Cdk4 and Cdk6 cooperate in countering the INK4 family of inhibitors during murine leukemogenesis

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Key Points

- A Cdk6 R31C knock-in mutation resistant to INK4 inhibitors cooperates with Cdk4 hyperactivity in the development of hematopoietic tumors.
- In Cdk6 R31C cells, p16INK4a increasingly binds and inhibits Cdk4, suggesting that both kinases cooperate in sequestering INK4 proteins in cancer.
- Cdk4 and Cdk6 are related protein kinases that bind D-type cyclins and regulate cell-cycle progression. Cdk4/6 inhibitors are currently being used in advanced clinical trials and show great promise against many types of tumors. Cdk4 and Cdk6 are inhibited by INK4 proteins, which exert tumor-suppressing functions. To test the significance of this inhibitory mechanism, we generated knock-in mice that express a Cdk6 mutant (Cdk6 R31C) insensitive to INK4-mediated inhibition. Cdk6R31CR31C mice display altered development of the hematopoietic system without enhanced tumor susceptibility, either in the presence or absence of p53. Unexpectedly, Cdk6 R31C impairs the potential of hematopoietic progenitors to repopulate upon adoptive transfer or after 5-fluorouracil-induced damage. The defects are overcome by eliminating sensitivity of cells to INK4 inhibitors by introducing the INK4-insensitive Cdk4 R24C allele, and INK4-resistant mice are more susceptible to hematopoietic and endocrine tumors. In BCR-ABL–transformed hematopoietic cells, Cdk6 R31C causes increased binding of p16INK4a to wild-type Cdk4, whereas cells harboring Cdk4 R24C and Cdk6 R31C are fully insensitive to INK4 inhibitors, resulting in accelerated disease onset. Our observations reveal that Cdk4 and Cdk6 cooperate in hematopoietic tumor development and suggest a role for Cdk6 in sequestering INK4 proteins away from Cdk4. (Blood. 2014;124(15):2380-2390)

Introduction

Cyclin-dependent kinases (Cdks) regulate multiple cellular processes including cell-cycle progression, transcription, or neuron biology.1 Cdk4 and Cdk6 are highly related (~70% homology) cell-cycle kinases that phosphorylate retinoblastoma protein (pRb) family members and regulate progression through G1 and entry into the DNA synthesis (S) phase. Cdk4 and Cdk6 bind D-type cyclins (D1, D2, and D3), which are transcriptionally induced by mitogenic signals, leading to Cdk4/6 activation and pRb phosphorylation. Cdk-dependent phosphorylation of pRb ultimately results in its inactivation and allows for the subsequent transcriptional induction of several genes required for S-phase or mitosis.2 These 2 kinases are thought to be at least partially redundant in regulating G1 transition when coordinately expressed. In vivo, lack of either of these kinases causes specific defects in a cell type–specific manner. Cdk4 deficiency results in defective proliferation of postnatal pancreatic β cells and pituitary cells,3,4 whereas lack of Cdk6 provokes specific defects in hematopoietic cells.5 Concomitant ablation of both Cdk4 and Cdk6 results in embryonic lethality from defective erythropoiesis.5

Most tumor types display functional upregulation of these kinases through overexpression (in some cases from amplification or translocation) of D-type cyclins or inactivation of the cyclin D-Cdk4/6–specific inhibitors: the INK4 proteins.7 This family is composed of 4 inhibitors—p16INK4a, p15INK4b, p18INK4c, and p19INK4d—which specifically inactivate Cdk4 and Cdk6 by provoking structural changes that avoid binding and activation by D-type cyclins.5 At least 3 of them, p16INK4a, p15INK4b, and p18INK4c, are inactivated in tumors by deletion, mutation, or promoter hypermethylation, and in vivo models suggest that concomitant inactivation of these inhibitors results in a stronger oncogenic effect.7,10 In particular, the 9p21 chromosomal region encoding p16INK4a and p15INK4b is one of the most commonly mutated loci in human cancer.7-11 Interestingly, a few tumor-associated point mutations have been found in p16INK4a and p18INK4c that avoid binding and therefore inhibition of Cdk4/ Cdk6.12,13 On the other hand, a specific point mutation in Cdk4 (Arg24 to Cys; R24C) prevents binding of the INK4 inhibitors in melanoma patients with sporadic or hereditary disease.14,15 Mice carrying this mutation in the endogenous Cdk4 locus develop


E.R.-D., V.Q., F.B., and M.P.-M. contributed equally to this work.

The data reported in this article have been deposited in the Gene Expression Omnibus database (accession number GSE59024).
a spectrum of tumors and are more susceptible to melanoma development. Lack of Cdk4 inhibition by INK4 inhibitors in Cdk4^R24C/R24C mice results in a stronger phenotype than lack of the individual INK inhibitors, as expected from the compensatory roles among these proteins. However, INK4 proteins may still be partially functional in Cdk4^R24C/R24C animals by inhibiting Cdk6.

To understand the unique or combined effect of lack of inhibition of Cdk4 and Cdk6 by INK4 inhibitors, we have generated knock-in mice expressing a Cdk6 mutant allele in which Arg31 (homologous to Arg24 in Cdk4) has been replaced by Cys (Cdk6^R31C or Cdk6^R). Preventing inhibition of Cdk6 by INK4 proteins has only a minor impact on mouse development or survival. In Cdk6^R^/R^ mice, more p16INK4a binds to Cdk4, potentially accounting for the limited oncogenic potential of the Cdk6 R31C mutation. The inhibitory function of INK4 proteins is completely abrogated in Cdk4^R/R; Cdk6^R^ double-mutant mice display a significant increase in hematopoietic tumors. These results suggest that the precise modulation of Cdk4/C6 activity by INK4 proteins contributes to maintaining the homeostasis of proliferation by sequestering INK4 inhibitors.

Methods

Mice colony and pathological analysis

To generate the targeting vector, we subcloned 5 kb of Cdk6 DNA (supplementary Figure 1 and supplemental Methods on the Blood Web site) into pBH48, and the GCCCGC (Ala30-Arg31) sequence located in exon 1 was replaced by GCATGC (Ala30-Cys31). Heterozygous mice carrying this allele were bred to CMV-Cre mice to generate "knock-in" Cdk6^−/−; R31C (Cdk6^−/−; R31C) animals. Cdk4^R/R; Cdk6^R^ mice were analyzed using standard methods for normalizing to the number of hematopoietic stem cells (HSCs) (population A: Lin− Scalo− CD117^+ CD150^− CD48^−) of wild-type within 4 × 10^6 bone marrow cells. Sixteen weeks later, recipient mice were euthanized and Ly5.2 donor cell contribution was analyzed using flow cytometry. Treatment was performed as described previously using 5-fluorouracil (5-FU; F 6627, Sigma-Aldrich).

Cell culture and tumor cell implants

Virus preparation, infection of hematopoietic cells, and establishment of cell lines were performed as described previously.22,23 Cells lines were characterized performing [3H]-thymidine incorporation assay and cell-cycle analysis as described previously.22,23 To analyze leukemogenesis, 1 × 10^5 cells from independently derived p18^RCRKO; AbL− transformed cell lines were intravenously injected into NSG mice. To study subcutaneous tumor formation, 1 × 10^6 cells were injected into the flank of NSG mice. Mice were euthanized on day 10 and tumor weights were analyzed. To analyze the response to the Cdk4/6 inhibitor PD-0332991 (Pflzer), cells were exposed to different doses of the compound and proliferation was quantified using a CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions.

Transcriptional profiling

Total RNA was extracted from asynchronous cultures using Trizol (Invitrogen). The quality of the RNA obtained was evaluated using the Laboratory-Chip technique (Agilent Bioanalyser). Samples were then fluorescent-labeled by transcription in vitro using commercial Two-Color Microarray-Based Gene Expression Analysis (Agilent), and the Mouse Gene Expression G3 60K (Agilent) containing ~56,000 60-mer probes was used. Images were acquired and quantified by means of confocal scanner and software (Agilent G2505C and Feature Extraction).

Biochemical analysis

Cells were lysed in eeg lysis buffer composed of 0.1% NP-40, 50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 250 mM NaCl, and 5 mM EDTA in the presence of a protease inhibitor cocktail (Roche). Protein concentrations were determined using the Roti-Quant kit (Roti). Immunoprecipitations were performed overnight at 4°C with 6 μg of serum against Cdk4 (a gift from Mariano Barbacid) and 25 μg Pierce Protein A/G Agarose beads (50% volume to volume ratio; Thermo Scientific). Equal amounts of protein (50 μg) from input, supernatant fractions, and from immunoprecipitation corresponding to 1.5 mg of input cell lysate were loaded per lane, respectively. The following antibodies were used for detection: Cdk4, Cdk6, p16INK4a, p15INK4b, p18INK4a, p19INK4a, insulin-like growth factor-1 (IGF-1), Trf2, and Hsc70 (all from Santa Cruz Biotechnology), nuclear factor-κB (NF-κB) p65 (from Merck Millipore), and phospho-pRB S780 and phospho-pRB S780/811 (from Cell Signaling).

Statistical and imaging analyses

Statistical analyses were performed using analysis of variance (ANOVA) or the Student t or log-rank tests (GraphPad Prism 5). All data are shown as mean ± standard deviation or ± standard error of the mean. Probabilities of P < .05 were considered significant. Images were quantified using ImageJ (National Institutes of Health, Bethesda, MD).

Results

Cdk6 R31C and Cdk4 R24C cooperate in tumor development

We targeted the mouse Cdk6 locus in mouse embryonic stem cells by homologous recombination with a targeting vector carrying the
first exon of Cdk6 and a loxP site-flanked PGK-neo cassette for selection (supplemental Figure 1). The GCCCGC (Ala30-Arg31) sequence located in exon 1 of Cdk6 was replaced by GCATGC, resulting in a Cdk6 protein in which the arginine residue (R31) is replaced by a cysteine (R31C mutation), known to prevent binding to INK4 inhibitors.26,27 Cdk6 R/R-homozygous mutants were born at the expected ratio, were fertile, and developed normally during the first months of life. These mutant mice displayed a slightly reduced lifespan when compared with wild-type littermates, which was mostly attributed to a higher incidence of angiosarcomas and epithelial tumors at a greater age (Figure 1A,B). No significant changes in survival or overall disease incidence were observed when maintaining Cdk6 R/R mice on a p53-null background, and all animals died within 40 weeks bearing sarcomas and hematopoietic tumors (Figure 1C). In contrast, the presence of p53 heterozygosity unmasked alterations in tumorigenesis resulting from the Cdk6 R31C mutation, as detected by the increased incidence of both hematopoietic tumors and sarcomas in Cdk6 R/R; Tp531/2 when compared with Cdk61/1; Tp531/2 mice or Cdk6 R/R; and Tp531/2 mice (Figure 1C-D; supplemental Table 1).

Because INK4 proteins function by inhibiting both Cdk4 and Cdk6, we next generated mice fully insensitive to INK4-mediated inhibition by combining the new Cdk6 R31C allele with the Cdk4 R24C allele.3 Cdk4 R24C homozygous mutants were born at the expected ratio, were fertile, and developed normally during the first months of life. These mutant mice displayed a slightly reduced lifespan when compared with wild-type littermates, which was mostly attributed to a higher incidence of angiosarcomas and epithelial tumors at a greater age (Figure 1A,B). No significant changes in survival or overall disease incidence were observed when maintaining Cdk6 R/R mice on a p53-null background, and all animals died within 40 weeks bearing sarcomas and hematopoietic tumors (Figure 1C). In contrast, the presence of p53 heterozygosity unmasked alterations in tumorigenesis resulting from the Cdk6 R31C mutation, as detected by the increased incidence of both hematopoietic tumors and sarcomas in Cdk6 R/R; Tp531/2 when compared with Cdk61/1; Tp531/2 mice or Cdk6 R/R; and Tp531/2 mice (Figure 1C-D; supplemental Table 1).

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Figure 1. Genetic analysis of Cdk6 R31C mice. (A) Tumor-free survival of wild-type and Cdk6 R31C mice. (B) Incidence of tumors in the indicated genotypes. (C) Tumor-free survival of Cdk6 R31C mice in p53-heterozygous or null backgrounds. (D) Incidence of tumors of different origins in the indicated genotypes. The presence of the Cdk6 R31C allele results in increased incidence of sarcomas and hematopoietic tumors only in a p53-heterozygous background. See supplemental Table 1 for details. (E) Tumor-free survival of Cdk4 R24C; Cdk6 R31C or double Cdk4 R24C; Cdk6 R31C mutant mice. (F) Tumor distribution in mutant mice with the indicated genotypes. See Table 1 for details. Long-rank test; *P < .05; **P < .01; ***P < .001.
combination of these 2 alleles, including hypertrophy of ventricular walls in the heart, abundant cysts in the kidney, and altered megakaryocyte morphology and number (Table 1; supplemental Figure 2).

### Cdk6 R31C mice display defective progenitor potential

We next focused our attention on the hematopoietic system because Cdk6 is known to modulate differentiation and proliferation in different hematopoietic compartments,1,5,6,27 and its activation in double-mutant mice resulted in hematopoietic neoplasias that are otherwise infrequent in Cdk4 R24C mice (Figure 1F; Table 1). Total cell numbers in spleen and bone marrow were comparable in all genotypes (supplemental Figure 2). On the other hand, single-mutant Cdk6 R31C mice displayed reduced numbers of T cells, B cells, and granulocytes in bone marrow, spleen, and peripheral blood of recipient mice upon transplantation of lethally irradiated Ly5.1 mice. Ly5.2 Cdk6R/R double-mutant mice resulted in a partial recovery in the numbers of these progenitor cells when compared with single mutants (Figure 2C). Similarly, the ratio of HSCs (CD150− CD48− CD34+), containing the most quiescent HSCs, was reduced in single and double mutants. Again, the combination of the Cdk4 R24C and Cdk6 R31C alleles partially rescued this defect as well as the reduced numbers in multipotent progenitors observed in single-mutant mice (Figure 2D).

We next performed bone marrow transplantation studies into lethally irradiated Ly5.1 mice. Ly5.2 Cdk6R/R progenitors displayed a reduced ability to repopulate as evident from the decreased numbers of LSK and HSC cells 16 weeks after transplantation (Figure 3A). In line with this, reduced numbers of donor-derived T cells, B cells, and granulocytes were found in bone marrow, spleen, and peripheral blood of recipient mice upon transplantation of Cdk6R/R bone marrow (Figure 3B). The concomitant presence of the Cdk4 R24C allele in a Cdk6 R31C background rescued these defects (Figure 3A-B). We also tested the potential of mutant hematopoietic progenitors on 5-FU treatment, which induces death of actively cycling but not quiescent stem cells. The percentage of LSK cells was significantly reduced in 8-week-old Cdk4R/R and Cdk6R/R single-mutant mice (Figure 2C and supplemental Figure 3). The combination of both alleles in Cdk4R/R, Cdk6R/R double-mutant mice resulted in a partial recovery in the numbers of these progenitor cells when compared with single mutants (Figure 2C). Similarly, the ratio of HSCs (CD150− CD48− CD34+), containing the most quiescent HSCs, was reduced in single and double mutants. Again, the combination of the Cdk4 R24C and Cdk6 R31C alleles partially rescued this defect as well as the reduced numbers in multipotent progenitors observed in single-mutant mice (Figure 2D).

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### Table 1. Pathologies in Cdk4 R24C; Cdk6 R31C compound mice

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Cdk4R/R, Cdk6R/R n = 20</th>
<th>Cdk4R/R, Cdk6R/R n = 12</th>
<th>Cdk4R/R, Cdk6R/R n = 16</th>
<th>Cdk4R/R, Cdk6R/R n = 18</th>
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<tr>
<td><strong>Tumoral or pretumoral phenotypes</strong></td>
<td></td>
<td></td>
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<tr>
<td>Acinar cell adenoma (pancreas)</td>
<td>5% (103)</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
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<tr>
<td>Angiosarcoma</td>
<td>0%</td>
<td>58% (68)</td>
<td>38% (89)</td>
<td>11% (72)</td>
</tr>
<tr>
<td>Bronchial adenocarcinoma</td>
<td>5% (118)</td>
<td>33% (71)</td>
<td>38% (114)</td>
<td>0%</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>5% (80)</td>
<td>8% (64)</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Histiocytic sarcoma</td>
<td>5% (85)</td>
<td>0%</td>
<td>0%</td>
<td>22% (54)</td>
</tr>
<tr>
<td>Leydig cell hyperplasia</td>
<td>0%</td>
<td>17% (71)</td>
<td>0%</td>
<td>50% (52)</td>
</tr>
<tr>
<td>Leydig cell tumor</td>
<td>0%</td>
<td>50% (69)</td>
<td>0%</td>
<td>50% (61)</td>
</tr>
<tr>
<td>Lung tumors</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>11% (61)</td>
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<tr>
<td>Lymphoma</td>
<td>10% (88)</td>
<td>0%</td>
<td>19% (83)</td>
<td>39% (59)</td>
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<td>Mammary gland hyperplasia</td>
<td>10% (92)</td>
<td>17% (63)</td>
<td>19% (83)</td>
<td>39% (59)</td>
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<tr>
<td>Pancreatic endocrine hyperplasia</td>
<td>20% (112)</td>
<td>33% (65)</td>
<td>19% (84)</td>
<td>50% (54)</td>
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<tr>
<td>Pancreatic endocrine tumor</td>
<td>0%</td>
<td>25% (70)</td>
<td>13% (97)</td>
<td>11% (72)</td>
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<tr>
<td>Pituitary gland hyperplasia</td>
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<td>17% (54)</td>
<td>13% (90)</td>
<td>22% (49)</td>
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<tr>
<td>Pituitary gland tumors</td>
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<td>25% (76)</td>
<td>13% (77)</td>
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<td>T-cell hyperplasia</td>
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<td>17% (65)</td>
<td>50% (114)</td>
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<td>Rhabdomyosarcoma</td>
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<td>13% (139)</td>
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<tr>
<td>Total incidence</td>
<td>50%</td>
<td>94%</td>
<td>88%</td>
<td>83%</td>
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<tr>
<td>Average latency</td>
<td>97.3</td>
<td>69</td>
<td>99</td>
<td>58.3</td>
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<td><strong>Nontumoral phenotypes</strong></td>
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<tr>
<td>Bronchus-associated lymphoid tissue</td>
<td>0%</td>
<td>0%</td>
<td>31% (101)</td>
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<td>Cardiac defects</td>
<td>20% (102)</td>
<td>0%</td>
<td>13% (85)</td>
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</tr>
<tr>
<td>Chronic hepatitis</td>
<td>5% (107)</td>
<td>0%</td>
<td>13% (70)</td>
<td>0%</td>
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<td>Chronic pancreatitis</td>
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<td>0%</td>
<td>13% (85)</td>
<td>10% (36)</td>
</tr>
<tr>
<td>Extramedullary hematopoiesis</td>
<td>5% (103)</td>
<td>58% (71)</td>
<td>19% (114)</td>
<td>22% (60)</td>
</tr>
<tr>
<td>Kidney disease</td>
<td>20% (96)</td>
<td>17% (66)</td>
<td>19% (104)</td>
<td>89% (57)</td>
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<tr>
<td>Liver steatosis</td>
<td>20% (91)</td>
<td>0%</td>
<td>0%</td>
<td>11% (44)</td>
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<tr>
<td>Megakaryocyte malformation</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>78% (60)</td>
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<tr>
<td>Splenic atrophy</td>
<td>0%</td>
<td>0%</td>
<td>13% (97)</td>
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<td>Vasculitis</td>
<td>5% (81)</td>
<td>0%</td>
<td>19% (90)</td>
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Bold-faced type indicates major differences compared with control groups.
treatment with 5-FU, Cdk6<sup>R/R</sup>, but not Cdk4<sup>R/R</sup>, mice displayed a significant defect in the recovery of red blood cells and hematocrit as well as white blood cells and lymphocytes in peripheral blood. These defects were also partially rescued in Cdk4<sup>R/R</sup>; Cdk6<sup>R/R</sup> double-mutant mice (supplemental Figure 4), suggesting that the alterations observed in Cdk6<sup>R31C</sup> mice could be mediated by the effect of INK4 inhibitors on Cdk4. In summary, these studies show cell intrinsic defects in progenitors in Cdk6<sup>R31C</sup> mice that are compensated for by the concomitant presence of a Cdk4<sup>R24C</sup> protein.

Cdk4<sup>R24C</sup> and Cdk6<sup>R31C</sup> cooperate in increasing proliferation and preventing cell death

To gain further insights in the mechanism underlying these observations and their relevance in cancer, we decided to make use of a well-established model in which bone marrow progenitors are transformed by the BCR-ABL1 oncogene. Bone marrow progenitors were derived from wild-type, Cdk4<sup>R/R</sup>; Cdk6<sup>R/R</sup>, or double Cdk4<sup>R/R</sup>; Cdk6<sup>R/R</sup> knock-in mice and transduced by viruses expressing the p185 form of the BCR-ABL1 oncogene. The presence of the Cdk4<sup>R24C</sup> or Cdk6<sup>R31C</sup> alleles enhanced DNA replication in these cell lines, especially when grown in low-serum conditions (Figure 4A). To analyze their effect during leukemia development, several independent cell lines were injected intravenously into recipient mice. As shown in Figure 4B, leukemia evolved rapidly, and the transformed cells infiltrated bone marrow, spleen, and liver (data not shown). The disease latency of mice injected with cells carrying either Cdk4<sup>R/R</sup> or Cdk6<sup>R/R</sup> single knock-in alleles was significantly reduced when compared with mice injected with wild-type tumors. Tumor cells harboring both the Cdk4<sup>R/R</sup> and Cdk6<sup>R/R</sup> alleles killed the animals even faster (Figure 4B). In line, Cdk4<sup>R/R</sup>; Cdk6<sup>R/R</sup> tumor cells were able to form significantly larger tumors when implanted subcutaneously into recipient mice (supplemental Figure 5). In this experimental system, the effect of Cdk6<sup>R31C</sup> was more pronounced, in line with the relevance of this kinase in the
hematopoietic system. Tumors derived from double-mutant clones displayed enhanced mitotic index (as monitored by phosphorylation of histone H3) and decreased number of apoptotic cells (as scored by antibodies against the active form of caspase 3; supplemental Figure 5). Thus, in these 2 independent assays, the concomitant presence of the 2 mutant Cdns was able to significantly accelerate tumor growth and progression.

The ability of Cdk4R/R; Cdk6R/R BCR-ABL1-transformed cell lines to grow in vitro allowed us to determine cell-cycle kinetics and revealed a reduced G0/G1 phase and high ratio of S/G2/M cells in the absence of a significant level of apoptosis when compared with wild-type or single-mutant mice (Figure 4C). Double knock-in cells were also more resistant than the single mutants or wild-type cells to the Cdk4/6 inhibitor PD-0332991 (Figure 4D), indicating the relevance of Cdk4/6 kinase activity for the growth of these leukemic cells.

We next compared the transcriptional profiles of asynchronous Cdk4R/R; Cdk6R/R to control cultures. Genes deregulated in these mutant cells (723 transcripts upregulated and 986 downregulated; fold-change >2; Figure 4E; supplemental Table 2) were enriched in targets of the transcription factors Egr1/2, Zf5, Rfx1, Atf4, Ctf1/2, Lrf, E12, or E2f, among others (Figure 4F). Gene ontology (GO) analysis of deregulated genes also indicated control of apoptosis as the major cellular process deregulated in double-mutant cultures (Table 2; supplemental Table 3). These INK4-insensitive cells also displayed significant differences in molecular pathways involved in cell adhesion and motion or regulation of cytokine signaling (Table 2; supplemental Table 3). The deregulation of some critical molecules involved in apoptosis or other major pathways of relevance in B-cell leukemias was validated using immunoblot (p65NFkB, IGF-1, Traf2) or cytometry analysis (interleukin-2Ra; Figure 4G). Altogether, these data suggest the involvement of multiple signaling pathways, in addition to the retinoblastoma/E2F route, in the response to INK4 insensitivity in Cdk4R/R; Cdk6R/R leukemic cells.

Cdk6 sequesters p16INK4a inhibitors away from Cdk4

The availability of BCR-ABL1-transformed cell lines carrying the different knock-in alleles allowed us to investigate and compare the levels of cell-cycle regulatory proteins side by side. The level of expression of Cdk4 R24C or Cdk6 R31C mutant proteins from the corresponding alleles was similar to the wild-type isoforms, although Cdk6 R31C levels were significantly reduced in double Cdk4R/R; Cdk6R/R knock-in cells. The levels of p16INK4a were increased in single Cdk4R/R or Cdk6R/R tumor cells (Figure 5A and supplemental Figure 5), in agreement with previous reports showing that p16INK4a is induced as...
a consequence of ectopic Cdk4 or Cdk6 activity. Importantly, the concomitant presence of the Cdk4 R24C and Cdk6 R31C alleles resulted in a significant increase in the phosphorylation of the Cdk4/6 target retinoblastoma protein (Rb; Figure 5B,C).

We next asked whether p16INK4a, in the presence of the Cdk6 R31C mutant allele, would increasingly bind wild-type Cdk4. As shown in Figure 5D, whereas p16INK4a was almost undetectable in Cdk4 complexes in wild-type BCR-ABL1 cells as well as in Cdk4(R/R); Cdk6(R/R) clones, it efficiently bound Cdk4 in Cdk4(+/+); Cdk6(R/R) cells. In control Cdk4(+/+); Cdk6(+/+) BCR-ABL1-transformed clones, Cdk6 complexes contained p16INK4a (Figure 5E), whereas, as expected, this inhibitor was not present in clones carrying the Cdk6 R31C mutant. Again, the amount of p16INK4a bound to Cdk6 increased in Cdk4(R/R); Cdk6(+/+) clones. Similar results were obtained in experiments in which we performed direct immunoprecipitation of p16INK4a and analyzed attached kinases (Figure 5F). Whereas p16INK4a did not form complexes with Cdk4 in control cells, it was readily detected in Cdk4 complexes in Cdk4(+/+); Cdk6(R/R) cells. Vice versa, we observed an enrichment in Cdk6-p16INK4a complexes in Cdk4(+/+); Cdk6(R/R) cells. The absence of p16INK4a in Cdk4 or Cdk6 complexes in Cdk4(R/R); Cdk6(R/R) cells correlated with increased phosphorylation of Rb (Figure 5B,C), the major substrate of these kinases. Overexpression of exogenous wild-type Cdk6 in Cdk4(+/+); Cdk6(R/R) cells was sufficient to reduce the levels of Cdk4-bound p16INK4a (Figure 5G), suggesting the relevance of Cdk4/6 protein levels in counteracting the activity of INK4 inhibitors. Taken together, our findings reveal that expression of single Cdk4 R24C or Cdk6 R31C mutants suffices to shift binding of the cell-cycle inhibitor p16INK4a to the remaining wild-type Cdk6 or Cdk4, respectively, exerting a compensatory inhibitor effect.
Only the presence of both INK4a-insensitive alleles enables the spontaneous development of hematopoietic tumors by completely preventing the tumor suppressor activity of INK4 proteins.

### Discussion

Cdk4 and Cdk6 are 2 highly related Cdns activated by D-type cyclins and inhibited by INK4 proteins. Despite their similarity, specific functions have been proposed in different cell types, such as astrocytes, osteoblasts, or prostatic cells. In vivo, whereas genetic ablation of Cdk4 results in specific deficiencies in pancreatic and pituitary endocrine cells, disruption of the Cdk6 gene results in reduced red blood cells and altered proliferation of T cells, suggesting differential functions for this kinase in the hematopoietic system. Cdk6 and Cdk4 also have separate activities in erythroblast differentiation, with nuclear activity of Cdk6 and cytoplasmic activity of Cdk4. Cdk6 blocks erythroid differentiation, but as cells commit to differentiation and Cdk6 declines, increasing amounts of Cdk4 appear in the nucleus, suggesting that Cdk6 controls proliferation of erythroblasts, whereas Cdk4 controls cell division in differentiated cells. Cdk4 is highly expressed in thymocytes, and Cdk6-cyclin D2 complexes are proposed to be involved in the acquisition of the competent state in human T lymphocytes. Cdk6 depletion causes reduced thymocyte cell numbers with a specific block in DN3 cells, a phenotype reminiscent of that observed in cyclin D3-null mice. Moreover, Cdk6 is also highly expressed in the cytoplasm of CD8 memory cells, favoring rapid division and expansion of these mature cells.

Cdk4 and Cdk6 are frequently deregulated in human cancer through inactivation of INK4 inhibitors, and several INK4 family members are codeleted in specific tumor types, suggesting cooperation between family members. In the mouse, individual genetic ablation of INK4 inhibitors display limited effect in tumor formation, and codeletion of 2 family members results in some additive effects, at least in sarcomas and endocrine tumors. However, these assays have been limited by the complexity of combining mutant alleles for all the INK4 genes. The analysis of Cdk6 knockout mice (Figure 1; Table 1) suggests additional compensatory roles among the INK4 family, at least in hematopoietic and endocrine tumors.

Whereas specific functions for Cdk4 or Cdk6 have been clearly established in normal tissues, their separate or synergistic functions during tumor development are less understood. Cdk4 is mostly amplified in sarcomas, whereas the Cdk4 R24C mutation is specifically found in melanoma. Cdk6, on the other hand, is translocated and amplified in multiple hematopoietic neoplasms such as T- and B-cell lymphomas and leukemias and acute myeloid leukemias. Our study uncovers an oncogenic role for Cdk6 R31C in a Cdk6 knockout background for hematopoietic tumors (Figures 1 and 4). As suggested recently, Cdk6 may have specific roles in these hematopoietic neoplasias because this kinase functions as a member of transcription complexes that stimulate different pathways, including angiogenesis.

#### Table 2. GO analysis of transcripts deregulated in Cdk4R; Cdk6R BCR-ABL1–transformed cells

<table>
<thead>
<tr>
<th>Annotation clusters</th>
<th>Description</th>
<th>Representative genes upregulated</th>
<th>Representative genes downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>GO:0008216, positive regulation of apoptosis; GO:0043067, regulation of programmed cell death; GO:0060167, induction of apoptosis; GO:0060166, anti-apoptosis; GO:0043066, negative regulation of apoptosis</td>
<td>APL1, ANK2, CDK5R1, CDKN2A, CHST11, DFFA, E2F2, FGFR3, IFIH1, IGF1, IL2RB, NOTCH2, NR4A1, PIHLA3, SPP1, TCF7, TM2, TRAF2</td>
<td>BBC3, BCL2A10, BCL2A1C, BCL2L11, BCL3, BNP3L, BTOG, CARD11, CD74, CDH1, CEBPB, CUL7, DMT3, DMRT2, FAS, FGFR1, GCH1, IL18, IL2RA, LST1, LT, MAP2K5, PIP4C3, PRK11, RRM2B, SPPH1, TRP53, TSC22D3, UACA, VDR</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>GO:0007155, cell adhesion; PIR: cell–cell adhesion</td>
<td>ALCAM, APP, CD33, CD97, CDK5R1, CELSR1, ESAM, F11R, FBLN7, HSP20, IBSP, ITGAM, L1CAM, LGALS3BP, NCAM2, NRP2, PCDH17, PCDHB16, PCDHB17, PCDHB20, PGM5, SPP1, SSPO</td>
<td>ARHGA06, BCL2L11, CD66, CDH1, CLDN12, COL6A6, DSG2, FAT1, FND3A, ITG8, LM07, MEGF10, MYBPC2, NLGN2, PCDH11X, PVR1L2, ROBO2, SELL, THY1, TRO</td>
</tr>
<tr>
<td>Cell projection and motion</td>
<td>GO:0007411, axon guidance; GO:003030, cell projection organization; GO:0048558, cell projection morphogenesis; GO:0032888, cellular component morphogenesis; GO:0006928, cell motion</td>
<td>ALCAM, ANK3, APP, CDK5R1, DNAJ1, EPHD1, GNA77B, ITGAM, L1CAM, NR2F1, NRP2, SEMA3A, SHROOM4, SOX6, TBR1, TNNT2, TNS3, VAV3, WWTR1</td>
<td>ARID5B, BBS4, CHRN2B, EFN5A, EGR2, ETV1, FAT1, FEZ2, FGD2, FG5, HAX2, LPS1, LST1, NEURO2, PDGFA, PLEXK5, PT2, PHOX5, ROBO2, ROBO3, RYK, TTC8, VANG3L2</td>
</tr>
<tr>
<td>Regulation of neurological processes</td>
<td>GO:0001969, regulation of transmission of nerve impulse; GO:004057, regulation of system process; GO:0031644, regulation of neurological system process; GO:0001926, transmission of nerve impulse</td>
<td>APP, CELSR1, DGL2, IJX5, KCNMA1, MOA, PCDHB16, SEMA3A, TNNT2, TRF</td>
<td>CHRN2B, COLQ, DRD5, EGR2, FKBP18, GRI2A, GRIK5, GRM6, HEXA, LST1, LT, NLGN2, P2RX3, PARK2, PROK2, PTK2</td>
</tr>
<tr>
<td>Regulation of cytokine signaling</td>
<td>GO:0004896, cytokine receptor activity; GO:0019226, cytokine–mediated signaling pathway</td>
<td>IL1R1, IL1RAP, IL2RB</td>
<td>ACVR2B, CCR2, CD74, CXCR3, CXCR5, EB3, IL1R2, IL1RB2, LFIR</td>
</tr>
</tbody>
</table>

*Most significantly deregulated functional pathways and their GO codes are shown first. See supplemental Table 2 for further details.*
(supplemental Figure 6). Our study provides the rationale for concomitant overexpression of both Cdk4 and Cdk6 in hematopoietic tumors, especially in those cases in which at least an INK4 protein is still present. In the presence of the Cdk6 R31C allele, p16INK4a is increasingly bound to Cdk4, leading to limited phosphorylation of pRb (Figure 5). Concomitant expression of the INK4-insensitive allele Cdk4 R24C results in enhanced phosphorylation of pRb and proliferation of hematopoietic precursors and tumoral cells (Figures 2-5), suggesting that these 2 cyclin D-dependent kinases cooperate not only by phosphorylating common or specific substrates but also by maintaining inhibitors away from the other family member.

The relevance of Cdk4/6 activity in cancer therapy has been recently highlighted by the success of Cdk4/6 inhibitors in phase 2 clinical trials in hormone-positive breast cancer.53 In fact, research in the past decade using mouse models has suggested the possible benefit of inhibiting Cdk4/6 kinases in breast, lung, and hematopoietic tumors, among others.54 Whereas Cdk4/6 inhibition results in the induction of senescence in epithelial tumors, hematopoietic cells are more susceptible to apoptotic cell death. Our expression data in Cdk4R/R; Cdk6R/R BCR-ABL1-transformed cells suggests that Cdk4/6 activity controls several apoptotic routes in addition to other molecular pathways (Table 2 and Figure 4). Further work on these pathways may provide new biomarkers of clinical use and will be instrumental to understand the effect of these cell-cycle kinases in leukemogenesis.

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Cdk4 and Cdk6 cooperate in counteracting the INK4 family of inhibitors during murine leukemogenesis

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