MYELOID NEOPLASIA

Requirement for CDK6 in MLL-rearranged acute myeloid leukemia

Theresa Placke,1,2 Katrin Faber,3 Atsushi Nonami,4 Sarah L. Putwain,5 Helmut R. Salih,2,6 Florian Heidel,7 Alwin Krämer,2,8 David E. Root,9 David A. Barbie,4,9 Andrei V. Krivtsov,10 Scott A. Armstrong,10 William C. Hahn,4,9 Brian J. Huntly,5 Stephen M. Sykes,11 Michael D. Milsom,12 Claudia Scholl,3, and Stefan Fröhling1,2

1Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center, Heidelberg, Germany; 2German Consortium for Translational Cancer Research, Heidelberg, Germany; 3Department of Internal Medicine III, Ulm University, Ulm, Germany; 4Department of Medical Oncology, Dana–Farber Cancer Institute, Boston, MA; 5Department of Haematology and Oncology, Cambridge Institute of Medical Research, and Wellcome Trust – Medical Research Council Cambridge Stem Cell Institute, University of Cambridge, Cambridge, United Kingdom; 6Department of Hematology and Oncology, Eberhard Karls University, Tübingen, Germany; 7Department of Hematology and Oncology, Otto von Guericke University, Magdeburg, Germany; 8Clinical Cooperation Unit Molecular Hematology and Oncology, German Cancer Research Center, and Department of Internal Medicine V, Ruprecht Karls University, Heidelberg, Germany; 9Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA; 10Leukemia Center, Human Oncology and Pathogenesis Program and Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, New York, NY; 11Immune Cell Development and Host Defense Program, Fox Chase Cancer Center, Philadelphia, PA; and 12Heidelberg Institute for Stem Cell Technology and Experimental Medicine and Division of Stem Cells and Cancer, Experimental Hematology Group, German Cancer Research Center, Heidelberg, Germany

Chromosomal rearrangements involving the H3K4 methyltransferase mixed-lineage leukemia (MLL) trigger aberrant gene expression in hematopoietic progenitors and give rise to an aggressive subtype of acute myeloid leukemia (AML). Insights into MLL fusion-mediated leukemogenesis have not yet translated into better therapies because MLL is difficult to target directly, and the identity of the genes downstream of MLL whose altered transcription mediates leukemic transformation are poorly annotated. We used a functional genetic approach to uncover that AML cells driven by MLL-AF9 are exceptionally reliant on the cell-cycle regulator CDK6, but not its functional homolog CDK4, and that the preference for growth inhibition induced by CDK6 depletion is mediated through enhanced myeloid differentiation. CDK6 essentiality is also evident in AML cells harboring alternate MLL fusions and a mouse model of MLL-AF9-driven leukemia and can be ascribed to transcriptional activation of CDK6 by mutant MLL. Importantly, the context-dependent effects of lowering CDK6 expression are closely phenocopied by a small-molecule CDK6 inhibitor currently in clinical development. These data identify CDK6 as critical effector of MLL fusions in leukemogenesis that might be targeted to overcome the differentiation block associated with MLL-rearranged AML, and underscore that cell-cycle regulators may have distinct, noncanonical, and nonredundant functions in different contexts. (Blood. 2014;124(1):13-23)

Introduction

A substantial proportion of acute myeloid leukemia (AML) cases harbor balanced translocations of chromosome 11q23, and AML with t(9;11)(p22;q23) is recognized as a distinct entity by the World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues.1,2 On the molecular level, t(11q23) results in fusion of the MLL gene, which encodes an H3K4 methyltransferase, to a broad spectrum of partner genes, such as MLLT3 (also called AF9), MLLT4 (AF6), MLLT1 (ENL), and MLLT10 (AF10) on chromosomes 9p22, 6q27, 19p13.3, and 10p12, respectively.3,4 A key functional feature of mixed-lineage leukemia (MLL) rearrangements is their ability to confer leukemia-initiating activity to hematopoietic stem and progenitor cells (HSPC).5,6 MLL fusions are characterized by loss of the C-terminal H3K4 methyltransferase domain, and their leukemogenic activity is dependent on both features of the remaining N-terminal portion, such as a binding motif for the menin tumor suppressor that mediates the contact between MLL and chromatin as well as aberrant transcriptional activation of target genes through heterologous domains contributed by the various partner proteins.7 For example, MLL fusions involving AF9, ENL, and AF10, which account for the majority of MLL-rearranged AML, recruit multiprotein complexes essential for transcriptional activation/elongation, such as that comprising the H3K79 methyltransferase DOT1L.8,9 Other factors required for establishing leukemogenic gene expression programs in MLL-rearranged AML include the polycomb group protein CBX8, the bromodomain protein BRD4, the H3K4/K9 demethylase KDM1A, and signaling through the nuclear factor κB (NF-κB) pathway.10-15 Certain MLL target genes, such as HOXA9 and MEIS1, have

Key Points

• CDK6 is a critical effector of MLL fusions in myeloid leukemogenesis.
• Genetic and pharmacologic inhibition of CDK6 overcome the differentiation block associated with MLL-rearranged AML.

Plenary Paper
established roles in MLL-mediated leukemogenesis, whereas the relevance of others remains elusive.

Current treatment of MLL-rearranged AML consists of chemotherapy and, in select cases, allogeneic stem cell transplantation, and results in long-term survival rates of less than 10% to approximately 50% depending on the MLL fusion partner and additional risk factors. Insights into MLL-mediated leukemogenesis have spurred efforts to develop novel, molecular mechanism-based therapeutic strategies, such as targeting the mnn–MLL interaction, DOT1L, or BRD4. However, these and other targeted approaches have not yet been translated into the clinic, and treatment of MLL-rearranged AML remains challenging.

Functional genetic screens provide a means to search for essential signaling pathways in preclinical models of cancer in an unbiased fashion. From a translational perspective, such an approach can be particularly valuable in the context of oncogenic mutations that are difficult to target directly, such as MLL rearrangements. Here, we have used a functional genetic approach based on large-scale RNA interference (RNAi) to identify dependence on a noncanonical and nonredundant function of CDK6 as specific liability of MLL-rearranged AML that could be exploited for therapeutic benefit.

Materials and methods

Cell culture

AML cell lines, 293T cells, and Ba/F3 cells were maintained under standard conditions. Cell lines derived from mouse HSPC were maintained in RPMI-1640 supplemented with 20% fetal bovine serum and 20% WEHI-conditioned medium as a source of interleukin-3. Cell line identity and purity were verified using the Multiplex Cell Authentication and Contamination Tests (Multiplexon). PD-0332991 was obtained from Selleck.

RNAi screening

Large-scale arrayed screening was conducted with a subset of the Broad Institute TRC short hairpin RNA (shRNA) library as described previously. Screens were performed in a 384-well format. Each well contained a single shRNA species, and each transcript was covered, on average, by 5 different shRNAs. Assay conditions (cell number per well, viral dose, puromycin concentration) were optimized for each cell line before high-throughput screening. Cells were seeded, incubated for 24 hours, infected with lentivirus, and incubated for 6 days. All lentiviral infections were performed in quadruplicate; 2 replicates were selected with puromycin during the final 5 days of incubation, whereas the other 2 replicates were left untreated. Cell viability and proliferation were measured 6 days after lentiviral infection using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Raw data for each shRNA were corrected using the B-score, an analog of the Z-score that uses a 2-way median polish to minimize row/column effects, and normalization to the median absolute deviation to account for plate-to-plate variation. A gene was defined as "hit" if 1 shRNA was associated with a B-score of less than −1.2 and at least 1 additional shRNA was associated with a B-score of less than −0.85.

Plasmds and viral transduction

RNAi experiments were performed using pLKO.1 shRNAs obtained from the TRC-Hs 1.0 (human) and TRC-Mm 1.0 (mouse) shRNA libraries through Open Biosystems or Sigma-Aldrich, or a custom shRNA against the MLL-AF9 fusion breakpoint. See supplemental Methods on the Blood Web site for details. The CDK6 and CDK4 complementary DNAs (cDNAs) were obtained from Open Biosystems and polymerase chain reaction (PCR)-amplified from an AML cell line, respectively, and cloned into the pLent6.2/V5-DEST or pLent7.3/V5-DEST lentiviral vectors (Invitrogen) for expression in human cells. The CDK6K43M mutant was generated using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). The MLL-AF9 cDNA was cloned into pLent6.2/V5-DEST or the pMSCV-PGK-neo and pMSCV-IRES-GFP retroviral vectors for expression in murine cells. For knockdown of Cdk6 in vivo, shRNA TRCN23153 was cloned into the LeGO-C2 lentiviral gene ontology vector. Generation of viral supernatants and viral transduction were performed as described previously. Vector particles were titrated based on virion RNA by measuring the abundance of the HIV-1 Rev response element using quantitative reverse-transcription PCR (qRT-PCR), and cells were infected with equivalent amounts of recombinant viruses to ensure comparability between different knockdown experiments.

In vitro studies

Determination of viable cell numbers, RNA isolation, cDNA synthesis, qRT-PCR, immunoblotting, flow cytometry, and colony assays were performed using standard procedures. See supplemental Methods for details. Chromatin immunoprecipitation-sequencing (ChIP-seq) was performed as described.

Murine bone marrow transplantation assays

Transplantation experiments were performed as described previously. Eight- to 10-week-old C57BL/6J mice (Jackson Laboratory) were housed in individually ventilated cages and preconditioned with 6 Gy total body irradiation (137Cs source) before administration of transduced hematopoietic cells via IV injection.

Statistics

Experiments were performed at least 3 times; unless otherwise indicated, 1 representative experiment is shown. Error bars represent mean ± standard error of the mean. Statistical analysis was performed using paired or unpaired 2-tailed Student t test, Kaplan–Meier survival estimates, or log-rank test as appropriate. Computations were performed using GraphPad Prism.

Study approval

Human AML samples and normal CD34+ cells were obtained under institutional review board–approved protocols following written informed consent. This study was conducted in accordance with the Declaration of Helsinki. Animal experiments were performed after approval and in accordance with the guidelines of the Animal Care and Use Committee at the Regierungspräsidium Karlsruhe.

Results

RNAi screens for essential genes in MLL-AF9–expressing AML cells

We performed loss-of-function RNAi screens in 5 AML cell lines (supplemental Table 1) using a lentivirally delivered shRNA library targeting genes encoding most protein kinases, selected protein phosphatase genes, and known cancer-related genes. To nominate candidates that are required specifically in the context of rearranged MLL, we identified genes whose depletion by at least 2 shRNAs inhibited MLL-AF9pos NOMO-1 and THP-1 cells, followed by elimination of genes that also scored in any of the 3 remaining, wild-type (WT) MLL-expressing cell lines with 2 or more shRNAs (Figure 1A). This approach yielded 8 genes potentially involved in MLL-rearranged AML (Figure 1B). We next validated the top-ranking genes by knockdown in NOMO-1, THP-1, and 2 MLL-AF9pos cell lines using shRNAs that scored exclusively in MLL-AF9pos cell lines in the RNAi screens, followed by monitoring of viable cell numbers. The knockdown efficiency of each shRNA was determined by qRT-PCR. Of the 7 genes tested, 2 (DGGH, MAP3K12) were not efficiently suppressed, whereas knockdown of 4 genes (CAMK1, SRPK2, THOC4,
Figure 1. RNAi screens for genes required by MLL-AF9<sup>pos</sup> AML cell lines. (A) Schematic of RNAi screens. Numbers in circles indicate genes scoring as positive. Genes scoring exclusively in MLL-AF9<sup>pos</sup> AML cell lines are indicated in red. (B) Candidate genes preferentially required in MLL-AF9<sup>pos</sup> AML cell lines. For each of the top-ranking genes, the shRNAs scoring in NOMO-1 and/or THP-1 are shown. Negative B-scores indicate reduced viable cell numbers. (C) Validation of candidate genes. Shown are the effects of 2 shRNAs per candidate gene on cell viability and proliferation of AML cell lines with and without an MLL-AF9 fusion and the knockdown efficiency of each shRNA in NOMO-1 cells, as determined by qRT-PCR.
UNCI3B) was growth inhibitory regardless of genotype. In contrast, depletion of CDK6, a serine/threonine kinase essential for passage through the cell-cycle G1/S phase restriction point, selectively impaired MLL-AF9pos cell lines (Figure 1C).

**Requirement for CDK6 in MLL-transformed hematopoietic cells**

To confirm the differential requirement for CDK6, we suppressed CDK6 in an expanded panel of AML cell lines using 3 different shRNAs. CDK6 knockdown, as determined by qRT-PCR and immunoblotting (supplemental Figure 1A), strongly inhibited MLL-AF9pos cells, whereas there was little to no effect in MLL-AF9neg cells (Figure 2A). CDK6 was also required by AML cell lines (MV4-11, ML-2) harboring other MLL fusions (MLL-AF4/AFF1, MLL-AF6; Figure 2B; supplemental Figure 1B).

To further ensure the specificity of these results, we performed rescue experiments with an shRNA targeting the 3’ untranslated

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**Figure 2. Requirement for CDK6 in MLL-rearranged hematopoietic cells.** (A) Effects of CDK6 suppression in MLL-AF9pos and MLL-AF9neg AML cell lines. (B) Effects of CDK6 suppression in MLL-AF4pos MV4-11 and MLL-AF6pos ML-2 cells. (C) Rescue of viable cell number by expression of the CDK6 coding sequence in NOMO-1 cells transduced with an shRNA targeting the CDK6 3’ UTR. (D) CDK6 protein expression of cells used in panel C. (E) Effects of Cdk6 suppression in suspension cultures of Ba/F3 cells and murine HSPC transduced with MLL-AF9. (F) Effects of Cdk6 suppression in methylcellulose cultures of murine HSPC transduced with MLL-AF9 or MOZ-TIF2. Original magnification, ×25.
region (UTR) of the CDK6 messenger RNA (mRNA). We first transduced MLL-AP9pos NOMO-1 cells with an empty control vector or the CDK6 coding sequence, which lacks the 3’ UTR. Subsequent knockdown of endogenous CDK6 inhibited the growth of empty control vector–transduced cells, whereas the RNAi–induced phenotype was countered by expression of the shRNA–resistant CDK6 cDNA (Figure 2C,D). Together, these data indicate that MLL-rearranged human AML cells are dependent on CDK6 expression.

To corroborate our results in genetically defined systems based on primary HSPC, we employed 2 cell lines generated through retroviral transduction of 5-fluorouracil-mobilized murine bone marrow (BM) with MLL-AP9 and selection for stable transgene expression during serial replating in methylcellulose. Consistent with the results obtained in human AML cell lines, Cd6 knockdown decreased the growth of MLL-AP9–transformed HSPC in suspension culture, whereas highly proliferative, WT MLL-expressing Ba/F3 murine pro-B cells were unaffected (Figure 2E; supplemental Figure 1C). Similarly, Cd6 knockdown substantially inhibited colony formation in methylcellulose of MLL-AP9–transduced murine BM compared with cells expressing another leukemogenic fusion, MYST3-NCOA2 (also called MOZ-TIF2; Figure 2F; supplemental Figure 1C). Thus, MLL-AP9 is dependent on Cd6 to transform primary HSPC in vitro.

Induction of myeloid differentiation by CDK6 suppression in MLL-rearranged AML cells

To examine the basis for the disparate effects of Cd6 depletion according to MLL status, we analyzed cell cycle and apoptosis in AML cell lines 6 days after shRNA knockdown of Cd6. Propidium iodide staining and measurement of 5-bromo-2′-deoxyuridine incorporation showed that MLL-AP9pos cells slightly accumulated in the G0/G1 phase after Cd6 suppression; however, this was also observed in MLL-AP9pos cells (Figure 3A; supplemental Figure 2A-C). Cd6 suppression caused no significant increase in apoptosis regardless of genotype (Figure 3B; supplemental Figure 2D). We next considered whether Cd6 depletion might induce differentiation preferentially in MLL-AP9pos cells. Consistent with this idea, MLL-AP9pos cells upregulated CD11b expression and assumed a more mature, macrophage-like morphology upon Cd6 knockdown, whereas these effects were not observed in WT MLL-expressing cells (Figure 3C,D). These phenotypic changes were paralleled by increases in the expression of CEPBA, SPI1 (also called PU.1), and, to a lesser extent, IRF8, which encode transcription factors involved in myelomonocytic differentiation (supplemental Figure 2E). The inhibitory function of Cd6 on differentiation was rescued by expression of the Cd6 coding sequence in MLL-rearranged cells transduced with an shRNA targeting the 3’ UTR of the Cd6 mRNA (Figure 3E,F; supplemental Figure 2F,G). In contrast, expression of a Cd6K43M mutant with disrupted kinase function or Cd4 was unable to block the differentiation induced by shRNA–mediated depletion of Cd6 (supplemental Figure 2F,G). These data indicate that MLL-rearranged AML cells are disproportionately reliant on Cd6 to maintain an immature phenotype and support the conclusion that the catalytic activity of Cd6 is crucial for the dependence of MLL-rearranged AML cells on Cd6 expression.

Targeting of Cd6 by rearranged MLL

To investigate whether the context-dependent requirement for Cd6 reflects a transcriptional link between rearranged MLL and Cd6, we measured Cd6 mRNA and protein in response to modulation of MLL-AP9 expression in AML cell lines. We transduced THP-1, an MLL-AP9pos cell line with low endogenous AP9 levels (supplemental Figure 2H), with shRNAs against the MLL-AP9 fusion breakpoint or AP9 exon 7, which is retained in the MLL-AP9 fusion transcript. A nontargeting shRNA and an shRNA targeting AP9 exon 5, which is absent in the MLL-AP9 fusion, served as controls (Figure 4A). Suppression of MLL-AP9 caused a decrease in Cd6 mRNA and protein, resembling the effects on HoxA9, a known MLL-AP9 target gene. In contrast, expression of Cd4, a closely related kinase that cooperates with Cd6 to promote cell-cycle progression, was not altered (Figure 4B,C). Conversely, introduction of MLL-AP9 into WT MLL-expressing AML cells, normal human CD34pos cells, and Ba/F3 cells increased Cd6 mRNA levels (Figure 4D,E). Furthermore, ChIP-seq demonstrated that MLL-AP9 binds to the Cd6 locus in mouse BM cells transduced with MLL-AP9 and in MLL-AP9–driven murine AML (Figure 4F). These findings indicate that Cd6 is rendered essential via direct targeting by truncated MLL.

Sensitivity of MLL-rearranged AML cells to pharmacologic Cd6 inhibition

Our genetic data suggested that Cd6 might be a therapeutic target in MLL-rearranged leukemias. We therefore evaluated colony formation of AML cells in the presence of palbociclib (also called PD-0332991), an orally available inhibitor of Cd4/Cd6 kinase activity that is in clinical development as an anticancer agent. For the MLL-AP9pos cell lines NOMO-1 and THP-1, we observed a strong, dose-dependent reduction in the number of colonies, whereas MLL-AP9pos HL-60 and K562 cells were affected to a substantially lesser extent (Figure 5A; supplemental Figure 3A).

Because PD-0332991 also inhibits Cd4, which acts redundantly with Cd6 to promote cell-cycle progression, it seemed possible that Cd4 blockade contributes to the effect of this compound in MLL-rearranged AML. We therefore suppressed Cd4 in 8 AML cell lines. Consistent with our initial RNAi screens, which did not identify Cd4 as essential gene in MLL-AP9pos cell lines, Cd4 knockdown had no or only a marginal effect on viable cell numbers in all cell lines investigated (Figure 5B; supplemental Figure 3B), implying that the effect of PD-033299 in MLL-rearranged cells is mainly attributable to Cd6 inhibition.

We next sought confirmation of Cd6 inhibitor activity in primary human MLL-rearranged AML. Consistent with the cell line results, PD-0332991 strongly inhibited colony formation in methylcellulose of mononuclear cells from 7 AML patients harboring 5 different MLL rearrangements (Figure 5C-D; supplemental Figure 3C-D).

Given that Cd6 knockdown induces maturation of MLL-rearranged AML cells, we evaluated whether the effect of pharmacologic Cd6 inhibition was also the result of myeloid differentiation. Consistent with the RNAi results, PD-0332991 induced CD11b expression, changes in cell morphology, and dose-dependent increases in the expression of CEPBA, SPI1, and more modestly IRF8, preferentially in MLL-rearranged cells (Figure 5E,F; supplemental Figure 3E). Collectively, these data show that MLL-rearranged AML cells are sensitive to pharmacologic Cd6 inhibition, indicating that their overreliance on Cd6 may provide a therapeutic opportunity. Furthermore, the results demonstrate that the catalytic activity of Cd6 contributes to the myeloid differentiation arrest in MLL-rearranged AML.
Figure 3. Effects of CDK6 suppression in AML cells. (A) Flow cytometric analysis of cell-cycle progression. Numbers indicate percentages of cells in G0/G1. (B) Flow cytometric analysis of apoptosis. Numbers indicate percentages of cells. (C) Flow cytometric analysis of myeloid differentiation in AML cell lines. Numbers indicate percentages of cells. (D) Microscopic analysis of May-Gr¨ unwald-Giemsa–stained cytospin preparations of AML cell lines. Original magnification, ×400. Insets show twofold magnified details of the corresponding photographs. (E) Inhibition of myeloid differentiation by expression of the CDK6 coding sequence in NOMO-1 cells transduced with an shRNA targeting the CDK6 3’ UTR. (F) Immunoblot analysis of cells shown in panel E.
Requirement for CDK6 in MLL-AF9–driven murine AML

To investigate whether Cdk6 is required for AML development and propagation in vivo, we generated highly aggressive GFP<sup>pos</sup> MLL-AF9–induced leukemias in a murine BM transplantation model and transduced leukemic cells from secondary transplant recipients with an shRNA against Cdk6 linked to mCherry (Figure 6A). Cells with high mCherry expression were sorted by flow cytometry 40 hours after transduction (supplemental Figure 4) (ie, at an early time point when the shRNA was already expressed but Cdk6 knockdown most likely had not yet occurred) and transplanted into tertiary recipients, which were monitored for development of disease and survival. In addition, we evaluated the effects of Cdk6 knockdown on clonogenic activity and myeloid differentiation (Figure 6A).

Ex vivo experiments demonstrated that Cdk6 knockdown impaired colony formation of leukemic cells from mice with secondary MLL-AF9–induced AML (Figure 6B). Microscopic and flow cytometric analysis of sorted GFP<sup>pos</sup>/mCherry<sup>pos</sup> cells cultured for 4 days demonstrated that the differentiation block of MLL-AF9<sup>pos</sup> leukemic cells was reversed by Cdk6 depletion, which induced morphological maturation and increased expression of myeloid differentiation antigens F4/80 and CD115 (Figure 6C).

Flow cytometry 4 weeks after transplantation demonstrated expansion of GFP<sup>pos</sup>/mCherry<sup>pos</sup> leukemic cells in the blood of control mice, whereas GFP<sup>pos</sup>/mCherry<sup>pos</sup> cells were barely detectable in mice transplanted with Cdk6 knockdown cells (Figure 6D). The decrease in leukemic burden translated into a significant survival advantage because mice transplanted with control cells died after a median of 28 days compared with 55 days in mice transplanted with Cdk6 knockdown cells (Figure 6E). Control mice developed acute leukemia with frequent blasts in the blood and extensive infiltration of BM and spleen by a prominent population of immature myeloid cells and blast forms (Figure 6F, top panels). In Cdk6 knockdown mice, the disease was characterized by expanded granulocyte and monocye populations as well as maturing myeloid forms in the blood, BM, and spleen, reminiscent of human myeloproliferative neoplasm; furthermore, histopathology demonstrated less BM and spleen infiltration with partially preserved splenic architecture (Figure 6F, bottom panels). These data demonstrate that depletion of Cdk6 overcomes the differentiation block of MLL-AF9–driven AML and prolongs survival in vivo, further supporting CDK6 inhibition as novel strategy to target MLL-rearranged AML cells.

Discussion

Only a minority of patients with MLL-rearranged AML attain long-term disease-free survival, indicating that more effective, molecular mechanism-based therapies are needed.α,β,γ Because interfering with rearranged MLL itself has proved challenging, many studies have focused on downstream events that may be therapeutically tractable. However, although it is firmly established that MLL fusion proteins cause a characteristic pattern of transcriptional changes,α,β it is less well defined which of the many deregulated genes are critical to the leukemic phenotype and would need to be blocked for therapeutic benefit. Furthermore, individual MLL target genes that are known to be required for AML development and maintenance, such as HOXA9 and MEIS1,α,β,γ,δ,ε are not amenable to pharmacologic inhibition. Thus, treatment of MLL-rearranged AML remains a challenge in clinical practice.

We used a functional genetic approach based on a series of shRNA screens to identify that the serine/threonine kinase CDK6 represents a targetable effector of MLL fusion proteins. In particular, we found that CDK6 activity is preferentially required by MLL-rearranged AML cells compared with other AML subtypes, and that this association is due to direct transcriptional regulation of CDK6 by mutant MLL because the Cdk6 locus is bound by MLL-AF9 in vitro and in vivo and modulation of MLL-AF9 levels resulted in concordant changes in CDK6 expression in different human and murine experimental models. The latter conclusion is also supported by data from previous ChIP-chip and ChIP-seq screens showing that CDK6
Figure 5. Pharmacologic inhibition of CDK6 in human AML cells. (A) Colony formation of AML cell lines treated with PD-0332991. (B) Effects of CDK4 suppression in AML cell lines. (C) Relative colony numbers of primary human MLL-rearranged AML specimens cultured in the presence of PD-0332991. (D) Cumulative analysis of normalized colony data shown in panel C. (E) Flow cytometric analysis of myeloid differentiation in AML cell lines treated with PD-0332991. Numbers indicate percentages of cells. (F) Microscopic analysis of May-Grünwald-Giemsa–stained cytospin preparations of AML cell lines treated with PD-0332991. Original magnification, ×400. Insets show twofold magnified details of the corresponding photographs.
is bound by MLL fusion proteins and displays abnormal H3K79 methylation patterns in human MLL-rearranged leukemic cell lines and a mouse model of MLL-AF9–driven AML.30,36,37 Remarkably, the data support a link between CDK6 and different MLL fusions, pointing to CDK6 essentiality as unifying feature of MLL-rearranged leukemias.

CDK6 acts redundantly with CDK4 to promote cell-cycle progression. Accordingly, impaired cell-cycle control in hematopoietic malignancies has been linked to combined deregulation of CDK4 and CDK6 through mechanisms such as inactivation of CDKN2 (also called Ink4) CDK inhibitors and aberrant cyclin D expression.38,39 In contrast, our findings indicate that MLL-rearranged leukemias require CDK6, whereas CDK4 is dispensable, substantiating that individual cancers may require particular CDKs depending on their developmental origin and/or pattern of acquired mutations. For example, CDK6 is an oncogene that is amplified in subsets of patients with glioblastoma and medulloblastoma,40,41 and overexpression of CDK6 has been observed in lymphoid malignancies,42–44 whereas CDK4 is affected by DNA copy number gain or a rare activating mutation in patients with melanoma,45,46 and was selectively essential in mouse models of RAS-driven melanoma and lung adenocarcinoma.47,48

Supporting the idea that MLL-mediated leukemogenesis involves noncanonical functions of CDK6 not shared by CDK4, the preferential growth inhibition of MLL-rearranged cells by CDK6 depletion is not due to increased cell-cycle arrest but myelomonocytic differentiation, as evidenced by changes in cell morphology, immunophenotype, and lineage-specific gene expression that are not observed in other AML subtypes. CDK6 has been shown to block maturation of normal murine myeloid progenitors through...
a noncatalytic mechanism.\textsuperscript{49} We now extend these findings by demonstrating that CDK6 possesses antidifferentiation activity in an aggressive subtype of human AML and demonstrate that this function requires its catalytic activity. More generally, our data underscore that an increasing repertoire of “atypical” and nonredundant CDK6 functions contribute to tumorigenesis in a context-specific manner. For example, recent studies indicate that cytokine-triggered chromatin association of CDK6 can modulate NF-κB–dependent gene expression in various cell types, including epithelial cancer and glioblastoma cell lines, and that CD6 is part of a transcription complex that induces the expression of Cdkn2A and the proangiogenic factor vascular endothelial growth factor receptor A in a murine model of B-cell acute lymphoblastic leukemia.\textsuperscript{50,51}

The effects of CDK6 knockdown are closely phenocopied by a small-molecule CDK4/6 inhibitor, PD-0332991. This observation not only links CDK6 kinase activity to arrested myeloid differentiation in MLL-rearranged AML, but also identifies CDK6 blockade as a therapeutic strategy with immediate translational potential, as PD-0332991 is already being evaluated clinically in other cancers and has received Breakthrough Therapy designation for the treatment of breast cancer.\textsuperscript{31} Importantly, Cdk6 knockout mice and mice expressing a kinase-dead Cdk6 allele are viable and develop normally with only a minor defect in erythropoiesis and decreased thymic cellularity,\textsuperscript{52,53} indicating that CDK6 is not essential under physiological conditions. In line with this, PD-0332991 was associated with manageable toxicity in clinical trials that enrolled patients with solid-organ and hematologic malignancies.\textsuperscript{54,56} Thus, targeting CDK6 may provide a clinically applicable therapeutic window in MLL-rearranged leukemia. Notably, estrogen receptor positivity, and not cyclin D1 amplification and/or loss of CDKN2A, is the best predictor of response to PD-0332991 in breast cancer (R.S. Finn, J.P. Crown, I. Lang, K. Boer, I.M. Bondarenko, S.O. Kulyk, J. Ettl, R. Patel, T. Pinter, M. Schmidt, Y. Shparyk, A.R. Thummala, N.L. Voytko, A. Breazna, S.T. Kim, S. Randolph, D.J. Slamon, unpublished data, Cancer Therapy & Research Center-American Association for Cancer Research San Antonio Breast Cancer Symposium, December 4-8, 2012), underscoring the importance of biomarkers beyond cell-cycle regulation. Our data indicate that rearranged MLL represents such an alternative determinant of response to CDK6 inhibition.

Although our studies demonstrate that targeting Cdk6 significantly prolongs survival of mice with highly aggressive MLL-AF9–driven AML, it is likely that the context-specific properties of CDK6 inhibition may be best exploited when coupled with chemotherapy or other targeted agents, similar to the combination chemotherapy or other targeted agents, similar to the combination of CDK6 inhibition should consider these contextual requirements, and suggest that patients with MLL-rearranged leukemias may benefit from CDK6 blockade, a notion that can readily be tested in the clinic.

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Authorship


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Correspondence: Stefan Fröhling, National Center for Tumor Diseases and German Cancer Research Center, Im Neuenheimer Feld 460, 69120 Heidelberg, Germany; e-mail: stefan.froehling@nct-heidelberg.de; and Claudia Scholl, Ulm University, Albert-Einstein-Allee 23, 89081, Ulm, Germany; e-mail: claudia.scholl@uni-ulm.de.

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