MLL fusion-driven activation of *CDK6* potentiates proliferation in *MLL*-rearranged infant ALL

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Abbreviations: ALL, acute lymphoblastic leukemia; *CDK4*, cyclin-dependent protein kinase 4; *CDK6*, cyclin-dependent protein kinase 6; *EZH2*, enhancer of zeste homolog 2; *MLL*, mixed lineage leukemia; *RB1*, retinoblastoma protein 1 (*RB1*); WBC, white blood cell

Acute lymphoblastic leukemia in infants (<1 year-of-age) is characterized by a high incidence of *MLL* rearrangements. Recently, direct targets of the *MLL* fusion protein have been identified. However, functional validation of the identified targets remained unacknowledged. In this study, we identify *CDK6* as a direct target of the *MLL* fusion protein and an important player in the proliferation advantage of *MLL*-rearranged leukemia. *CDK6* mRNA was significantly higher expressed in *MLL*-rearranged infant ALL patients compared with *MLL* wild-type ALL patients ($P < 0.001$). Decrease of *MLL-AF4* and *MLL-ENL* fusion mRNA expression by siRNAs resulted in downregulation of *CDK6*, affirming a direct relationship between the presence of the *MLL* fusion and *CDK6* expression. Knockdown of *CDK6* itself significantly inhibited proliferation in the *MLL-AF4*-positive cell line SEM, whereas knockdown of the highly homologous gene *CDK4* had virtually no effect on the cell cycle. Furthermore, we show in vitro sensitivity of *MLL*-rearranged leukemia cell lines to the *CDK4/6*-inhibitor PD0332991, inducing a remarkable G₁ arrest, and downregulation of its downstream targets *pRB1* and *EZH2*. We therefore conclude that *CDK6* is indeed a direct target of *MLL* fusion proteins, playing an important role in the proliferation advantage of *MLL*-rearranged ALL cells.

Introduction

Obtaining successful treatment results in infant (<1 y-of-age) acute lymphoblastic leukemia (ALL) remains a major challenge in pediatric cancer. Infant ALL is characterized by chromosomal translocations involving the mixed lineage leukemia (*MLL*) gene,¹ which occur in approximately 80% of the cases.² As a result of such translocations, the N-terminal portion of the *MLL* gene becomes fused to the C-terminal region of one of its many translocation partner genes.³ The most recurrent *MLL* translocations found among infant ALL patients are t(4;11), t(11;19), and t(9;11),^{2,4} giving rise to the fusion proteins *MLL-AF4*, *MLL-ENL*, and *MLL-AF9*, respectively. To date, long-term event-free survival (EFS) rates for *MLL*-rearranged infant ALL range between ~30% and 50%, depending on the treatment protocol.² In infant ALL, the presence of *MLL* rearrangements, and young age are the strongest predictors of an unfavorable outcome.² Patients diagnosed with ALL below the age of 1 month have a 5-y overall survival of ~17%.⁵

MLL-rearranged ALL cells display unique gene expression signatures.^{6,7} Although this embodied an important finding, the overwhelming number of differentially expressed genes⁸ made it difficult to distinguish between the actual “drivers”

of the leukemia and by-stander effects. Recently, however, the biology underlying *MLL* fusion driven regulation of gene expression became better understood. *MLL*, a histone methyltransferase, normally catalyzes trimethylation of the fourth residue of histone 3 (H3K4me3).^{9,10} In contrast, *MLL* fusion proteins have lost this ability.¹¹ Nonetheless, most of the recurrent *MLL* translocation partners, like *AF4*, *ENL*, and *AF9*, are able to connect to another histone methyltransferase, i.e., *DOT1L*. Consequently, *MLL* fusion proteins recruit *DOT1L*, which leads to dimethylation of lysine 79 on histone 3 (H3K79me2) instead of H3K4me3.^{12–15} Both H3K4me3 and H3K79me2 are associated with transcriptional activation.¹⁶ Hence, genomic regions displaying aberrant H3K79me2 enrichment are prone to mark genes transcriptionally activated by the *MLL* fusion protein itself.¹⁷ With this in mind, Guenther et al. and Krivtsov et al. independently identified signatures consisting of such genes, including, for instance, *MEIS1*, *FLT3*, and several *HOXA*-cluster genes.^{17–19} Strikingly, based on H3K79 methylation profiles alone, *MLL*-rearranged ALL could be distinguished from other ALL subtypes lacking *MLL* rearrangements.¹⁹ Thus, we now have access to smaller and more specific gene sets consisting of genes abnormally regulated by

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the *MLL* fusion protein itself. However, the question remains which of these genes contributed the most to leukemogenesis and/or leukemia maintenance. From this perspective, we here studied one of the identified *MLL* fusion target genes, i.e., *CDK6* (encoding human cyclin-dependent kinase 6).¹⁸ Like its functional homolog *CDK4*, *CDK6* is a serine/threonine kinase that is activated upon association with D-type cyclins (i.e., cyclin D1, D2, and D3) during the G₁ phase of the cell cycle.²⁰ Activation of this complex leads to partial inactivation (by phosphorylation) of retinoblastoma protein 1 (RB1),²¹ allowing progression to the S phase.²² Activation of *CDK4* and *CDK6* can be prevented by complex formation with cyclin-dependent kinase inhibitors *CDKN2A* and *CDKN2B*. In human cancers, deregulation of the cell cycle is often mediated by alterations in

CDK activity (including that of *CDK4* and *CDK6*), inducing unscheduled proliferation as well as genomic and chromosomal instability.^{23,24} For instance, *CDK4* or *CDK6* overexpression has been demonstrated in several malignancies, including certain types of leukemia and lymphomas.²⁵

As *CDK6*, but not *CDK4*, has been proposed as a direct target of *MLL* fusion proteins, we postulated that *MLL*-rearranged ALL cells may gain a proliferative advantage from *MLL* fusion-driven upregulation of *CDK6*. A major complication in studying *MLL*-rearranged ALL is that there are no human models recapitulating the presence of the *MLL*-AF4 fusion protein.²⁶⁻²⁹ Therefore, we set out to explore to what extent *MLL*-rearranged ALL cells are dependent on *CDK6* in terms of cell proliferation using the *MLL*-AF4 fusion protein-positive cell line SEM.

Table 1. All *CDK6* probe sets significantly higher expressed in *MLL*-r infant ALL patients

ID	logFC	T	P value	adj. P value	B
224848_at	1.035591	7.564293	7.52E-12	3.29E-09	16.59254
243_g_at	0.546159	7.028207	1.22E-10	3.11E-08	13.91603
224847_at	0.892852	7.00689	1.36E-10	3.40E-08	13.81122
235287_at	1.072996	6.819905	3.53E-10	7.54E-08	12.89764
224851_at	0.961323	6.218592	6.99E-09	8.41E-07	10.03769
231198_at	0.566643	5.643918	1.07E-07	7.03E-06	7.433588
207143_at	0.573842	4.655778	8.15E-06	0.000215	3.320232
214160_at	0.438097	3.898921	0.000157	0.002164	0.547444

Table representing all probe sets of *CDK6* on the HGU133plus2.0 microarray with results from linear modeling for microarrays (LIMMA) searching for differentially expressed probe sets between *MLL*-rearranged infant ALL patients (n=68) and *MLL* wild-type samples (consisting of *MLL* wild-type infant ALL patients [n = 18], pediatric (non-infant) B-ALL patients [n = 16], and healthy bone marrow samples [n = 13]). *MLL*-r, *MLL*-rearranged; ID, probe set; logFC, estimate of the log2-fold-change; T, moderated T-statistic; P value, raw P value; adj. P value, FDR-adjusted P value or Q value; B, log odds.

Figure 1. *CDK6* is marked by occupation of *MLL*, *AF4*, *H3K79me2*, and *H3K4me3* in SEM. Graph visualizing number of annotated 36-base pair reads obtained from ChIP-seq analysis at the *CDK6* locus (left panel) and *CDK4* locus (right panel) using antibodies against the N-terminal domain of *MLL* and the C-terminal domain of *AF4*, *H3K4* trimethylation, and *H3K79* dimethylation in *MLL*-AF4-positive cell line SEM. An additional 1 kb upstream and downstream of *CDK4* and an additional 10 kb upstream and downstream of *CDK6* is given.

Results

The *CDK6* locus is occupied by MLL and AF4, and associated with H3K79me2

Confirming recent observations,^{18,19} we validated binding of the MLL-AF4 fusion protein at the genomic localization of *CDK6*. For this, ChIP-sequencing analyses was performed on chromatin precipitations (using antibodies against MLL, AF4, H3K4me3, and H3K79me2) obtained from MLL-AF4-positive SEM cells. We found pronounced co-occupancy of both the N-terminal domain of MLL and the C-terminal domain of AF4 at the transcriptional start site of *CDK6*. In contrast, at the *CDK4* locus, binding of MLL and AF4 appeared largely absent, suggesting MLL-AF4 occupancy at the *CDK6*, but not the *CDK4* locus (Fig. 1). Moreover, the presence of MLL and AF4 at the genomic *CDK6* locus appeared to be associated with both H3K4me3, as well as H3K79me2, whereas the *CDK4* locus did not seem to be marked by these histone modifications (Fig. 1). In addition, these histone marks were present on broad domains throughout *CDK6*. Similar spreading of both histone modifications at key leukemia and stem cell-associated genes has been described by Guenther et al.¹⁸

Elevated *CDK6* expression in MLL-rearranged infant ALL

Using our recently published gene expression profiling data (Affymetrix HU133plus2.0 GeneChips),⁸ we evaluated *CDK6* and *CDK4* expression levels in MLL-rearranged infant ALL. *CDK6* appeared significantly more highly expressed in the MLL-rearranged infant ALL patients when compared with infant ALL patients ($P = 0.001$), pediatric non-infant (>1 y-of-age) precursor B-ALL patients ($P < 0.0001$) carrying wild-type MLL genes, or healthy bone marrow samples ($P < 0.0001$) (Fig. 2A). All probe sets of *CDK6* had an FDR-adjusted P value of 0.002 or smaller (Table 1). In contrast, *CDK4* expression was not upregulated in MLL-rearranged infant ALL compared to infant ALL with wild-type MLL (Fig. 2B). Among MLL-rearranged infant ALL patients, *CDK6* expression is higher than the expression of *CDK4* ($P < 0.0001$) (Fig. 2A and B). The downstream *CDK4/CDK6* target *RB1* also appeared significantly more highly expressed in MLL-rearranged infant ALL patients when compared with pediatric precursor B-ALL patients and healthy bone marrow samples ($P < 0.0001$). However, *RB1* expression levels in MLL wild-type infant ALL patients appeared comparable to that of the MLL-rearranged infant ALL cases (Fig. 2C).

WBC higher in patients with high *CDK6* mRNA expression

Although the white blood cell (WBC) count at diagnosis obviously is subjected to many variations,

Figure 2. *CDK6* mRNA expression values are higher in MLL-rearranged infant ALL patients. Graphical representation of VSN-normalized expression values of *CDK6* mRNA (224848_at) (A), *CDK4* mRNA (202246_s_at) (B), and *RB1* mRNA (203132_at) (C) from gene-expression profiling data (Affymetrix HU133plus2 GeneChips) in patient material. *CDK6* is significantly higher expressed in MLL-rearranged infant ALL patients (MLL-r infant ALL, $n=68$) compared to MLL wild-type infant ALL (MLL wt infant ALL, $n=18$), pediatric (non-infant) precursor B-ALL patients (pre-B-ALL, $n=16$) and healthy bone marrow (healthy bone marrow, $n=13$). *CDK4* is only significantly higher expressed in MLL-rearranged infant ALL patients compared to pediatric B-ALL patients. *RB1* is significantly higher expressed compared to pediatric B-ALL patients and healthy bone marrow samples, but not compared to wild-type MLL infant ALL patients. The differences between patient groups were statistically analyzed using the Mann-Whitney U test. Probe sets with the largest difference between MLL-rearranged and MLL wild-type are shown in the graph; all probe sets of *CDK6* had an FDR-adjusted P value of 0.002 or smaller (Table 1). Error bars represent the mean $\pm$ standard error of the mean.

this parameter may reflect some degree of leukemic cell proliferation and disease aggressiveness. For instance, among infant ALL patients, high WBC counts are highly characteristic and represent an independent predictor of an adverse outcome.² Thus, with WBC counts as a suggestive marker for leukemic blast proliferation, we determined whether high-level *CDK6* expression was associated with increased WBC at diagnosis. For this, we compared *MLL*-rearranged infant ALL patients with “low” (< median) and “high” ($\geq$ median) *CDK6* expression, and found that patients expressing high levels of *CDK6* presented with significantly higher WBC counts at diagnosis ($P = 0.027$) (Fig. 3, left panel). A similar association with the expression of *CDK4* was not observed (Fig. 3, right panel).

CDK6 expression is regulated by the MLL fusion

To confirm that *CDK6* indeed is regulated by the MLL fusion itself, we transfected the *MLL*-rearranged ALL cell lines KOPN-8 and SEM with siRNAs directed against *MLL-ENL* or *MLL-AF4*, respectively. Transfection with si*MLL-ENL* in KOPN-8 resulted in a 92% decrease of the *MLL-ENL* transcript as compared with transfections with non-silencing siRNAs directed against *AGF1*. Similar experiments using si*MLL-AF4* in SEM cells showed a 67% reduction in *MLL-AF4* mRNA expression (Fig. 4A). Knockdown of *MLL-ENL* and *MLL-AF4* led to pronounced decreases in *CDK6* expression in both KOPN-8 and SEM cells, whereas *CDK4* expression largely remained unaffected (Fig. 4B). Similarly, upon knockdown of the MLL fusion, protein levels of CDK6 were affected to a greater extent than that of CDK4 (Fig. 4C). Hence, these data are in line with our confirmatory ChIP-seq data (Fig. 1) and clearly demonstrate that transcription of *CDK6*, but not *CDK4*, is driven by the MLL fusion itself.

CDK6 drives proliferation in MLL-rearranged ALL

To assess to what extent proliferation in *MLL*-rearranged ALL cells depends on the elevated expression of *CDK6*, we separately knocked down *CDK6* and *CDK4* in the t(4;11)-positive ALL cell line SEM. This led to the transient reduction of CDK6 and CDK4 protein expression to approximately 50% for 24 to 48 h, whereupon protein expression was almost completely recovered

(Fig. 5A). Cell cycle analysis showed that suppression of CDK4 hardly affected proliferation. In contrast, knockdown of CDK6 led to marked increases in the number of cells present in the G_0/G_1 phase, at the expense of the percentage of cells in the S phase or G_2/M phase (Fig. 5B). Cell cycle analysis alternatively assessed by BrdU staining at 48 h after transfection, showed similar results with an increase in number of cells in the G_1 phase and decrease of number of cells in the S phase after transfection with *CDK6*, while knockdown of *CDK4* practically had no effect on the cell cycle (Fig. 5C). These data establish a role for CDK6, but not for its homolog CDK4, in the proliferation of *MLL*-rearranged ALL cells. Moreover, neither CDK4 nor CDK6 suppression affected cell viability as determined by Annexin V/PI staining (Fig. 5D).

In vitro sensitivity of MLL-rearranged ALL cell lines to PD0332991

Next we tested the in vitro sensitivity to the CDK4/CDK6 inhibitor PD0332991 of *MLL*-rearranged ALL cell lines SEM, KOPN-8, BEL-1, and RS4;11. All *MLL*-rearranged ALL cell lines tested appeared highly responsive to PD0332991 treatment, with IC_{50} values (i.e., the concentration required to inhibit 50% of the viable cells) ranging from ~ 0.08 – $8 \mu M$ (Fig. 6). Next we exposed t(4;11) SEM cells and t(11;19) KOPN-8 cells to $1 \mu M$ of PD0332991 for 48 h. In SEM and KOPN-8 cells, this resulted in a considerable G_1 arrest, almost doubling the percentage of cells residing in the G_0/G_1 phase, while the number of cells in the S phase dropped to less than 20% as compared to unexposed control cells (Fig. 7). Likewise, the number of cells in G_2/M phase was also markedly reduced.

PD0332991 inhibits downstream targets pRB1 and EZH2 in MLL-rearranged ALL cell lines

As pRB and EZH2 are downstream targets of CDK6, we determined protein levels of the pRB and EZH2 in the *MLL*-rearranged ALL cell lines SEM and KOPN-8 in the presence and absence of PD0332991 ($1 \mu M$). Exposure to PD0332991 did not affect CDK6 protein levels itself, which is hereditary to the working mechanism of PD0332991, but effectively suppressed RB1 phosphorylation and EZH2 expression in all 3 cell lines (Fig. 8).

Figure 3. WBC count is higher in patients with high *CDK6* mRNA expression. Graph shows white blood cell (WBC) count (y-axis) in *MLL*-rearranged patients with low (<median, $n = 31$) and high ($\geq$ median, $n = 31$) expression of *CDK6* (left panel) and *CDK4* (right panel). The difference between patient groups were statistically analyzed using the Mann-Whitney U test.

Discussion

We here confirm that, as proposed by previous reports,^{18,19} the cell cycle-dependent kinase CDK6 represents a direct target of the MLL fusion protein in *MLL*-rearranged ALL. Furthermore, we show that the transcriptional activation of *CDK6* provides *MLL*-rearranged ALL cells with a proliferative advantage, which can effectively be counteracted by inhibiting CDK6. Hence, CDK6 may be an attractive therapeutic target for this aggressive type of leukemia, with the CDK4/CDK6 inhibitor PD0332991 as a potential active drug. Furthermore, this study demonstrates the importance of public databases (e.g., ChIP-seq and gene expression databases) for furthering research faster.

PD0332991 is a well-tolerated oral *CDK4/6*-inhibitor with myelosuppression³⁰ and neutropenia³¹ as dose-limiting toxicities. Clinical benefit of PD0332991 has been shown, with progression-free survival for over a year in 5 out of 17 mantle cell lymphoma patients,³² and partial response in patients with a progressive teratoma;³³ both tumors are characterized by high *CDK4* and *RBI* expression, respectively. PD0332991 also significantly reduced tumor load in several Rb-positive human xenograft models in vivo.³⁴ In concordance with our results, Wang et al.³⁵ showed that PD0332991 established a cell cycle block in AML cell line MV4;11, containing the *MLL*-AF4 fusion protein as well as an internal tandem duplication (ITD) of *FLT3*. They showed that CDK4/6 activation is a downstream

Figure 4. *CDK6* is downregulated after knockdown of MLL fusion by siRNA. Graphical representation of (A) expression values of MLL-ENL mRNA in KOPN-8 (left) and MLL-AF4 mRNA in SEM (right) after knockdown by siRNA of MLL-ENL and MLL-AF4, respectively, (white bars) measured by RT-PCR relative to control siRNA AGF1 (siAGF1, grey bars) and (B) expression values of *CDK6* mRNA (left) and *CDK4* mRNA (right) in MLL fusion knockdown samples (white bars) measured by RT-PCR relative to control siAGF1 (grey bars). Expression of mRNA was measured relative to housekeeping gene *B2M* as loading control. Error bars represent the mean $\pm$ standard error of the mean. (C) Protein levels of *CDK4* and *CDK6* after transfection with siRNA directed against *MLL-ENL* and *MLL-AF4* as shown by western blot (left panel) and graph visualizing quantification of western blot (right panel). β -actin is shown as loading control.

Figure 5. Proliferation of the MLL-rearranged cell line SEM depends on CDK6 but not on CDK4. **(A)** Protein levels of CDK4 (upper panel) and CDK6 (lower panel) followed over 3 d (D0 to D3) after transfection with siRNA directed against CDK4 and CDK6, respectively, as shown by western blot (left panel) and graphic visualization of quantification of western blot (right panel). β -actin is shown as loading control. **(B)** Graph visualizing number of cells in the G₀/G₁ phase (grey), S phase (white), and G₂/M phase (black) after knockdown by siRNA of CDK4 and CDK6 and transfection with siAGF1 and empty pulse control measured over 4 d (represented on the x-axis). Cell cycle phase assessment was done by DNA content measurement by means of propidium iodide staining quantified by flow cytometry. Bars represent mean – standard error of the mean. **(C)** Graph visualizing cell cycle analysis results (G₀/G₁ phase, grey; S phase, white; G₂/M phase, black) through bromodeoxyuridine (BrdU) staining quantified by flow cytometry at 48 h after transfection with either siRNAs directed against CDK4, CDK6, nonsense target AGF1 and empty pulse in MLL-AF4 cell line SEM. **(D)** Quantitative measurement of alive, early apoptotic, late apoptotic, and dead cells 48 h after transfection with siRNAs directed against CDK4 (upper left), CDK6 (upper right), nonsense target AGF1 (lower left), and empty pulse control (lower right) as measured by Annexin V (discriminating between alive and apoptotic/dead cells) on the x-axis and propidium iodide (PI, discriminating between apoptotic and dead cells) on the y-axis.

effector of FLT3-ITD-mediated oncogenic pathways, and argue that sensitivity of MV4;11 to PD0332991 may be explained by activation of this pathway. Additionally we now argue that it is very well imaginable that MLL-AF4 is able to bind and upregulate CDK6 directly also in *MLL*-rearranged AML, and via this contributing to the sensitivity of MV4;11 to PD0332991. Further studies with PD0332991 now are warranted to assess whether it can also effectively inhibit proliferation of *MLL*-rearranged ALL cells in vivo. Similarly to other inhibitors moving into the clinic, cautious testing of the effect on normal residual hematopoietic stem cells and progenitors is required due to the high bone marrow involvement of ALL.

Where CDK6 plays an important role in the proliferation of *MLL*-rearranged ALL, the highly homologous CDK4 does not seem to affect proliferation to a great extent. Non-overlapping functional roles of CDK4 and CDK6 have been demonstrated earlier and may be tissue-specific and depend on different complexes each kinase forms.³⁶⁻³⁹ Also, the present study suggests that CDK4 and CDK6 are not functionally redundant, with only CDK6 playing a critical role in the proliferation of *MLL*-rearranged ALL. Another possible explanation for a lack of effect on the proliferation after abrogation of CDK4 may lie in a compensatory mechanism, where the highly

expressed CDK6 in *MLL*-rearranged ALL may possibly surmount the absence of CDK4.

Further, this study raises questions about the role of the polycomb group protein *EZH2* in *MLL*-rearranged leukemia. Although we demonstrate a significant decrease in the protein levels of *EZH2* and proliferation arrest after exposure to the *CDK4/6* inhibitor, recent studies in *MLL*-AF9-rearranged acute myeloid leukemia (AML) have shown that inactivation of this gene can compromise, but not fully abrogate, leukemic growth.^{40,41} This would suggest either the implication of other downstream targets of *CDK6* and the *RBI* gene, as specific effects of PD0332991 or possibly a more important role of *EZH2* in *MLL*-rearranged ALL. Experiments using inactivation of *EZH2* in *MLL*-rearranged ALL could possibly shed more light on this specific issue.

There has been debate whether the “two-hit” model—one hit activating an oncogene and one hit inactivating a tumor suppressor gene^{42,43}—applies to *MLL*-rearranged leukemia. Yu et al.⁴⁴ already noted that the *MLL* rearrangement as a single event can result in 2 hits; an *MLL* fusion product with a partner gene could confer gain-of-function activity, and simultaneous *MLL* haplo-insufficiency would contribute to the disordered cell fate of the leukemia. In this study we present an

Figure 6. In vitro sensitivity of *MLL*-rearranged cell lines to *CDK4/6*-inhibitor PD0332991. Graph showing results of 4-d MTT assay with PD0332991 (in μM on x-axis) on *MLL*-rearranged ALL cell lines KOPN-8 (*MLL-ENL*), RS4;11, SEM, and BEL-1 (*MLL-AF4*). Dotted line represents concentration needed to inhibit 50% of the viable cells (IC50).

alternative way in which the *MLL* fusion confers gain-of-function activity; the *MLL* fusion upregulates expression of *CDK6* and by this drives the continued proliferation in *MLL*-rearranged leukemia.

In conclusion, *CDK6* is a direct target of the *MLL* fusion protein and plays an important role in the proliferation advantage of *MLL*-rearranged ALL cells. Additionally, we show that the proliferation advantage of *MLL*-rearranged ALL gained by *CDK6* can be effectively targeted in vitro by the *CDK4/6* inhibitor PD0332991.

Materials and Methods

RNA extraction and cDNA synthesis

Total RNA was extracted from a minimum of 2×10^6 cells using TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer's guidelines. The quality of the extracted RNA was assessed on 1.5% agarose gels and cDNA was prepared for quantitative real-time PCR analysis as described earlier.⁸

Gene expression data

Bone marrow or peripheral blood samples were used from untreated infants (younger than 1 y) diagnosed with ALL included in the INTERFANT-99 treatment protocol.² For all primary patient samples used in this study, approval was obtained from the Erasmus MC Institutional Review Board, and authorization was acquired from the parents or legal guardians of the children via informed consent in accordance with the Declaration of Helsinki. All samples were processed within 24 h after sampling as described recently.⁴⁵ All leukemia samples used in this study contained more than 90% leukemic cells. Total RNA was synthesized into biotinylated cRNA. Labeled cRNA was then fragmented and hybridized to HU133plus2.0 GeneChips (Affymetrix) according to the manufacturer's guidelines. The infant ALL gene expression data presented in this study have been deposited in National Center for Biotechnology Information Gene Expression Omnibus⁴⁶ and is accessible via GEO Series accession number GSE19475,⁸ and the pediatric precursor B-ALL profiles were deposited as GSE13351.⁴⁷

Quantitative real-time PCR analysis

CDK4, *CDK6*, *MLL-AF4*, and *MLL-ENL* mRNA expression levels were determined by quantitative real-time PCR analysis⁴⁸ using the DyNAmo SYBR Green qPCR kit (Finnzymes, #F-400) as described before. Oligonucleotide primers used for PCR amplification were purchased from Eurogentec. Primer sequences were as follows: *CDK4* forward: 5'-TGGGCAGAAG TCTGTTTT-3', reverse: 5'-GGAGGGGAAT GTCATTAAG-3'; *CDK6* forward: 5'-ACTGCCAAGA ACTATGACTG T-3', reverse: 5'-CTGCTGGGAT TTGTTTTATT-3'; *MLL-AF4* forward: 5'-CCCCGCCCAA GTATC-3',

reverse: 5'-GGCGGCCATG AATG-3'; *MLL-ENL* forward: 5'-CCCCGCCCAA GTATC-3', reverse: 5'-GCTCGAAGTC TGAGTCTGA-3'. *B2M* was used as a reference gene: forward: 5'-GGAGCATTCA GACTTGTCTT-3', reverse: 5'-ATGCGGCATC TTCAAA-3'.

Cell culturing and in vitro sensitivity testing

The ALL cell lines SEM, BEL-1, RS4;11 (translocation t[4;11]-positive; generating fusion protein MLL-AF4), and KOPN-8 (translocation t[11;19]-positive generating the MLL-ENL fusion protein), were maintained as suspension cultures in RPMI 1640

Figure 7. G₁-arrest in *MLL*-rearranged ALL cell lines after PD0332991 treatment. (A) Visualization of cell cycle phases as measured by DNA content through propidium iodide (PI) staining quantified by flow cytometry after exposure of MLL-AF4-positive cell line SEM to 1 μM PD0332991 (lower panel) and blank control (upper panel) for 48 h. Cell cycle phases are pointed out by arrows in the graph. Both S phase and G₂/M phase are significantly reduced, indicating a G₁ arrest. (B) Graphical representation of the number of cells that are in G₀/G₁ phase (grey), S phase (white), or G₂/M phase (black) after treatment with 1 μM PD0332991 and blank control for 48 h as measured by DNA content in KOPN-8 (left bars) and SEM (right bars). MLL-AF4-positive cell line SEM and MLL-ENL-positive cell line KOPN-8 have a significant increase in number of cells in G₀/G₁ phase and decrease of cells in the S and G₂/M phase.

with glutamax (Invitrogen, #61870036) supplemented with 10% (v/v) FCS and 2% penicillin/streptomycin/fungizone (PSF; Invitrogen, #15140-122) at 37 °C in humidified air-containing 5% CO₂. In vitro cytotoxicity to PD0332991 (Pfizer) was determined by 4-d MTT assays as described before.⁴⁹ Drug exposure treatment was performed using 1 μ M PD0332991 for 48 h under standard culture conditions, unless mentioned otherwise.

ChIP and ChIP-seq procedure

ChIPs on SEM cells (50×10^6) were performed using the SimpleChIP Enzymatic Chromatin IP kit (Cell signaling technology, #9003) according to the manufacturer's instructions with minor modifications. The cells were fixed in RPMI medium containing 1% formaldehyde with gentle rotation for 10 minutes at room temperature, and the reaction was stopped by glycine quenching (125 mM final concentration). Nuclei were collected and digested with micrococcal nuclease (2 μ L, provided by the SimpleChIP kit) followed by 8 min of sonication (8 cycles of 30 s of sonication and 30 s without sonication) using a BioRuptor UCD-200 (Diagenode). Pull downs were performed on DNA fragments (ranging from 100–600 bp) using antibodies against N-terminal MLL (Bethyl laboratories Inc, #A300-086A), C-terminal AF4 (AbCam, #ab60054), H3K4me3 (Millipore, #cs200554), and H3K79me2 (AbCam, #ab3594). ChIP-Seq samples were sequenced (36-bp reads) on the illumine GII platform and analyzed by NARWHAL.⁵⁰ The data was visualized using the UCSC genome browser (hg19).

DNA content and apoptosis measurement

DNA content (i.e., cell cycle distribution) measurement was done using the CycleTEST PLUS DNA Reagent Kit (BD Biosciences, #340242). 0.5×10^6 cells were treated with 225 μ L of solution A (containing trypsin) for 5 min at room temperature, 175 μ L of solution B (containing trypsin inhibitor) for 10 min at room temperature, and 175 μ L of solution C (containing propidium iodide (PI) and spermine tetrahydrochloride) for 15 min on ice.

Induction of apoptosis was assessed by an Annexin V/PI assay. For this, 0.2×10^6 cells were incubated in annexin binding buffer

(10 mM HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM CaCl₂) and centrifuged at 1500 RPM at 4 °C for 5 min. Buffer was removed and a solution of Annexin V (1:1000) and PI (2 μ g/ml) (BD Biosciences, #556570) was added to the cell culture.

For both assays, detection of positive staining was performed on a FACSCalibur (Becton Dickinson) flow cytometer.

Transfection with siRNA

Leukemic cells (4×10^6) were transfected with specific siRNAs by electroporation in 4 mm electroporation cuvettes (Bio-Rad Laboratories, #1652081) containing 400 μ L of RPMI medium supplemented with 10% fetal calf serum, in the presence of 10 μ L of siRNAs (20 μ M) directed against *CDK4*, *CDK6* (ON-TARGET plus SMARTpool, Dharmacon), *MLL-AF4*, or *AML1-MTG8* fusion protein (*AGF1*) as nonsense control (as described previously⁵¹), or in the presence of 50 μ L of siRNAs (20 μ M) directed against *MLL-ENL*: sense 5'-CCAAAAGAAA AGUCUGCCCA G-3'; antisense 5'-CUGGGCAGAC UUUUCUUUUG GUU-3'. For the latter experiment, control cells were transfected with 50 μ L si*AGF1* (20 μ M). Electroporation was carried out applying a rectangular pulse of 300 V for 10 milliseconds, using a Gene Pulser MXcell Electroporation System (Bio-Rad Laboratories). After incubating for 15 min at room temperature, the cells were diluted to 1×10^6 cells/ml and cultured under standard culture conditions. Transfection of si*MLL-ENL* and si*MLL-AF4* was repeated after 2 d to reach maximum knockdown, and cells were harvested and RNA isolated after 96 h. Transfection experiments were repeated multiple times with similar results to those published in this manuscript.

Western blot

Cell pellets were snap-frozen in liquid nitrogen and stored at -80 °C until further use. After thawing, cells were resuspended in 50 μ L of lysis buffer containing: 5 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate (Merck), 25 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM glycerolphosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 10 mM sodium fluoride (Sigma-Aldrich), and 50 μ M of freshly prepared sodium pervanadate. Cells were lysed for 30 min on ice. After lysing cells were centrifuged for 15 min at 13 000 rpm and 4 °C. Protein concentration was determined by BCA protein assay (Pierce Biotechnology, #23225) with different concentrations of bovine serum albumin as standards. Cell lysates containing 25 μ g of protein were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell). Western blots were probed with mouse anti-*CDK4*, anti-*CDK6*, anti-p*RB1* S608, and anti-*EZH2* (Cell Signaling, #2906, #3136, #2181, and #4905) and mouse anti-beta actin (as a control for equal loading) (Abcam) for 2 h at room temperature. Next the western blots were incubated in fluorescently labeled secondary antibodies (LI-COR Biosciences) for 1 h and subsequently detected using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Figure 8. Treatment with *CDK4/6*-inhibitor PD0332991 affects downstream targets *pRB* and *EZH2*. Protein levels of *CDK6*, *RB1* phosphorylation at serine 608 (*pS608*) and *EZH2* in *MLL-ENL*-positive cell line KOPN-8 and *MLL-AF4*-positive cell line SEM after 0 h, 24 h, and 48 h of treatment with 1 μ M *CDK4/6*-inhibitor PD0332991 as shown by western blot. β -actin is shown as loading control.

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Authorship

M.H.v.d.L. designed the research, performed research and statistical analysis, and wrote paper. M.W., P.S., and L.S. performed research. E.v.R. performed research and statistical analysis. R.P. and R.W.S. designed and supervised the research, and reviewed the manuscript.

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