figure 7D). These results suggest that high cyclin D1 expression leads to increased sensitivity to transcription inhibitors.

To test whether this synthetic lethality interaction could be observed in primary MCL cases we treated cells from MCL patients with DRB and Triptolide. We observed that primary MCL cells with higher levels of cyclin D1 were more sensitive to DRB and Triptolide than MCL cells with lower amount of cyclin D1 (Figure 7E-F). Overall, these results suggest the existence of a potential synthetic lethality interaction between the overexpression of cyclin D1, responsible of a global transcription downmodulation, and transcription inhibitors.

DISCUSSION

We investigated whether the oncogenic overexpression of cyclin D1 is responsible for a transcriptional dysregulation in lymphoid tumor cells. As a model we used MCL cells characterized by the constitutive overexpression of cyclin D1 due to the t(11;14) translocation. We found that endogenous cyclin D1 showed widespread binding to promoter regions of active genes and its overexpression was responsible for a global transcriptional downmodulation in these malignant B cells. This dysregulation seems to be mediated by the interference of the overexpressed cyclin D1 with the release of promoter-proximally paused Pol II. The interaction of overexpressed cyclin D1 with the transcription machinery and the consequent global RNA downmodulaton rendered the tumor cells more sensitive to transcription inhibitors.

Our genomic analysis of cyclin D1 chromatin interaction revealed an outstanding and somewhat unexpected number of cyclin D1 binding sites that positively correlated with cyclin D1 protein levels and preferentially occurred at promoters close to the TSS. The analysis of the relationship between the cyclin D1 promoter occupancy and chromatin states together with the transcriptional output demonstrated that cyclin D1 binds globally to active promoters and these interactions are proportional to the mRNA abundance of targeted genes. Our results agree with the observation that exogenous cyclin D1 targets promoters of genes highly expressed during retina development in cyclin D1 knock-in mouse embryos (39). The density of cyclin D1 peaks in MCL cells was higher than the reported recently for exogenous cyclin D1 in mouse cancer cell lines and retinal tissue (39-41). This difference could be explained by the extremely high cyclin D1 protein levels present in MCL cell lines. Furthermore, we observed that exogenous cyclin D1 was recruited to promoters that

are pre-configured in an active state in the absence of cyclin D1, and the recruitment seems to be proportional to the expression levels of the targeted genes. This chromatin behavior was strikingly similar to that reported for MYC in human tumor cells and mouse primary lymphocytes (26-28, 42). Recent studies have associated MYC chromatin binding profile to its capacity to globally enhance transcription at all active loci giving rise to the model of MYC as a global transcriptional amplifier (26, 27).

Surprisingly, despite the significant similarities between the chromatin binding behavior of cyclin D1 and MYC, the overexpression of cyclin D1 in lymphoid cells was responsible for a significant reduction in the cell RNA content that was proportional to cyclin D1 protein levels, an effect that was cell cycle independent and was related to a reduced transcription rate. The analysis of MCL and MM cell lines and primary MCL cases confirmed the negative correlation between cyclin D1 protein levels and the total RNA cell content. Moreover, the digital quantification of two independent gene sets showed that cyclin D1 overexpression also determined a downregulation of most expressed mRNA transcripts. These results suggest that the widely spread binding of cyclin D1 to promoters decreases the nascent transcription of bound genes producing a significant reduction of the cellular mRNA content. Thus, cyclin D1 seems to act as global transcriptome regulator decreasing the overall gene transcription program of the cells. As far as we know, this is the first time that a global transcription downregulation effect has been reported for a protein outside the basal transcription machinery.

The phosphorylation state of the C-terminal domain (CTD) of Pol II is critical in determining its activity during the transcription process (43, 44). Initially, Pol II is recruited into the pre-initiation complex with a hypophosphorylated CTD (IIa) and later, during initiation, the CTD is phosphorylated on Ser-5 mainly by CDK7 and on

Ser-2 during active elongation by CDK9, the kinase component of the positive elongation factor b (P-TEFb), resulting in Pol II hyperphosphorylation (IIo). We observed that the overexpression of cyclin D1 determined a shift in the phosphorylation pattern of the Pol II CTD from a productive elongation to a polymerase paused pattern(35). Moreover, primary MCL cases with higher levels of cyclin D1 showed increased proportion of hypophosphorylated forms of Pol II (IIa).

These phosphorylation patterns were consistent with the observed accumulation in promoter-proximal regions of Pol II paused following cyclin D1 overexpression. Conversely, the global MYC dependent transcription amplification was related to the capacity of MYC to increase the Pol II processivity at all transcribed genes (26, 27, 45, 46). In light of these results, we postulated that the phosphorylation pattern shift and the global transcriptional downmodulation we observed could be mediated by the physical interaction of cyclin D1 with the transcription machinery. This scenario is supported by the co-immunoprecipitation of cyclin D1 with RNA Pol II and CDK9 observed in MCL cells lines and in 293T cells. Previous studies have reported the interaction of cyclin D1 with other CDKs, besides the canonical CDK4/6, in different cell models including CDK1, CDK2, CDK3, CDK5 and CDK11 (39, 47-49). Furthermore, the binding of cyclin D1 to non-canonical CDK partners has been described to inhibit the targeted CDK (50). In a similar way, the binding of cyclin D1 could sequester CDK9 in inactive complexes compromising the CDK9 dependent Ser-2 phosphorylation required for active elongation. This would give rise to the accumulation of paused RNA Pol II and the global transcriptional downmodulation we identified (Figure 8). Further analyses should clarify in more detail how cyclin D1 interferes with the activation of the transcription machinery, whether other CDK9 functions are compromised, and

whether off-target effects may be a common phenomena following the pathogenic overexpression of other cyclins.

The increased sensitivity to a CDK9 inhibitor displayed by lymphoid cells following cyclin D1 overexpression, together with the significantly greater sensitivity to transcription inhibitors observed in MCL and MM cell lines and in primary MCL cases with higher cyclin D1 levels suggest the existence of a synthetic lethality between cyclin D1 overexpression and transcription inhibition. This lethal interaction opens opportunities for new treatment strategies in cyclin D1 overexpressing tumors. In this sense, a recent study has reported that SNS-032, a CDK7/9 inhibitor, induces cytotoxicity in MCL cells (51). Interestingly, in line with our results, the SP-53 MCL cell line, which displays very low levels of cyclin D1 (52), was the less sensitive cell line to SNS-032 (51).

The identification of a cyclin D1 dependent global transcriptional donwmodulation may look at first glance inconsistent with a potential oncogenic effect of cyclin D1 in MCL and it raises the question about its potential significance. We may hypothesize that this transcriptional downmodulation could be a mechanism that participates in the transcriptional shut-down during the S phase following the G1/S transcriptional wave or during M-phase in a normal cell cycle (53, 54). This dysregulation also could correspond to a non-physiological collateral effect produced by the very high levels of cyclin D1. Further studies should clarify whether this downmodulation respond to a physiological role of cyclin D1.

The hypothesis that the transcriptional downregulation mediated by high levels of cyclin D1 plays a potential oncogenic role in MCL is suggested by the fact that MCL cells, in addition to the t(11;14)(q13;q32) translocation, accumulate other genetic alterations that lead to increased cyclin D1 levels, emphasizing that cyclin D1

dependent phenotypes might be important in MCL pathogenesis. Interestingly, the presence of more stable cyclin D1 transcripts and higher proteins levels correlates with increased aggressiveness of the tumors (22). Although this aspect requires further studies, we may speculate that a reduction of the transcriptome program of a cell could have an oncogenic impact by decreasing the levels of active TSG, including cell cycle checkpoint genes, which are frequently inactivated or downregulated during oncogenesis. In that sense, 91% of TSG expressed in the analyzed cancer panel were found downmodulated following cyclin D1 overexpression. Moreover, a significant fraction of TSG showed cyclin D1 promoter binding in MCL cell lines. These results would support the idea that the global transcription downmodulation could play an oncogenic role through the downregulation of TSG. The effect on proto-oncogenes would be limited since the activation of these genes usually requires dominant mutations or genetic alterations that lead to increased activity (Figure 8). Also, we may hypothesize that the accumulation of paused Pol II due to cyclin D1 overexpression might facilitate the generation of genomic instability by increasing the probability of conflicts between DNA replication and transcription machineries. The collision between these machineries may cause an increase in DNA breaks as a consequence of replication fork stalling and collapse leading to recombination and chromosome rearrangements (Figure 8) (55). In this sense, it has been recently shown that artificially stabilized transcription initiation complexes can impede replication fork progression (56). Further studies should clarify the potential conflicts between promoter-proximal paused Pol II mediated by cyclin D1 overexpression and DNA replication forks.

In conclusion, we have identified that oncogenic cyclin D1 overexpression produces a global transcriptome downmodulation. This dysregulation might, at least

in part, be mediated by the interaction of the cyclin D1 with the transcription machinery that would compromise the release of promoter-proximal paused Pol II. This global transcriptional downmodulation induced by cyclin D1 overexpression seems to generate a synthetic lethality interaction that may be exploited therapeutically.

METHODS

Cell lines and primary MCL cells

For this study, we used five well-characterized MCL cell lines (Z-138, GRANTA-519, JeKo-1, UPN-1 and JVM-2), seven multiple myeloma cell lines (JJN-3, RPMI 8226, NCI-H929, U266, KMS-12-PE, KMM-1 and ARP-1), a lymphoblastoid leukemic cell line JVM-13 cell line and HEK-293T. All cells were cultured at 37ºC and 5% CO2 in RPMI (Gibco) but GRANTA-519 and HEK-293T in DMEM (Gibco). Media was supplemented with 10% fetal bovine serum, 500 µg/ml of streptomycin and 500 units/mL of penicillin (Gibco).

Primary MCL samples (> 80% tumor cells) were obtained from peripheral blood samples of 14 MCL patients diagnosed according to the WHO criteria. Tumor cells were isolated by centrifugation on a Ficoll-Hypaque (GE Healthcare) gradient, cryopreserved and maintained within the Hematopathology collection registered at the Biobank from Hospital Clínic-IDIBAPS (R121004-094).

Plasmids and infection procedures

Plasmids for constitutive infection used the backbone pBABE-puro retroviral vector. pBABE-puro was a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene, plasmid number # 1764). pBABE puro-cyclinD1 (tagged with the 27-base pair sequence HA) was a gift from William Hahn (Addgene, plasmid number # 9050). pBABE-Flag-Cdk9-IRES-eGFP was a gift from Andrew Rice (Addgene plasmid # 28096). Mutation T286A in the cyclin D1 gene was inserted under the manufacturer's guidelines using the kit QuikChange II Site-Directed Mutagenesis Kit (Agilent). All

constructs were validated by sequencing. For Cyclin D1 knockdown, we used the MISSION pLKO.1-puro plasmids (Sigma-Aldrich) (Supplemental Table 4).

Constitutive overexpression models were achieved by spin-infection of JVM-13 or HEK-293T exponential growing cells (1350g, 90 min, 27ºC) in the presence of polybrene (Sigma-Aldrich) and supernatants containing viral particles produced in HEK-293T cells. HEK-293T CDK9-FLAG-GFP infected cells were sorted by cell cytometry (BD LSRFortessa, BD Biosciences). Cyclin D1 constitutive models were selected after one week of puromycin treatment at 0.4µg/mL (Sigma-Aldrich). Inducible cell lines JVM-2 and JVM-13 were developed under the manufacturer's guidelines using the plasmids of the kit Retro-X™ Tet-On® Advanced Inducible Expression (Clontech Laboratories). Silencing of cyclin D1 in exponential growing GRANTA-519 cells was performed by lentiviral spin-infection (1000g, 90 min, 32ºC) in presence of polybrene, followed by additional 3 hours incubation; the protocol was repeated for two consecutive days. Cells were selected with 0.5 µg/ml of puromycin (Sigma-Aldrich) 24 hours after infection.

In vitro transcription inhibition experiments

Exponential growing MCL cell lines and cyclin D1 inducible cells incubated for 24 hours in the presence of doxycicline (0.1µg/mL) were treated with 5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB**,** Sigma-Aldrich) or with Triptolide (Selleck Chemicals) at the indicated final concentrations. The annexin positive fraction was determined by cell cytometry 48 hours after DRB treatment or 72 hours after triptolide treatment using Annexin V Apoptosis Detection Kit FITC (eBioscience) according to the manufacturer's guidelines. Nine MCL primary samples were treated for 72 hours with DRB or triptolide at indicated concentrations and apoptosis was

analyzed in the CD19-positive fraction (CD19-PE,Beckton Dikinson) using Annexin V Apoptosis Detection Kit FITC (eBioscience).

Chromatin Immunoprecipitation (ChIP)

For cyclin D1 crosslinked chromatin was fragmented with the Covaris S220 instrument using the truChIP™ HighCell Chromatin Shearing Kit with Non-ionic Shearing Buffer. Inmmunoprecipitation was performed with anti-cyclin D1 antibody or control IgG. Chromatin was decrosslinked and purified with Agencourt AMPure beads (Beckman Coulter) according to the manufacturer's guidelines. 1% of sheared DNA was used as input control, for qPCR validation and for analyzing sonication efficiency. Equal volumes of eluted chipped DNA were amplified using SYBR Green PCR Master Mix (Applied Biosystems) under the manufacturer's guidelines. Specific primers were designed for the peaks obtained in the ChIP-seq analysis (Supplemental Table 3). Primers for negative regions were also designed to test the specificity of the enrichment. For Pol II crosslinked chromatin was sonicated with a Bioruptor (Diagenode). Shared chromatin was immunoprecipitated using anti-Pol II antibody or control IgG Chromatin was decrosslinked and purified using the phenol-chloroform method. 1% of sheared DNA was used as input control, for qPCR validation and for analyzing sonication efficiency.

ChIP-seq and RNA-seq

Library preparation for ChIP-seq was performed using the NEBNext Ultra DNA Library Prep kit (New England BioLabs). Briefly, the immunoprecipitated DNA (10 ng) was end-repaired, and A-nucleotide overhangs were then added, followed by adapter ligation, PCR enrichment (15 cycles) and purification with AMPure beads

(ratio 1.25) (Beckman Coulter). The purified DNA library products were evaluated and quantified using Bioanalyzer (Agilent, High Sensitivity DNA Kit) and the KAPA Library Quantification Kit (KapaBiosystems), respectively. Sequencing was performed on the Illumina MiSeq instrument using 50 cycles V2 kit. For RNA-seq total RNA was isolated using Trizol (Zymo Research). Preparation of RNA-seq libraries was carried out using the TruSeq RNA Sample Preparation Kit (Illumina) according to the manufacturer's standard protocol. The libraries were 75 bp paired-end sequenced at ~ 80 million reads per library with Illumina technology

RNA extraction and quantification by flow cytometry

Cells were counted in duplicate from cell cultures using FACS (Attune Nxt Thermo Fisher Scientific). Total RNA was extracted from one million cells using RNeasy Mini Kit - QIAGEN under manufacturer's guidelines. RNA extracts were quantified by Nanodrop N-1000 (Thermo Fisher Scientific). Cell pellets of one million cells were fixed in 0.5% formaldehyde for 15 min on ice. Cells were pelleted, washed with cold PBS and resuspended in 300µL cold PBS. Then 700µL of 100% cold ethanol dropwise was added while vortexing. The cells were incubated overnight at -20ºC. Cell pellets were washed once with PBS at room temperature and resuspended in 250mL of HBSS medium with calcium and magnesium. This suspension was incubated with a final concentration of 2ng/mL of Hoechst 33342 (Sigma-Aldrich) and 4 ng/mL of Pyronin Y (Sigma-Aldrich) for 20 mins at RT. Cells were analyzed using BD LSRFortessa SORP (BD Biosciences). Hoechst 33342 was excited by 355 nm laser and emitted at 450 nm. Pyronin Y was excited by 488 nm laser, emitted at 560 nm.

5-ethylnyl-uridine (EU) incorporation assay

EU incorporation assays were performed using the Click-iT RNA Alexa Fluor 594 Imaging Kit (Invitrogen) according to the manufacturer's instructions. Briefly, cells were incubated with 1 mM EU for 90 mins, fixed with 3.7% PFA for 15 min at room temperature, permeabilized with 0.5% Triton X-100 for 15 min and Click-iT reaction was performed. DNA was counterstained with Hoechst 33342. Experiments were performed in duplicate and in each independent experiment; at least four different fields and 150 cells were measured per condition. Images were acquired on A Leica AF6000 LX microscope and Hamamatsu digital camera (C4742-80) and analyzed using ImageJ. Nuclear masks were generated based on Hoestch staining and mean AlexaFluor 594 fluorescence intensities per pixel were quantified per nucleus.

Nanostring gene expression analysis

Cells were counted in duplicate from cell cultures using FACS (Attune Nxt Thermo Fisher Scientific). Pellets of $1x10^5$, $2x10^5$ or $4x10^5$ cells were lysed in 40µL Buffer RLT (Qiagen) for one minute at room temperature with strong vortexing and frozen immediately in dry-ice. We followed the manufacturer's guidelines for the nCounter cell lysate hybridization and used two different gene expressions panels: nCounter Human Cancer Reference Kit (GXA-CR1-12) and nCounter CAE Kit (GXA-CAE-12, NanoString Technologies). The signals for each gene were obtained with nSolver software 3.0 (NanoString Technologies).

Immunoprecipitation and Western blot

Protein extracts preparation and western blot analysis were performed as previously described (Palomero et al, 2014). The primary antibodies and the working dilutions are described in Supplemental Table 5. Flag immunoprecipiation of CDK9-Flag complexes was performed using ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich) following manufacturer's guidelines.

Genomic and Transcriptomic analysis

ChIP-seq reads were aligned using Bowtie (http://bowtie-bio.sourceforge.net/index.shtml, v4.3.4) against the human GRCh37/Hhg19 reference genome. Peak calling was performed with the Model-based Analysis for ChIP-seq (MACS, http://liulab.dfci.harvard.edu/MACS/, v1.4.2). Peaks were visualized on the UCSC browser (https://genome.ucsc.edu/). Significant cyclin D1-bound intervals were annotated regarding the overlap to genomic features. Sequentially, intervals were annotated as promoter peaks (from -3000 to +1000 around the TSS), then as gene body (from +1000 to TTS), further classified as exonic or intronic. Finally, intervals non-overlapping with any of these features were considered as intergenic peaks. Average score profiles were performed using the module Sitepro of the cis-regulatory element annotation system (CEAS, http://liulab.dfci.harvard.edu/CEAS/ v0.9.9.7). SeqMINER v1.3 was studied to visualize ChIP-seq clusters. Peaks were visualized with the Integrative Genomics Viewer (v2.3.81) and Venn diagrams were done using Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/). Pausing ratio for a gene is calculated dividing the number of tags between (-30, +300) around TSS and the number of tags in gene body (from +300 of TSS to TTS).

The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (https://david-d.ncifcrf.gov/) application was used to define the functional pathways associated to cyclin D1 bound genes. We performed hierarchical cluster analysis using two databases (GOTERM_BP_FAT and KEGG_PATHWAY) and the EASE score (modified Fisher exact p-value). The Enrichment Score was calculated as the geometric mean of -log EASE score and the percentage of enrichment was calculated as described before (40), based on the enrichment score of the top hits (Enrichment Score>5).

Affymetrix Expression Console was used to compute Robust Multichip Average (RMA) expression values from 122 MCL patients investigated with Affymetrix HG U133 plus 2.0 gene expression arrays previously deposited in GEO (accession no. GSE93291). For each gene, the probe set with the highest average signal was selected for the different analysis. RNA-seq reads were aligned using Bowtie as described above. To quantify the expression of each gene we used TopHat v2.0.9 with default parameters and the human GRCh37/Hhg19 as a reference genome. Gene expression values (quantified as reads per kilobase of transcript per million reads, RPKMs) were calculated using edgeR Bioconductor package. Analysis of transcription factor binding sites was obtained using ChIP-seq data from the ENCODE Project in GM12878 (https://www.encodeproject.org/) and available through the UCSC Genome Browser.

Statistics

Statistics and graphical results were done in R statistical computing language (R v3.1.3) and GraphPad Prism version 4.00 for Windows (GraphPad Software, La Jolla California USA). Data are mainly presented as the mean ± SEM. Differences in mean

were analyzed using parametric (paired and unpaired Student's t test and ANOVA) and non-parametric tests (Mann-Whitney, Kolmogorov-Smirnov and χ^2 test). Correlations were evaluated calculating the Pearson or Spearman correlation coefficient or mixed effects models among replicate measures, when appropriate. Multiple testing was dealt with by Holm–Bonferroni method. Two-sided tests were applied throughout, and a *P* value below 0.05 (after multiple-testing if required) was considered significant. Statistical tests used are indicated in the figure legend.

Study Approval

The study was approved by institutional review boards at the Hospital Clinic of Barcelona. Written informed consent was obtained from all participants and the ethics committees approved this consent procedure in accordance with the principles of the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

A.R., E.A. and S-C. H. performed ChIP-seq and RNAseq experiments. D.S. generated lentiviral silencing models. A.R., D.S., G.N. and C.C. performed gene expression experiments, western blotting and cell culture procedures. Bioinformatics and statistics data analyses were performed by C.G., A.R. and C.G. P.M performed Nanostring experiments. A.R. and K.K. generated the constitutive and inducible models. A.R, S.M, M.L. and D.C. performed transcription inhibition experiments. B.S. and M-S.I. contributed to discussion and data interpretation. E.A., C.E. and J.P. conceived the study, designed experiments, supervised the project and interpreted the data. A.R., E.A., D.S. and J.P. wrote the paper.

ACKNOWLEDGEMENTS

This study was supported by Grant No. PI14/00355, Fondo de Investigaciones Sanitarias-Instituto de Salud Carlos III (to P.J.); Grant No. SAF2015-64885-R, Ministerio de Economía y Competitividad (to E.C.; Grant No. AGAUR 2014-SGR-795 (to E.C.), Generalitat de Catalunya Suport Grups de Recerca; Grant No. RD12/0036/0036, the Red Temática de Investigación Cooperativa en Cáncer (RTICC); the European Regional Development Fund “Una manera de fer Europa”CERCA Programme / Generalitat de Catalunya (to E.C.). E.C. is an Academia Researcher of the "Institució Catalana de Recerca i Estudis Avançats" (ICREA) of the Generalitat de Catalunya. R.A. was supported by an FPU fellowship (Ministerio de Educación). S.D. was supported by a Juan de la Cierva fellowship (Ministerio de Educación). This work was mainly developed at the Centre Esther Koplowitz (CEK), Barcelona, Spain. We are indebted to the Genomics core facility of the Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) for the technical help. We are grateful to A. Davies, A. Girò, L. Plà, M. Sánchez, C. Muro and N. Villahoz for their excellent technical assistance.

REFERENCES

1. Dickson, C., Fantl, V., Gillett, C., Brookes, S., Bartek, J., Smith, R., Fisher, C., Barnes, D., and Peters, G. 1995. Amplification of chromosome band 11q13 and a role for cyclin D1 in human breast cancer. *Cancer Lett* 90:43-50.
2. Zukerberg, L.R., Yang, W.I., Gadd, M., Thor, A.D., Koerner, F.C., Schmidt, E.V., and Arnold, A. 1995. Cyclin D1 (PRAD1) protein expression in breast cancer: approximately one-third of infiltrating mammary carcinomas show overexpression of the cyclin D1 oncogene. *Mod Pathol* 8:560-567.
3. Jares, P., Fernandez, P.L., Campo, E., Nadal, A., Bosch, F., Aiza, G., Nayach, I., Traserra, J., and Cardesa, A. 1994. PRAD-1/cyclin D1 gene amplification correlates with messenger RNA overexpression and tumor progression in human laryngeal carcinomas. *Cancer Res* 54:4813-4817.
4. Callender, T., el-Naggar, A.K., Lee, M.S., Frankenthaler, R., Luna, M.A., and Batsakis, J.G. 1994. PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. *Cancer* 74:152-158.
5. Bosch, F., Jares, P., Campo, E., Lopez-Guillermo, A., Piris, M.A., Villamor, N., Tassies, D., Jaffe, E.S., Montserrat, E., Rozman, C., et al. 1994. PRAD-1/cyclin D1 gene overexpression in chronic lymphoproliferative disorders: a highly specific marker of mantle cell lymphoma. *Blood* 84:2726-2732.
6. Pruneri, G., Fabris, S., Baldini, L., Carboni, N., Zagano, S., Colombi, M.A., Ciceri, G., Lombardi, L., Rocchi, M., Buffa, R., et al. 2000. Immunohistochemical analysis of cyclin D1 shows deregulated expression in multiple myeloma with the t(11;14). *Am J Pathol* 156:1505-1513.
7. Benzeno, S., Lu, F., Guo, M., Barbash, O., Zhang, F., Herman, J.G., Klein, P.S., Rustgi, A., and Diehl, J.A. 2006. Identification of mutations that disrupt phosphorylation-dependent nuclear export of cyclin D1. *Oncogene* 25:6291-6303.
8. Moreno-Bueno, G., Rodriguez-Perales, S., Sanchez-Estevez, C., Hardisson, D., Sarrio, D., Prat, J., Cigudosa, J.C., Matias-Guiu, X., and Palacios, J. 2003. Cyclin D1 gene (CCND1) mutations in endometrial cancer. *Oncogene* 22:6115-6118.
9. Zack, T.I., Schumacher, S.E., Carter, S.L., Cherniack, A.D., Saksena, G., Tabak, B., Lawrence, M.S., Zhsng, C.Z., Wala, J., Mermel, C.H., et al. 2013. Pan-cancer patterns of somatic copy number alteration. *Nat Genet* 45:1134-1140.
10. Jares, P., Colomer, D., and Campo, E. 2007. Genetic and molecular pathogenesis of mantle cell lymphoma: perspectives for new targeted therapeutics. *Nat Rev Cancer* 7:750-762.
11. Coqueret, O. 2002. Linking cyclins to transcriptional control. *Gene* 299:35-55.
12. Casimiro, M.C., Velasco-Velazquez, M., Aguirre-Alvarado, C., and Pestell, R.G. 2014. Overview of cyclins D1 function in cancer and the CDK inhibitor landscape: past and present. *Expert Opin Investig Drugs* 23:295-304.
13. Pestell, R.G. 2013. New roles of cyclin D1. *Am J Pathol* 183:3-9.
14. Fu, M., Rao, M., Bouras, T., Wang, C., Wu, K., Zhang, X., Li, Z., Yao, T.P., and Pestell, R.G. 2005. Cyclin D1 inhibits peroxisome proliferator-activated receptor gamma-mediated adipogenesis through histone deacetylase recruitment. *J Biol Chem* 280:16934-16941.
15. Horstmann, S., Ferrari, S., and Klempnauer, K.H. 2000. Regulation of B-Myb activity by cyclin D1. *Oncogene* 19:298-306.

16. Reutens, A.T., Fu, M., Wang, C., Albanese, C., McPhaul, M.J., Sun, Z., Balk, S.P., Janne, O.A., Palvimo, J.J., and Pestell, R.G. 2001. Cyclin D1 binds the androgen receptor and regulates hormone-dependent signaling in a p300/CBP-associated factor (P/CAF)-dependent manner. *Mol Endocrinol* 15:797-811.
17. Zwijsen, R.M., Wientjens, E., Klompmaker, R., van der Sman, J., Bernards, R., and Michalides, R.J. 1997. CDK-independent activation of estrogen receptor by cyclin D1. *Cell* 88:405-415.
18. Jares, P., and Campo, E. 2008. Advances in the understanding of mantle cell lymphoma. *Br J Haematol* 142:149-165.
19. Gruszka-Westwood, A.M., Atkinson, S., Summersgill, B.M., Shipley, J., Elnenaei, M.O., Jain, P., Hamoudi, R.A., Kaeda, J.S., Wotherspoon, A.C., Matutes, E., et al. 2002. Unusual case of leukemic mantle cell lymphoma with amplified CCND1/IGH fusion gene. *Genes Chromosomes Cancer* 33:206-212.
20. Bea, S., Salaverria, I., Armengol, L., Pinyol, M., Fernandez, V., Hartmann, E.M., Jares, P., Amador, V., Hernandez, L., Navarro, A., et al. 2009. Uniparental disomies, homozygous deletions, amplifications, and target genes in mantle cell lymphoma revealed by integrative high-resolution whole-genome profiling. *Blood* 113:3059-3069.
21. Rosenwald, A., Wright, G., Wiestner, A., Chan, W.C., Connors, J.M., Campo, E., Gascoyne, R.D., Grogan, T.M., Muller-Hermelink, H.K., Smeland, E.B., et al. 2003. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell* 3:185-197.
22. Wiestner, A., Tehrani, M., Chiorazzi, M., Wright, G., Gibellini, F., Nakayama, K., Liu, H., Rosenwald, A., Muller-Hermelink, H.K., Ott, G., et al. 2007. Point mutations and genomic deletions in CCND1 create stable truncated cyclin D1 mRNAs that are associated with increased proliferation rate and shorter survival. *Blood* 109:4599-4606.
23. Mohanty, A., Sandoval, N., Das, M., Pillai, R., Chen, L., Chen, R.W., Amin, H.M., Wang, M., Marcucci, G., Weisenburger, D.D., et al. 2016. CCND1 mutations increase protein stability and promote ibrutinib resistance in mantle cell lymphoma. *Oncotarget*. 7:73558-73572.
24. Beà, S., Valdés-Mas, R., Navarro, A., Salaverria, I., Martín-Garcia, D., Jares, P., Giné, E., Pinyol, M., Royo, C., Nadeu, F., et al. 2013. Landscape of somatic mutations and clonal evolution in mantle cell lymphoma. *Proc Natl Acad Sci U S A* 110:18250-18255.
25. Queiros, A.C., Beekman, R., Vilarrasa-Blasi, R., Duran-Ferrer, M., Clot, G., Merkel, A., Raineri, E., Russinol, N., Castellano, G., Bea, S., et al. 2016. Decoding the DNA Methylome of Mantle Cell Lymphoma in the Light of the Entire B Cell Lineage. *Cancer Cell* 30:806-821.
26. Lin, C.Y., Loven, J., Rahl, P.B., Paranal, R.M., Burge, C.B., Bradner, J.E., Lee, T.I., and Young, R.A. 2012. Transcriptional amplification in tumor cells with elevated c-Myc. *Cell* 151:56-67.
27. Nie, Z., Hu, G., Wei, G., Cui, K., Yamane, A., Resch, W., Wang, R., Green, D.R., Tessarollo, L., Casellas, R., et al. 2012. c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. *Cell* 151:68-79.
28. Sabo, A., Kress, T.R., Pelizzola, M., de Pretis, S., Gorski, M.M., Tesi, A., Morelli, M.J., Bora, P., Doni, M., Verrecchia, A., et al. 2014. Selective transcriptional regulation by Myc in cellular growth control and lymphomagenesis. *Nature* 511:488-492.

29. Consortium, E.P. 2012. An integrated encyclopedia of DNA elements in the human genome. *Nature* 489:57-74.
30. Loven, J., Orlando, D.A., Sigova, A.A., Lin, C.Y., Rahl, P.B., Burge, C.B., Levens, D.L., Lee, T.I., and Young, R.A. 2012. Revisiting global gene expression analysis. *Cell* 151:476-482.
31. Consortium, M. 2006. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol* 24:1151-1161.
32. Yang, J., Chen, L., Kong, X., Huang, T., and Cai, Y.D. 2014. Analysis of tumor suppressor genes based on gene ontology and the KEGG pathway. *PLoS One* 9:e107202.
33. Xie, S.Q., Martin, S., Guillot, P.V., Bentley, D.L., and Pombo, A. 2006. Splicing speckles are not reservoirs of RNA polymerase II, but contain an inactive form, phosphorylated on serine2 residues of the C-terminal domain. *Mol Biol Cell* 17:1723-1733.
34. Zeitlinger, J., Stark, A., Kellis, M., Hong, J.W., Nechaev, S., Adelman, K., Levine, M., and Young, R.A. 2007. RNA polymerase stalling at developmental control genes in the Drosophila melanogaster embryo. *Nat Genet* 39:1512-1516.
35. Brookes, E., and Pombo, A. 2009. Modifications of RNA polymerase II are pivotal in regulating gene expression states. *EMBO Rep* 10:1213-1219.
36. Bensaude, O. 2011. Inhibiting eukaryotic transcription: Which compound to choose? How to evaluate its activity? *Transcription* 2:103-108.
37. Carter, B.Z., Mak, D.H., Schober, W.D., McQueen, T., Harris, D., Estrov, Z., Evans, R.L., and Andreeff, M. 2006. Triptolide induces caspase-dependent cell death mediated via the mitochondrial pathway in leukemic cells. *Blood* 108:630-637.
38. Wang, Y., Lu, J.J., He, L., and Yu, Q. 2011. Triptolide (TPL) inhibits global transcription by inducing proteasome-dependent degradation of RNA polymerase II (Pol II). *PLoS One* 6:e23993.
39. Bienvenu, F., Jirawatnotai, S., Elias, J.E., Meyer, C.A., Mizeracka, K., Marson, A., Frampton, G.M., Cole, M.F., Odom, D.T., Odajima, J., et al. 2010. Transcriptional role of cyclin D1 in development revealed by a genetic-proteomic screen. *Nature* 463:374-378.
40. Casimiro, M.C., Crosariol, M., Loro, E., Ertel, A., Yu, Z., Dampier, W., Saria, E.A., Papanikolaou, A., Stanek, T.J., Li, Z., et al. 2012. ChIP sequencing of cyclin D1 reveals a transcriptional role in chromosomal instability in mice. *J Clin Invest* 122:833-843.
41. Casimiro, M.C., Di Sante, G., Crosariol, M., Loro, E., Dampier, W., Ertel, A., Yu, Z., Saria, E.A., Papanikolaou, A., Li, Z., et al. 2015. Kinase-independent role of cyclin D1 in chromosomal instability and mammary tumorigenesis. *Oncotarget* 6:8525-8538.
42. Walz, S., Lorenzin, F., Morton, J., Wiese, K.E., von Eyss, B., Herold, S., Rycak, L., Dumay-Odelot, H., Karim, S., Bartkuhn, M., et al. 2014. Activation and repression by oncogenic MYC shape tumour-specific gene expression profiles. *Nature* 511:483-487.
43. Egloff, S., and Murphy, S. 2008. Cracking the RNA polymerase II CTD code. *Trends Genet* 24:280-288.

44. Chapman, R.D., Heidemann, M., Hintermair, C., and Eick, D. 2008. Molecular evolution of the RNA polymerase II CTD. *Trends Genet* 24:289-296.
45. Rahl, P.B., Lin, C.Y., Seila, A.C., Flynn, R.A., McCuine, S., Burge, C.B., Sharp, P.A., and Young, R.A. 2010. c-Myc regulates transcriptional pause release. *Cell* 141:432-445.
46. Wolf, E., Lin, C.Y., Eilers, M., and Levens, D.L. 2015. Taming of the beast: shaping Myc-dependent amplification. *Trends Cell Biol* 25:241-248.
47. Jirawatnotai, S., Hu, Y., Michowski, W., Elias, J.E., Becks, L., Bienvenu, F., Zagozdzon, A., Goswami, T., Wang, Y.E., Clark, A.B., et al. 2011. A function for cyclin D1 in DNA repair uncovered by protein interactome analyses in human cancers. *Nature* 474:230-234.
48. Jirawatnotai, S., and Sittithumcharee, G. 2016. Paradoxical roles of cyclin D1 in DNA stability. *DNA Repair (Amst)* 42:56-62.
49. Musgrove, E.A. 2006. Cyclins: roles in mitogenic signaling and oncogenic transformation. *Growth Factors* 24:13-19.
50. Higashi, H., Suzuki-Takahashi, I., Saitoh, S., Segawa, K., Taya, Y., Okuyama, A., Nishimura, S., and Kitagawa, M. 1996. Cyclin-dependent kinase-2 (Cdk2) forms an inactive complex with cyclin D1 since Cdk2 associated with cyclin D1 is not phosphorylated by Cdk7-cyclin-H. *Eur J Biochem* 237:460-467.
51. Chen, R., Chubb, S., Cheng, T., Hawtin, R.E., Gandhi, V., and Plunkett, W. 2010. Responses in mantle cell lymphoma cells to SNS-032 depend on the biological context of each cell line. *Cancer Res* 70:6587-6597.
52. Wang, M., Sun, L., Qian, J., Han, X., Zhang, L., Lin, P., Cai, Z., and Yi, Q. 2009. Cyclin D1 as a universally expressed mantle cell lymphoma-associated tumor antigen for immunotherapy. *Leukemia* 23:1320-1328.
53. Bertoli, C., Skotheim, J.M., and de Bruin, R.A. 2013. Control of cell cycle transcription during G1 and S phases. *Nat Rev Mol Cell Biol* 14:518-528.
54. Meryet-Figuiere, M., Alaei-Mahabadi, B., Ali, M.M., Mitra, S., Subhash, S., Pandey, G.K., Larsson, E., and Kanduri, C. 2014. Temporal separation of replication and transcription during S-phase progression. *Cell Cycle* 13:3241-3248.
55. Gaillard, H., Herrera-Moyano, E., and Aguilera, A. 2013. Transcription-associated genome instability. *Chem Rev* 113:8638-8661.
56. Mirkin, E.V., Castro Roa, D., Nudler, E., and Mirkin, S.M. 2006. Transcription regulatory elements are punctuation marks for DNA replication. *Proc Natl Acad Sci U S A* 103:7276-7281.

FIGURE LEGENDS

Figure 1. Cyclin D1 binds genome-wide in MCL cell lines. A) Venn diagram representing cyclin D1 ChIP-seq peaks in four MCL cell lines. B) Distribution of cyclin D1 interacting regions over specific genomic regions in MCL cell lines. Box plots showing cyclin D1 tag density of the different genomic regions and pie charts displaying the genomic distribution of genomic intervals with a number of tags higher than the mean. The distribution across the human genome is represented as a control. C) Venn diagram representing cyclin D1 targeted genes identified by ChIP-seq in MCL cell lines. Genes were considered targets when displayed cyclin D1 binding sites located within 1kb upstream of their TSS. D) Average signal profiling of cyclin D1 around the TSS (+/- 3kb) in MCL cell lines. E) Top hits of the functional annotation clustering analysis of common cyclin D1 target genes among the four MCL cell lines. Only the genes with the most significant peaks in their promoters (-log *P* >350) were considered for the analysis. F) Genome browser view of the ChIP-seq tag density plots of four representative cyclin D1 target genes. G) ChIP-qPCR validation of eight selected cyclin D1 target genes in GRANTA-519. The fold change enrichments relative to a negative region are represented (mean ± SEM) (n=2).

Figure 2. Cyclin D1 occupancy correlates with active promoter marks and open chromatin conformation. A) Heatmap showing the ChIP-seq tag density of cyclin D1, H3K27ac, H3K4me3, H3K4me1 and DNAse I cutting sites around all genomic TSS in Z-138. Each row represents a gene centered on the TSS (+/− 5kb). Promoters are sorted by cyclin D1 number of tags. Cyclin D1 bound (top) and unbound (bottom) cyclin D1 genes are shown. B) Pie chart representing common regions bound by cyclin D1, H3K27ac and H3K4me3 marks. Only cyclin D1 peaks at promoters (-5kb TSS) in Z-138 are shown. C) Cyclin D1 occupancy in active promoters and enhancers. Percentage of active promoters (H3K4me3+) and enhancers (H3K4me1+, H3K4me3-) colocalizing with cyclin D1 in active regions (defined by H3K27ac presence) are shown. D) Boxplot showing cyclin D1 number of tags in active promoters and active enhancers. The number of all cyclin D1 peaks is represented as control. *** *P*<2.2E-16, Student's t-test, Holm Bonferroni correction.

Figure 3. Cyclin D1 binding correlates with gene expression levels. A) Distribution of genes showing cyclin D1 peaks within their promoters (5Kb upstream of the TSS) according to their respective gene expression levels. All genes were sorted into 50 equal bins based on their expression levels. Results are shown as mean ± SEM of all four MCL cell lines. B) Linear correlation between cyclin D1 binding and transcription. Genes were sorted like in (A). The average of cyclin D1 ChIP-seq normalized tag densities at promoters and the RPKM normalized expression levels are shown for each bin. Spearman correlation, rho= 0.98, *P*<2.2E-16. C) Profile of cyclin D1 occupancy around the TSS in Z-138. Genes were divided in ten groups based on their expression levels (from higher to lower expression). The distribution of the cyclin D1 ChIP-seq tag density average around the TSS (+/- 1kb) is displayed for each group. D) Linear correlation between cyclin D1 binding in MCL cell lines and gene expression in MCL primary samples (n=122). Genes were sorted into 50 equal bins based on their expression in MCL samples. For each bin the cyclin D1 ChIP-seq tag density average in the MCL cell lines and the gene expression mean in primary samples are shown. Spearman correlation, rho= 0.97, *P*<2.2E-16. E) Heatmap showing the cyclin D1 ChIP-seq tag density within gene promoters of JVM13-cD1^{T286A} and MCL cell lines. Each row represents a gene centered on the TSS (+/− 5kb). Promoters are sorted by the number of cyclin D1 tags in Z-138. F) Linear correlation between cyclin D1 binding and gene expression in JVM13-cD1^{T286A}. Genes were sorted into 50 equal bins like in (B). Spearman correlation, rho= 0.97, *P*<2.2E-16.

Figure 4. Cyclin D1 overexpression results in a reduction of the total RNA content in malignant lymphoid cells. A) Cyclin D1 protein in JVM13-Ctrl, JVM13-D1 and JVM13-D1^{T286A}. α-Tubulin was used as loading control. B) Total RNA content extracted from 10^6 cells. Results are shown relative to the Ctrl (mean +/- SEM, n=9). **P*<0.05, Student's t-test. C) RNA quantification by pyronin Y staining in JVM13 inducible cell lines. Only cells in G1 phase were analyzed. Top panel: FACS profile of a representative experiment. Bottom panel: bar graph displaying the pyronin Y mean signal. Results are shown relative to the Ctrl (mean +/- SEM, n=3). **P*<0.05, ***P*<0.01, Student's t-test. D) Cyclin D1 expression in control (shCtrl) and Cyclin D1-depleted (shCycD1 #1 and #2) GRANTA-519 cells. α-Tubulin was used as

loading control. E) Total RNA content in cyclin D1-depleted GRANTA-519 cells like in (A). Results are shown relative to the Ctrl (mean +/- SEM, n=8), **P<0.01, ***P<0.001, Student's t-test. F) RNA quantification by pyronin Y staining in control and cyclin D1-depleted GRANTA-519 cells like in (B). Top panel and bottom panel like in (C). Results are shown relative to the Ctrl (mean +/- SEM,n=4) **P<0.01, ***P<0.001, Student's t-test. G) Correlation between cyclin D1 protein levels and pyronin Y staining in MCL cell lines and cell models. (mean +/- SEM, n=4) P=4.77E-4, mixed effects models. H) Pyronin Y intensity of seven multiple myeloma (MM) cell lines. The cell lines are colored according cyclin D1 levels. I-J) Quantification of nuclear EU intensity after 24h of cyclin D1 induction in JVM13 cell models (n=2) (I) or following cyclin D1 silencing in GRANTA-519 cell line (n=2) (J). **P<0.01, ***P<0.001, Student's t-test. Holm Bonferroni correction for multiple comparisons was applied to panels B, C, E and F.

Figure 5. Cyclin D1 overexpression produces a global downmodulation of mRNAs in lymphoid cells

A) Boxplot displaying nCounter-based gene expression data of a 48 gene panel analyzed in JVM13-Ctrl and JVM13-cD1^{T286A} cells. Cell extracts from three different amounts of cells, counted by cell cytometry, are represented on the X axis. The nCounter counts of expressed transcripts (counts > 30) are shown in log2 scale on the Y axis (n=2). ***P<2E-16, Student's paired t test. B) Boxplot displaying the mean gene expression level of expressed genes in Cancer Panel of the JVM13cD1^{T286A} according to cyclin D1 tag density at promoters (-5Kb,TSS) distributed in four quartiles . Cell extracts from 4x10^4 cells were analyzed (n=2). P=1.7E-06, ANOVA test. C) Boxplot displaying the mean gene expression level of expressed genes in Cancer Panel of JVM13-Ctrl and JVM13-cD1^{T286A} inducible cell lines. Cell extracts from 4x10^4 cells were analyzed (n=2). ***P<2E-16, Student's paired t test. D) Barplots displaying the gene expression ratio between JVM13-Ctrl and JVM13-cD1^{T286A} inducible cell lines. Genes are sorted from the highest to the lowest expression ratio, both upregulated genes (grey) and downregulated genes (red) over JVM13-Ctrl.

Figure 6. Cyclin D1 co-localizes with RNA Polymerase II and promotes an increase of the Pol II pausing index. A) Correlation between normalized cyclin D1 ChIP-seq tag density in JVM13-D1^{T286A} and Pol II ChIP-seq tag density at promoters in JVM13-Ctrl and JVM13-D1^{T286A}. Promoters are sorted into 50 equal-size groups based on ChIP-seq tag densities of cyclin D1. X-axis represents mean cyclin D1 normalized tags of the promoters in JVM13-D1^{T286A}. Y-axis represents Pol II tag density in both cell lines. The linear regression line between cyclin D1 and Pol II presence in promoters is shown. B) Average signal profiling of Pol II occupancy around the TSS (+/- 3kb) in JVM13-Ctrl and JVM13-D1^{T286A} inducible cell lines. Cyclin D1 binding profile in JVM13-D1^{T286A} is also shown. C) Western blot showing different phosphorylated forms of Pol II in JVM13-Ctrl, JVM13-D1^{T286A} and JVM13-D1 inducible cell lines. A representative western blot (n=3) for each antibody is represented. α-tubulin is used as loading control. D) Plot representing the pausing index. Lines illustrate right-handed shift of pausing ratio at all genes with cyclin D1 in their promoter (-5kb, TSS) after cyclin D1 induction in JVM13-Ctrl and JVM13-D1^{T286A} cells. ***P<2E-16, Kolmogorov-Smirnov test. E) Proportion of Pol II (IIo) and Pol (IIa) forms in primary MCL cases. P=0.01, non parametric Mann-Whitney test) F) Pol II (8WG16) antibody signal in primary MCL cases. P=0.03, non parametric Mann-Whitney test). G) Co-immunoprecipitation experiment in Z-138 using antibodies against cyclin D1 and control IgG. Immunoprecipitated proteins were analyzed by western blot analysis by blotting with cyclin D1 and Pol II antibody. Input at 1% was loaded as a control. H) Co-immunoprecipitation experiment in HEK293T-CDK9FLAG-cD1^{T286A} with anti-FLAG resins. Immunoprecipitated proteins were analyzed by western blot analysis by blotting with CDK9 and Cyclin D1 antibodies. HEK293T-cD1^{T286A} immunoprecipitation was used as negative control.

Figure 7. Cyclin D1 overexpression renders tumor cells sensitive to CDK9 inhibitors. A) Cell survival of cyclin D1 inducible model after treatment with a CDK9 inhibitor (DBR) at increasing concentrations. JVM13-Ctrl and JVM13-D1^{T286A} cells, after 24 hours of doxycilin induction, were treated during 48 hours with DBR. Results are shown as mean ± SEM respect to the untreated (mean ± SEM, n=3). *P<0.05, Student's t test. B) MCL cell lines were treated during 72 hours with DBR.

Results are shown respect to the untreated (mean ± SEM, n=4). *P<0.05; **P<0.01, ***P<0.001, Student's t test. C-D) Cell survival of MCL cell lines (C) and multiple myeloma cell lines (D) after treatment with Triptolide at 40nM. Exponential growing cell lines were treated and cell survival was measured at 48 h. Results are shown respect to the untreated (mean ± SEM, n=4) *P<0.05; **P<0.01, ***P<0.001, Student's t test (C) or mixed effects models (D). E-F) Cell survival of nine primary MCL cases after treatment with 60 μM of DBR (E) or 200nM of Triptolide (F). Cell survival was measured at 72 h after treatment. Survival is calculated respect to the untreated controls and the means for each group are represented. Non parametric Mann-Whitney test was applied. Holm Bonferroni correction for multiple comparisons was applied to panels A-C.

Figure 8. Proposed model for cyclin D1 dependent global transcriptional downregulation, a side effect of its oncogenic overexpression. A) Cyclin D1 canonical cell cycle role in normal cells. B) The overexpression of cyclin D1, in addition to the cell cycle induction, may interact with CDK9 and interfere with the normal release of Pol II paused compromising active elongation. This would lead to a global transcription downmodulation, including TSG. The dot lines indicate other potential oncogeneic effects, such increased genomic instability due to augmented conflicts between transcription and DNA replication machinery.

SUPPLEMENTARY DATA:

Cyclin D1oncogenic overexpression induces global transcriptional downregulation in malignant lymphoid cells

Robert Albero[1,7], Anna Enjuanes[2,4,7], Santiago Demajo[1], Giancarlo Castellano[3], Magda Pinyol[2,4], Noelia García[1], Cristina Capdevila[1], Guillem Clot[1], Helena Suárez-Cisneros[2], Mariko Shimada[5,6], Ken Karube[5,6], Mónica López-Guerra[1,4,6], Dolors Colomer[1,4,6], Sílvia Beà[1,4], José Ignacio Martin-Subero[1,4], Elías Campo[1,4,6] and Pedro Jares[1,4,6*]

[1]Lymphoid Neoplasm Program, August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain.

[2]Genomics Unit, August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain.

[3]Molecular Biology Core, Hospital Clinic of Barcelona, Barcelona, Spain

[4] Centro de Investigación Biomédica en Red de Cáncer (CIBERONC)

[5]Hematopathology Unit and Cell Biology, Graduate School of Medicine and Faculty of Medicine, University of the Ryukyus, Nishihara, Japan

[6] Haematopathology Unit, Department of Anatomic Pathology, Hospital Clınic, University of Barcelona, Barcelona, Spain

[7]These authors contributed equally to this work.

SUPPLEMENTARY DATA INDEX:

Supplemental Material and Methods

Cyclin D1 ChIP

Fifty million of exponential-growing cells were crosslinked for 10 minutes in 1% (v/v) formaldehyde at room temperature, neutralized with 125mM of glycine and washed twice with cold PBS. Cells were lysed according to the manufacturer's guidelines and chromatin was fragmented with the Covaris S220 instrument for 20 min (5% duty factor, 135w intensity, 200 cycles per burst) using the truChIP™ HighCell Chromatin Shearing Kit with Non-ionic Shearing Buffer (Covaris). Immunoprecipitation was performed with anti-cyclin D1 antibody or control IgG. Sheared chromatin was clarified by 10 mins of maximum speed centrifugation at 4°C. Sheared DNA was immunoprecipitated overnight at 4ªC with 20 µg of anti-cyclin D1 antibody or equivalent amount of control IgG. Antibody complexes were recovered with 80µL of equilibrated Protein G Dynabeads (Thermo Fisher Scientific) through co-incubation for 90 min at 4°C. After intensive washes in low salt buffer (1% Triton X-100, 150nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), high salt buffer (1% Triton X-100, 500nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), LiCl buffer (1% Nonidet 40, 0.25M LiCl, 10mM TrisHCl pH=8.0, 1% sodium deoxycholate, 1mM EDTA) and TE (10mM TrisHCl pH=8.0, 1mM EDTA), bound chromatin was eluted in ChIP elution buffer (1% SDS, 0.1M NaHCO3). Chromatin was decrosslinked and purified with for 4 hours at 65°C in the presence of NaCl and Proteinase K. Lastly, samples were treated with RNAse and Proteinase K before purification. AgencourtAMPure beads (Beckman Coulter) were used to purify DNA according to the manufacturer's guidelines. 1% of sheared DNA was used as input control, for qPCR validation and for analyzing sonication efficiency. Equal

volumes of eluted chipped DNA were amplified using SYBR Green PCR Master Mix (Applied Biosystems) under the manufacturer's guidelines. Specific primers were designed for the peaks obtained in the ChIP-seq analysis (Supplemental Table S3). Primers for negative regions were also designed to test the specificity of the enrichment.

Pol II ChIP

Twenty million of exponential-growing cells were crosslinked for 10 minutes in 1% (v/v) formaldehyde at room temperature, neutralized with 125Mm of glycine and washed twice with cold PBS. Cytoplasmic membranes were lysed in Pol II lysis buffer (1%SDS, 10mM EDTA, 50mM Tris-HCl pH=8) with protease inhibitors (Thermo Fisher Scientific) for five minuts incubation on ice and nuclei were pelleted by centrifugation (2000g, 5min, 4°C). Pol II lysis buffer was added to obtain a concentration of 25 million nuclei/mL and sonicated with a Bioruptor (Diagenode) for 18 mins (Cycles: 30''on/30''off, High Power, 4°C). In order to precipitate the SDS, sonicates were incubated on ice for 30-60 mins. Following 15 mins 4ºC centrifugation at maximum speed, the supernatants were diluted with ChIP buffer between 8-10 times. ChIP buffer was added to ensure that all samples had the same final volume. 30µg of total chromatin were immunoprecipitated overnight at 4ºC with 20 µg of anti-Pol II antibody or equivalent amount of control IgG. Antibody complexes were recovered after 90 mins of co-incubation with 50µL of equilibrated Protein G Dynabeads (Thermo Fisher Scientific) at 4°C. Beads were washed for five mins at 4ºC in low salt buffer (1% Triton X-100, 150nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), high salt buffer (1% Triton X-100, 500nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), LiCl buffer (1% Nonidet 40, 0.25M

LiCl, 10mM TrisHCl pH=8.0, 1% sodium deoxycholate, 1mM EDTA) and TE (10mM TrisHCl pH=8.0, 1mM EDTA). Bound chromatin was eluted in ChIP elution buffer (1% SDS, 0.1M NaHCO3). Chromatin was decrosslinked for 4 hours at 65°C in the presence of NaCl and Proteinase K. Lastly, samples were treated with RNAse A (Sigma Aldrich) and Proteinase K before purification. Samples were purified using the phenol-chloroform method. 1% of sheared DNA was used as input control, for qPCR validation and for analyzing sonication efficiency.

RNA Sequencing

Total RNA was isolated using Trizol (Zymo Research). Preparation of RNA-seq libraries was carried out using the TruSeq RNA Sample Preparation Kit (Illumina) according to the manufacturer's standard protocol. Briefly, mRNA molecules were purified from 800ng of total RNA using poly-T oligo attached magnetic beads. Following purification, mRNA was fragmented using divalent cations at 94ºC and copied into first strand cDNA using reverse transcriptase and random primers. Second strand cDNA synthesis was performed using DNA Polymerase I and RNase H. The cDNA fragments were end repaired and a single 'A' base overhang was added before adapter ligation. The products were then purified and amplified by PCR to generate the final cDNA library. The libraries were 75 bp paired-end sequenced at ~ 80 million reads per library with Illumina technology.

Immunoprecipitation and Western blot

Protein extracts preparation and western blot analysis were performed as previously described (Palomero et al, 2014). The primary antibodies and the working dilutions are described in Supplemental Table 5. For endogenous CDK9 and Cyclin D1

immunoprecipitation, approximately 15-20 million exponentially growing cells were washed in PBS RT (300g, 5 mins) twice. Protein extracts were done in immunoprecipitation buffer (0.5% Nonidet-40, 150nM NaCl, 10mM KCl, 1.5mM MgCL, 10mM Tris-HCl pH=8.0) with protease inhibitors (Thermo Fisher Scientific). Total protein was quantified and 1.5mg of total protein was used per co-immunoprecipitation and incubated overnight at 4°C with gently mixing with 7.5µg of CDK9, cyclin D1 antibody or IgG Mouse control antibody in a final volume of 350µL of Co-IP buffer. Then, for each sample 50µL of protein G-magnetic beads (Invitrogen) were equilibrated by washing twice with co-IP buffer. Beads were finally resuspended in 25µL of co-IP buffer and added to co-IPS. After 90 mins incubation, supernatants were recovered (Unbound fractions) and beads were washed twice for 5 mins with gentle rotating at 4°C with co-IP buffer. To elute, 40µL of Laemmli buffer (x2.5) without DTT were added to beads and heated at 70°C for 10 mins. A second elution was performed with 40µL of sample buffer (x2.5) and 5µL of DTT 1M. Finally, 5µL of DTT 1M were added to first elution and both elutions were boiled as usual for western blotting. Anti-Mouse light chain antibody-HRP conjugate (Millipore) was used to detect primary antibodies. Flag immunoprecipiation of CDK9-Flag complexes was performed using ANTI-FLAG® M2 Affinity Gel ((Sigma-Aldrich) following manufacturer's guidelines.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1

A) Western blot analysis of cyclin D1 in MCL cell lines. α-Tubulin was used as loading control. B) Linear correlation between cyclin D1 protein amount and the number of ChIP-seq peaks inMCL cell lines. Cyclin D1 intensity was corrected by α-Tubulin. Pearson correlation is shown. C) Genome browser views displaying the ChIP-seq tag density of the entire length of four representative cyclin D1 target genes in GRANTA-519 cell line.

Supplemental Figure 2

A) Profile of cyclin D1 occupancy around the TSS according to gene expression in UPN-1, JeKo-1 and GRANTA-519 cell lines. Genes were divided in ten groups based on their expression levels (from higher to lower expression). The average cyclin D1 ChIP-seq tag density distribution around the TSS (+/- 1kb) is displayed for each group. B) Western blot analysis of cyclin D1 expression in Z-138 MCL cell line (b), JVM13 transduced with empty-vector (JVM13-Ctrl) and JVM13 transduced with constitutive HA tagged Cyclin D1-T286A (JVM13-cD1^{T286A}) (a). β-Actin was used as loading control. C) Linear correlation between cyclin D1 protein amount and the number of ChIP-seqpeaks in four MCL cell lines and JVM13-cD1^{T286A} cells.D-E) Profile of cyclin D1 occupancy around the TSS in JVM13-cD1^{T286A} cells. Genes were divided in ten groups regarding gene expression in JVM13-cD1^{T286A} cells (D) or JVM13 control cells (E) from higher to lower expression. The average cyclin D1 ChIP-seq tag density distribution around the TSS (+/- 1kb) is displayed for each group.

Supplemental Figure 3

A) Kernel distribution of normalized cyclin D1 tag density showing a unimodal distribution in MCL cell lines. B) Heatmap showing the cyclin D1 ChIP-seq tag density within gene intervals of Z-138 and MYC ChIP-seq tag density in GM12878. Each row represents an interval of +/− 2kb around the summit part of the peak in Z-

138. Regions are sorted by the number of cyclin D1 tags in Z-138. C) Tag density of cyclin D1 peaks in Z-138 with or without MYC co-localization in GM12878 cells. Data is shown as mean ± SEM. ***P<2.2e-16,Student's t-test,Holm Bonferroni correction.

Supplemental Figure 4

A) RNA quantification by pyronin Y staining in JVM13 inducible cell lines. Only cells in G2/M phase were analyzed. Data isshown relative to the Ctrl (mean +/- SEM,n=3). *P<0.05,Student's t-test. B) RNA quantification by Nanodrop corresponding to total RNA extracted from one million cells in JVM2 inducible cell lines. Results are shown relative to the Ctrl (mean +/- SEM,n=9).C)RNA quantification by pyronin Y staining in JVM2 inducible cell lines. Only cells in G1 phase were analyzed. Pyronin Y mean signal is shown relative to the Ctrl (mean +/- SEM,n=3). *P<0.05,Student's t-test. D)RNA quantification by pyronin Y staining in cyclin D1-depleted GRANTA-519 cells. Only cells in G2/M phase were analyzed. Data is shown relative to the Ctrl (mean +/- SEM,n=4). ***P<0.001,Student's t-test.Holm Bonferroni correction for multiple comparisons was applied to panels A-D.E) Western blot showing cyclin D1 expression in different MCL cell lines and cyclin D1 overexpressing cell models. Endogenous cyclin D1 (b) or overexpressed-tagged cyclin D1 (a) are shown. α-Tubulin was used as loading control. F) Western blot showing cyclin D1 in different multiple myeloma cell lines. α-Tubulin was used as loading control. G) Correlation between cyclin D1 protein levels measured by western-blot and the total RNA content per cell quantified by pyronin staining in primary MCL cases. Pyronin signals represented are the mean of two technical replicates of G1 cell fraction. Cyclin D1 intensity was corrected by β-Actin.

Supplemental Figure 5

Representative images of EU staining (red) for the evaluation of nascent RNA synthesis after cyclin D1 induction for 2 days in JVM-13 cells (A) or following

cyclin D1 depletion in GRANTA-519 cells (B). DAPI (blue) was used to create the nuclear masks for quantification. Scale bars, 10 μm.

Supplemental Figure 6

A) Percentage of TSG and non-TSG genes with cyclin D1 peaks in their promoter regions (-5Kb from TSS) that were found expressed by the Cancer Panel in JVM13-cD1^{T286A} (P=0.038, χ^2 test). B) Percentage of TSG and non-TSG genes with peaks in the four MCL cell lines(P= P<3.3E-9, χ^2 test). C) Proportion of TSG genes distributed among the four quartiles corresponding to the gene expression levels in primary MCL cases. The group of highly expressed genes shows enrichment in TSGs. Color indicates percentage of TSG in each quartile.

Supplemental Figure 7

A) Pol II occupancy on three representative cyclin D1-bound genes. Pol II (8GW16) profiles are shown in JVM13-Ctrl and JVM13-D1^{T286A} inducible cell lines. Cyclin D1 binding pattern in JVM13-D1^{T286A} is also represented. B) Correlation between normalized Pol II ChIP-seq tag density at promoters in JVM13-Ctrl and JVM13-D1^{T286A}. Promoters were sorted into 50 equal-size groups based on ChIP-seq tag densities in JVM13-Ctrl. Dashed line marks diagonal. C) Western blot showing total Pol II (IIo and IIa isoforms), Pol II (8WG16) and cyclin D1 in 14 primary MCL cases. MCL cases with cyclin D1 expression above the mean were considered as high cyclin D1 (red) and samples below the mean as low cyclin D1 (green). β-Actin was used as loading control.

Supplemental Figure 8

A) Co-immunoprecipitation experiment in JeKo-1 using antibodies against cyclin D1 and control IgG. Immunoprecipitated proteins were analyzed by western blot analysis by blotting with cyclin D1 and Pol II antibody. Input at 1% was loaded as a control.B)

Endogenous CDK9 co-immunoprecipitation experiment in Z-138 and Jeko-1 (C) using antibodies against CDK9 (B) and control IgG. Immunoprecipitated proteins were analyzed by Western blot with cyclin D1 and CDK9 antibodies. Input at 1% was loaded as a control.

SUPPLEMENTAL TABLES

Supplemental Table 1. Enrichment of cyclin D1 ChIP regions in different genomic features (%)

Genomic feature	GRANTA-519	Jeko-1	UPN-1	Z-138	Genomicbackground
Promoter	26.0	26.7	24.8	18.9	3.5
Exon	4.0	5.2	3.5	3.5	1.9
Intron	23.9	24.0	26.2	28.6	42.4
Intergenic	46.1	44.1	45.5	49.0	52.2

Supplemental Table 2. Enrichment of cyclin D1 ChIP regions with tag density higher that the mean in each cell line in different genomic features (%)

Genomic feature	GRANTA-519	Jeko-1	UPN-1	Z-138	Genomicbackground
Promoter	56	56	49	42	3.5
Exon	5	6	5	5	1.9
Intron	12	12	15	19	42.4
Intergenic	27	26	31	34	52.2

Supplemental Table 3. Annotation clusters with Enrichment Score>5 in CycD1-bound regions.

Annotation_Cluster1	Enrichment Score: 11.575770882907724		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0006412~translation	6.11E-13	3.04E-16
KEGG_PATHWAY	hsa03010:Ribosome	3.21E-08	2.60E-11
GOTERM_BP_FAT	GO:0006414~translational elongation	4.35E-06	2.36E-09

Annotation Cluster2	Enrichment Score: 11.362884382236716		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0007049~cell cycle	2.65E-12	1.49E-15
GOTERM_BP_FAT	GO:0000278~mitotic cell cycle	4.68E-11	2.54E-14
GOTERM_BP_FAT	GO:0022402~cell cycle process	9.85E-11	5.35E-14
GOTERM_BP_FAT	GO:0022403~cell cycle phase	7.09E-10	3.85E-13
GOTERM_BP_FAT	GO:0000087~M phase of mitotic cell cycle	2.40E-08	1.30E-11
GOTERM_BP_FAT	GO:0051301~cell division	2.08E-07	1.12E-10
GOTERM_BP_FAT	GO:0007067~mitosis	2.80E-07	1.52E-10
GOTERM_BP_FAT	GO:0000280~nuclear division	2.80E-07	1.52E-10
GOTERM_BP_FAT	GO:0000279~M phase	5.17E-07	2.80E-10
GOTERM_BP_FAT	GO:0048285~organelle fission	5.76E-07	3.10E-10

Annotation Cluster3	Enrichment Score: 8.546639796201333		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0006396~RNA processing	4.11E-13	2.68E-16
GOTERM_BP_FAT	GO:0016071~mRNA metabolic process	5.33E-07	2.89E-10
GOTERM_BP_FAT	GO:0008380~RNA splicing	5.64E-07	3.06E-10
GOTERM_BP_FAT	GO:0006397~mRNA processing	2.86E-05	1.55E-08
GOTERM_BP_FAT	GO:0000398~nuclear mRNA splicing, via spliceosome	2.71E-04	1.47E-07
GOTERM_BP_FAT	GO:0000377~RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	2.71E-04	1.47E-07
GOTERM_BP_FAT	GO:0000375~RNA splicing, via transesterification reactions	2.71E-04	1.47E-07
KEGG_PATHWAY	hsa03040:Spliceosome	0.00439914	3.56E-06

Annotation Cluster4	Enrichment Score: 8.190837507398893		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	8.06E-07	4.37E-10
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	4.40E-06	2.39E-09
GOTERM_BP_FAT	GO:0006281~DNA repair	2.22E-05	1.20E-08
GOTERM_BP_FAT	GO:0033554~cellular response to stress	2.52E-04	1.36E-07

Annotation Cluster5	Enrichment Score: 7.557183124723839		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0022613~ribonucleoprotein complex biogenesis	9.06E-10	2.50E-13
GOTERM_BP_FAT	GO:0042254~ribosome biogenesis	2.10E-06	1.14E-09
GOTERM_BP_FAT	GO:0034470~ncRNA processing	1.28E-04	6.93E-08

GOTERM_BP_FAT	GO:0034660~ncRNA metabolic process	2.35E-04	1.27E-07
GOTERM_BP_FAT	GO:0006364~rRNA processing	0.01140151	6.19E-06
GOTERM_BP_FAT	GO:0016072~rRNA metabolic process	0.02713958	1.47E-05

Annotation Cluster6	Enrichment Score: 6.433945684537975		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0044265~cellular macromolecule catabolic process	6.96E-06	3.78E-09
GOTERM_BP_FAT	GO:0043632~modification-dependent macromolecule catabolic process	8.36E-05	4.54E-08
GOTERM_BP_FAT	GO:0019941~modification-dependent protein catabolic process	8.36E-05	4.54E-08
GOTERM_BP_FAT	GO:0006511~ubiquitin-dependent protein catabolic process	1.03E-04	5.61E-08
GOTERM_BP_FAT	GO:0009057~macromolecule catabolic process	1.59E-04	8.61E-08
GOTERM_BP_FAT	GO:0051603~proteolysis involved in cellular protein catabolic process	2.78E-04	1.50E-07
GOTERM_BP_FAT	GO:0044257~cellular protein catabolic process	3.65E-04	1.98E-07
GOTERM_BP_FAT	GO:0030163~protein catabolic process	0.00107267	5.82E-07
GOTERM_BP_FAT	GO:0006508~proteolysis	97.8774912	1.88E-06

Annotation Cluster7	Enrichment Score: 5.754700439254545		
Category	Term	FDR	EASE score
GOTERM_BP_FAT	GO:0051276~chromosome organization	1.15E-05	6.26E-09
GOTERM_BP_FAT	GO:0006325~chromatin organization	0.0408486	2.21E-05
GOTERM_BP_FAT	GO:0016568~chromatin modification	0.07203701	3.91E-05

Supplemental Table 4. Primers for cyclin D1 ChIP-seq validation and shSequences

Primers		
Gene	***Position***	***Sequence***
XPC	Forward	TTTAAGGAGGTCGCTCGAAG
XPC	Reverse	GGCCATTTTTCCTGAGTCTG
RFC3	Forward	TAGCCTTTCCGTCCAAAATC
RFC3	Reverse	GGCCTACGCTTGAAAATCC
CCNT1	Forward	CCGAGTTAACAGCCAATATGC
CCNT1	Reverse	GTTCTCGCGGGAAGATACAC
CDC5L	Forward	CTTTGGCCAGAGTGGTTTG
CDC5L	Reverse	GATATTGGGTGGCTGAAAGG
POLE	Forward	CGCTCCTCAGAGACATGGA
POLE	Reverse	CAAATTTCTCCCCTGAAGCA
TIPIN	Forward	CTCACCTCACGCAGAAAACA
TIPIN	Reverse	CCCAGGAGTTCCCGAGTATC
MRE11A	Forward	GCAGGATCCGTGAAAAGAA
MRE11A	Reverse	AGAGCCGAACTGGACTTGAA
RPL4	Forward	CCAAACCACTCCTATTCCCT
RPL4	Reverse	TAGCCAACTCGTAATAAGACCA
Chr12negativeregion	Forward	CCATTGTAGGAGCCAAATCC
Chr12negativeregion	Reverse	ATTGAACACCAGCTCCCAAC

sh_Sequences		
Identifier	***Reference***	***Sequence***
shCycD1 #1	(TRCN0000295873)	CCGGCCACAGATGTGAAGTTCATTTCTCGAGAAATGA ACTTCACATCTGTGGTTTTTG
shCycD1 #2	(TRCN0000295874)	CCGGACAACTTCCTGTCCTACTACCCTCGAGGGTAGTA GGACAGGAAGTTGTTTTTTG

Supplemental Table 5. Antibodies

Primary antibodies

Reference	Antigen detected	Company	W.B. Dilution	ChIP	Co- IP
sc-8396	Cyclin D1	Santa Cruz	1:1000	X	X
sc-20044	Cyclin D1	Santa Cruz	1:1000		
sc-753	Cyclin D1	Santa Cruz	1:1000		
13499	Phospho-Rpb1 CTD (Ser2) (E1Z3G)	Cell signalling	1:500		
sc-899	Pol II (N-20)	Santa Cruz	1:100		
sc-55492	Pol II (F-12)	Santa Cruz	1:500		
920101	RNA Polymerase II(8WG16)	Biolegend	1:500	X	
sc-13130	CDK9 (C-20)	Santa Cruz	1:1000		X
CP06-100UG	Alpha-Tubulin	Oncogene	1:10000		
A5341-100UL	Beta-actin	Sigma	1:5000		

Secondaryantibodies

Reference	Antigen detected	Company	W.B. Dilution
P0217	Anti-rabbit (HRP conjugated)	DAKO	1:3000
P0260	Anti-mouse (HRP conjugated)	DAKO	1:3000
AP200P	Anti-Mouse light chain antibody- HRP	Millipore	1:1000
7074	Anti-rabbit IgG, HRP-linked	Cell signalling	1:1000

ChIP/IP Control antibodies

Reference	Antigen detected	Company
sc-2025	Normal mouse IgG	Santa Cruz