

Cdk6 blocks myeloid differentiation by interfering with Runx1 DNA binding and Runx1-C/EBP α interaction

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Interactions between the cell cycle machinery and transcription factors play a central role in coordinating terminal differentiation and proliferation arrest. We here show that cyclin-dependent kinase 6 (Cdk6) is specifically expressed in proliferating hematopoietic progenitor cells, and that Cdk6 inhibits transcriptional activation by Runx1, but not C/EBP α or PU.1. Cdk6 inhibits Runx1 activity by binding to the runt domain of Runx1, interfering with Runx1 DNA binding and Runx1-C/EBP α interaction. Cdk6 expression increased myeloid progenitor proliferation, and inhibited myeloid lineage-specific gene expression and terminal differentiation *in vitro* and *in vivo*. These effects of Cdk6 did not require Cdk6 kinase activity. Cdk6-mediated inhibition of granulocytic differentiation could be reversed by excess Runx1, consistent with Runx1 being the major target for Cdk6. We propose that Cdk6 downregulation in myeloid progenitors releases Runx1 from Cdk6 inhibition, thereby allowing terminal differentiation. Since Runx transcription factors play central roles in hematopoietic, neuronal and osteogenic lineages, this novel, noncanonical Cdk6 function may control terminal differentiation in multiple tissues and cell types.

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Introduction

Coordinating terminal differentiation and cell cycle arrest involves coupling the activity of the transcriptional regulators that activate lineage-specific gene expression programs to the cell cycle machinery. The importance of such coordination is illustrated by the observation that ectopic expression of cell cycle promoting factors is able to interfere with differentiation of numerous cell types. Well-characterized examples

include the ability of the c-Myc oncoprotein to block the differentiation of adipocytes by repressing the transcription of C/EBP α , a key inducer of adipogenesis (Freytag and Geddes, 1992), and the ability of Cyclin D1/Cdk4 to inhibit myogenesis through binding to MyoD (Zhang *et al*, 1999). However, in other cases, the molecular mechanisms are not clear. E2F-1 can block granulopoiesis (Strom *et al*, 1998), adipogenesis (Porse *et al*, 2001) and myogenesis (Wang *et al*, 1995), but the relevant molecular targets are not defined. Cdk6 inhibits osteogenic differentiation by a mechanism that appears unrelated to its cell cycle function (Ogasawara *et al*, 2004). Conversely, lineage-specific transcription factors, such as C/EBP α and GATA-1, have in several cases been found to directly block cell cycle progression (Slomiany *et al*, 2000; Rylski *et al*, 2003). C/EBP α interacts with and represses the E2F complex (Slomiany *et al*, 2000), and this is required for the ability of C/EBP α to arrest the cell cycle and induce terminal adipocyte and granulocyte differentiation (Porse *et al*, 2001). In the case of GATA-1, the critical event appears to be downregulation of *Myc* expression (Rylski *et al*, 2003). Acquired mutations in *GATA1* and *CEBPA* are observed in acute myeloid leukemias (Wechsler *et al*, 2002; Nerlov, 2004), underscoring the important role of lineage-specific transcriptional regulators in controlling both cellular proliferation and differentiation, and ultimately function as tumor suppressors.

In this context, the Runx family of transcription factors poses a particular challenge. There is strong genetic evidence that Runx proteins are important for differentiation of multiple cell types, including osteoblasts (Komori and Kishimoto, 1998), neurons (Inoue *et al*, 2002; Levanon *et al*, 2002) and hematopoietic cells (Ichikawa *et al*, 2004). A tumor suppressor function for Runx proteins is indicated by the targeting of *RUNX1* by a wide range of chromosomal translocations in acute leukemias (Ito, 2004). However, Runx proteins are also capable of functioning as oncoproteins, as proviral activation of *Runx1*, *Runx2* and *Runx3* in mouse leukemia models has been observed (Ito, 2004). A possible explanation for this paradox is that Runx proteins have distinct molecular functions in progenitor cells, where they promote proliferation, and in terminally differentiating cells, where they act cooperatively with lineage-specific factors, such as PU.1 and C/EBP α (in hematopoiesis) (Zhang *et al*, 1996) or C/EBP β (in osteogenesis) (Gutierrez *et al*, 2002), to promote lineage-specific gene expression. It is not clear, however, which molecular mechanism would mediate such a switch in Runx function.

We here provide evidence that in the hematopoietic system Cdk6 is expressed in the proliferating progenitor compartment, and that Cdk6 blocks the differentiation-specific functions of Runx1 in immature proliferating cells by direct interaction with the Runx1 runt domain. The Cdk6–Runx1 interaction interferes with DNA binding of the Runx1/CBF β heterodimer and inhibits Runx1-C/EBP α interaction. This

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leads to inhibition of cooperative transcriptional activation by Runx1 and C/EBP α , increased progenitor proliferation and loss of lineage-specific gene expression and terminal granulocytic differentiation. Downregulation of Cdk6 therefore provides a molecular switch that allows the differentiation-promoting activity of Runx proteins to be selectively activated in terminally differentiating cells.

Results

Cdk6 downregulation is required for terminal granulocytic differentiation

In order to examine the possible involvement of G1 cyclin-dependent kinases (Cdks) in coordinating proliferation and differentiation of hematopoietic progenitors, expression of *Cdk4* and *Cdk6* was compared in Lin^{lo}c-Kit^{hi} progenitor cells, Lin^{lo}c-Kit⁺ immature committed cells and Lin⁺c-Kit⁻ terminally differentiating cells (Figure 1A). Interestingly, whereas *Cdk4* mRNA was uniformly expressed at the different stages of differentiation, *Cdk6* mRNA expression was specifically absent in terminally differentiating cells (Figure 1B), suggesting a link between *Cdk6* downregulation and terminal differentiation. In order to investigate whether downregulation of Cdk6 was a requirement for granulocytic differentiation, we generated stable 32D myeloid cells stably expressing Cdk6. The transfected cells express three- to fourfold higher levels of the Cdk6 protein compared with parental cells (Supplementary Figure S1). G-CSF efficiently induced the formation of segmented granulocytes in the empty vector-transfected cells (Figure 1C), and this was preceded by downregulation of *Cdk6* expression (Figure 1D). In contrast, G-CSF-induced granulocytic differentiation was markedly decreased in Cdk6-expressing cells (Figure 1C), leading to accumulation of blasts and early granulocyte progenitors (Figure 1E). Expression levels of mRNAs encoding myeloperoxidase (MPO) and neutrophil elastase (NE), both induced during granulocyte differentiation, were also decreased in Cdk6-expressing cells, as measured by semiquantitative RT-PCR (Figure 1F). This showed that sustained expression of Cdk6 inhibits G-CSF-induced granulocytic differentiation and lineage-specific gene expression in 32D myeloid progenitor cells.

Cdk6 specifically interferes with Runx1 function

The genes encoding C/EBP α , PU.1 and Runx1 are all targets of mutation or translocation in acute myeloid leukemia (AML), and myeloid-specific promoters generally depend on combined binding sites for these factors for their activity (Zhang *et al*, 1996; Tenen, 2003). To determine whether Cdk6 directly interfered with any of these factors, we examined the effect of Cdk6 on their ability to activate the macrophage-colony-stimulating factor receptor (M-CSF-R) promoter, which contains collaborating Runx1-, PU.1- and C/EBP α -binding sites (Petrovick *et al*, 1998). Cdk6 was found to inhibit M-CSF-R promoter activation by Runx1 in both NIH3T3 cells (Figure 2A) and K562 myeloid progenitors (data not shown). In contrast, we observed no effect of Cdk6 on the ability of PU.1 or C/EBP α to activate the M-CSF-R promoter in transiently transfected NIH3T3 cells (Figure 2B and C). Inhibition of Runx1 activity by Cdk6 was stronger than that observed with the closely related Cdk4 protein (Figure 2D). Titration experiments confirmed that significantly higher levels of Cdk4 were required to affect

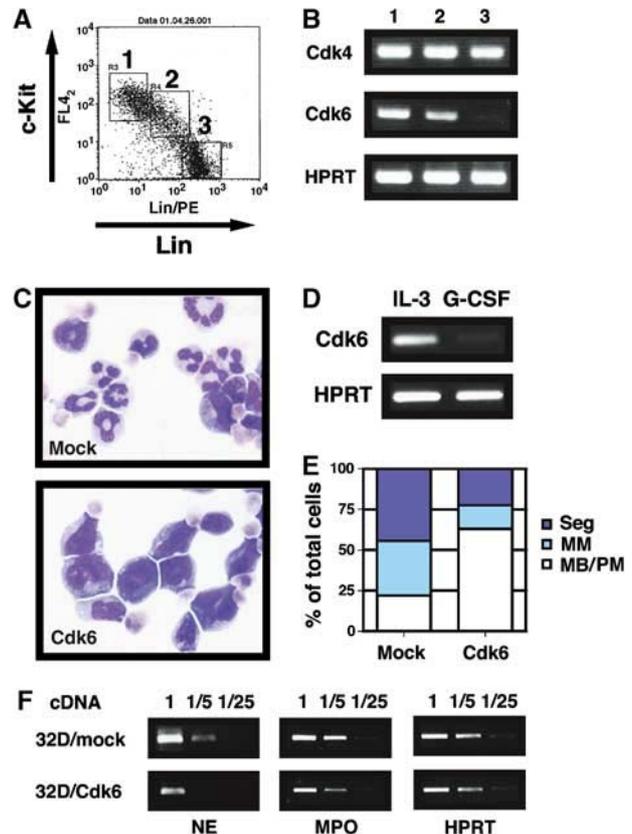


Figure 1 Cdk6 is expressed in the c-Kit⁺ progenitor compartment and inhibits granulocyte differentiation of 32Dcl.3 cells. (A) Fetal liver cells at E14 were fractionated into Lin⁻c-Kit^{hi} (1), Lin^{lo}c-Kit⁺ (2), and Lin⁺c-Kit⁻ (3) cells by FACS. (B) Expression of *cdk4* and *cdk6* in fractions from (A) was analyzed by RT-PCR. (C) Control and *cdk6*-transduced 32Dcl.3 cells were cultured in the presence of G-CSF. Cytospins were prepared from the cultured cells at day 7, and stained with May-Grünwald-Giemsa solution. (D) Cdk6 expression during G-CSF-induced differentiation of 32Dcl.3 cells. Cells cultured in the presence of IL-3 (lane 1) or G-CSF for 24 h (lane 2); total RNA was analyzed by RT-PCR. (E) Differential counts of cells from (C). In total, 100 cells were counted per sample. The averages from two independent experiments are shown. (MB: myeloblasts; PM: promyelocytes; MM: metamyelocytes; Seg: segmented granulocytes). (F) mRNA expression of myeloid differentiation marker genes in control and Cdk6-transduced 32Dcl.3 cells was analyzed by RT-PCR after 2 days of differentiation using two serial fivefold dilutions of cDNA. NE: neutrophil elastase; MPO: myeloperoxidase; HPRT: hypoxanthine-guanine phosphoribosyltransferase.

Runx1 activity compared to Cdk6 (Supplementary Figure S2). To determine whether Cdk6 kinase activity was required for the observed inhibition of Runx1 function, a kinase-dead Cdk6 mutant was tested and found to inhibit Runx1 with the same efficiency as wildtype Cdk6 (Figure 2E). Western blotting of extracts from transfected cells showed Runx1 levels in cells expressing Cdk6 equivalent to those found in the absence of Cdk6 (Figure 2D and E, lower panel). These results showed that Cdk6 specifically inhibits Runx1-dependent promoter activation among myeloid transcription factors by a mechanism that did not require the Cdk6 kinase activity and did not affect the level of Runx1 protein.

Cdk6 interacts with the Runx1 runt domain

To examine the possibility of a direct Cdk6-Runx1 interaction, we first investigated the ability of Cdk6 and Runx1 to form a complex by co-immunoprecipitation analysis. 293T

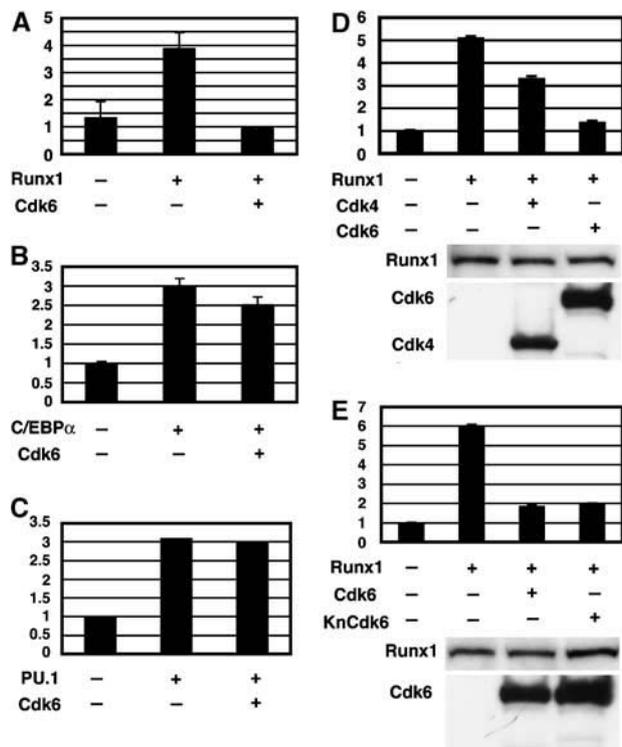


Figure 2 Cdk6 selectively inhibits Runx1 transactivation function. (A) NIH3T3 cells were transfected with 200 ng of pM-CSF-R-luc reporter and 1 ng of pRL-TK internal control plasmid, 80 ng of pCMV-MTPU.1 and 80 ng of pCMV-Cdk6 as indicated. (B) As in (A), but with 80 ng of pcDNA3-C/EBP α , cells were cotransfected as indicated. (C) As in (A), but with 80 ng of pEF-Runx1 and 50 ng pEF-CBF β , cells were cotransfected as indicated. (D) As in (C), with 80 ng of pCMV-Cdk4 and 80 ng of pCMV-Cdk6, cells were cotransfected as indicated. (E) As in (C), with 80 ng of pCMV-Cdk6 and 80 ng of pCMV-knCdk6, cells were cotransfected as indicated. Equal amount of total cell lysate from cells transfected as in (D) and (E) were electrophoresed on a 12% SDS-PAGE gel and subjected to Western analysis with anti-Runx1 polyclonal antibody or HA-7 monoclonal antibody (detecting the HA-tag on the Cdk4/6 protein; panels D and E). The (-) sign indicates addition of empty expression vector. The averages from two independent transfections are shown.

cells were transiently transfected with Flag-tagged Runx1 (Flag-Runx1) and HA-tagged Cdk6 (Cdk6-HA). Flag-Runx1 and associated proteins were co-immunoprecipitated with the M2 anti-flag monoclonal antibody. Cdk6-HA was readily detected in anti-Flag, but not in control, immunoprecipitates (Figure 3A). By similar analysis, using anti-Runx1 and anti-Cdk6 antibodies, we could detect association of endogenous Cdk6 and Runx1 in K562 cells (Figure 3B). Consistent with the transfection data, indicating a lower affinity of Cdk4 for Runx1 compared to that of Cdk6, we were unable to co-immunoprecipitate endogenous Cdk4 with Runx1 under these conditions. Together, these results indicate that Cdk6 preferentially interacts with and inhibits Runx1.

To identify the region of Runx1 responsible for interaction with Cdk6, Flag-tagged deletion mutants of Runx1 (Figure 3C) were expressed together with Cdk6-HA in 293T cells, and the cell extracts were subjected to co-immunoprecipitation assay. We found that all Runx1 fragments containing the runt domain were capable of interacting with Cdk6 (Figure 3D). This showed that Runx1 runt domain is suffi-

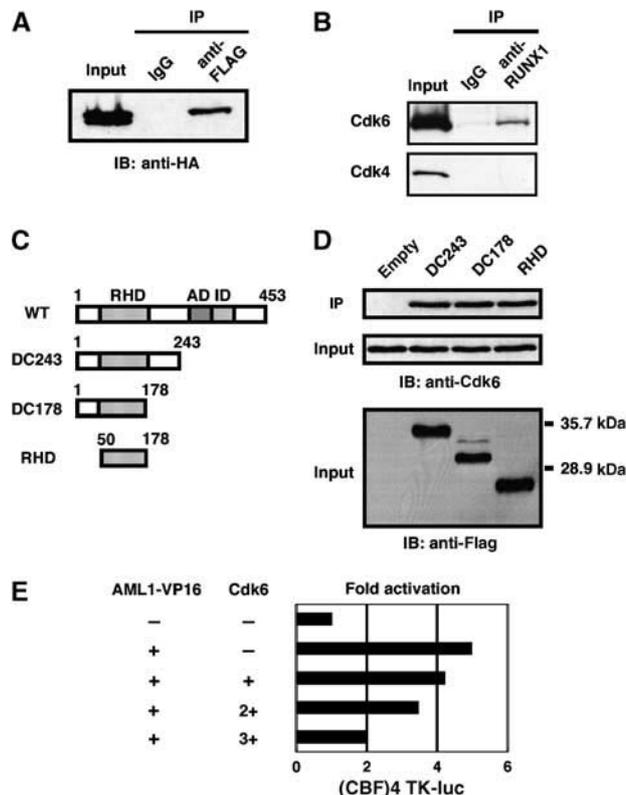


Figure 3 Cdk6 interacts with Runx1 Runt domain. (A) Lysates of 293T cells transfected with plasmids encoding Flag-Runx1 and cdk6-HA were immunoprecipitated with anti-flag mAb or control mouse IgG. In all, 5% of the input and precipitates were separated by SDS-12% PAGE and analyzed by Western blotting with anti-HA mAb. (B) Lysates of K562 cells were immunoprecipitated with anti-AML1 polyclonal antibody or rabbit IgG and analyzed as in (A) using an anti-Cdk4 or -Cdk6 polyclonal antibodies. (C) FLAG-tagged Runx1 deletion mutants: RHD: Runt homology domain; AD: activation domain; ID: inhibitory domain. (D) Interaction between Cdk6 and FLAG-Runx1 mutants was analyzed as in (A). (E) NIH3T3 cells were transfected with 200 ng of p(CBF)4TK-luc, 1 ng of pRL-TK, 200 ng of pVP16, 200 ng of pAML1-VP16 as indicated. Increasing amounts (100 or 200 ng) of pCMV-cdk6 were cotransfected as indicated. The means of two independent transfections are shown.

cient for interaction with Cdk6, and pointed to the interaction between Cdk6 and Runx1 runt domain as being instrumental to the observed repression. To confirm this, we used a hybrid protein in which the Runx1 runt domain was fused to the transactivation domain of the HSV VP16 protein, yielding Runx1-VP16. Cdk6 was able to efficiently repress the activity of Runx1-VP16 (Figure 3E) on a synthetic promoter containing four Runx-binding sites, demonstrating that the interaction of Cdk6 with the runt domain is sufficient for Cdk6 to antagonize Runx1 function.

Cdk6 inhibits Runx1 DNA-binding, but not Runx1-CBF β interaction

Runx1 is known to heterodimerize with CBF β through the runt domain, and heterodimerization with CBF β enhances Runx1 DNA-binding activity. Thus, interaction of Runx1 with CBF β is one of the important determinants of Runx1 function. We therefore investigated whether Cdk6 disrupts the association of Runx1 with CBF β . 293T cells were cotransfected with Flag-Runx1 and CBF β in either the absence or presence

of Cdk6-HA and the association of Runx1 with CBF β was monitored by co-immunoprecipitation. No inhibition of Runx1-CBF β interaction by Cdk6 was observed (Figure 4A). Another main function of the runt domain is DNA binding. To examine whether Cdk6 disrupts this Runx1 function, two types of experiments were performed. First, 293T cells were transfected with Flag-Runx1, CBF β and Cyclin D3 expression

vectors in either the absence or presence of Cdk6-HA, nuclear extracts prepared, and the DNA binding of Runx1 was monitored by pull-down using a biotinylated oligonucleotide containing Runx-binding sites. Whereas Runx1 bound to this probe in the absence of Cdk6, inhibition of Runx1 DNA binding by both Cdk6 and a kinase-dead Cdk6 mutant (knCdk6) was observed (Figure 4B), consistent with Cdk6

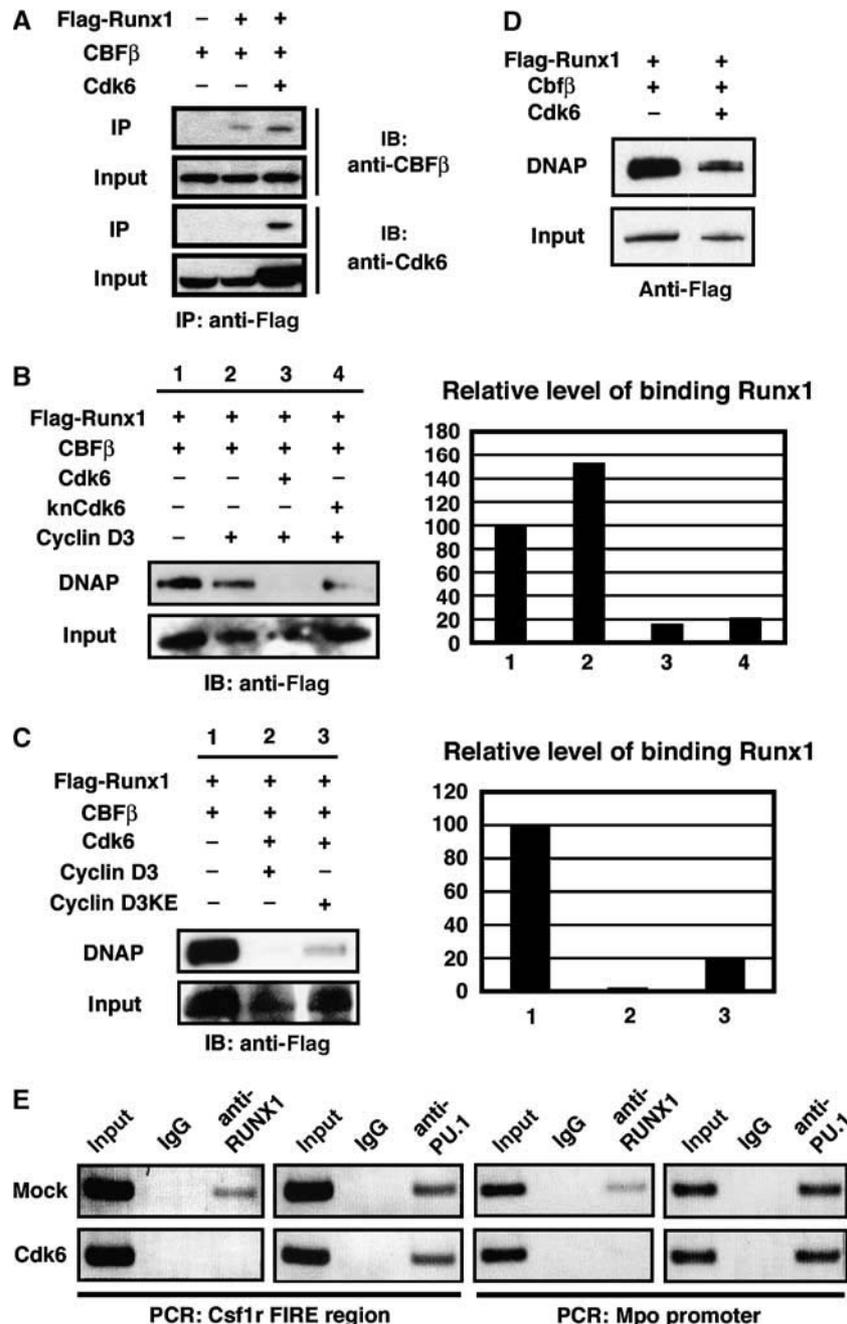


Figure 4 Cdk6 inhibits Runx1 DNA binding. (A) 293T cells were transfected with 1 μ g pCMV-Flag-Runx1, 1 μ g pEF-CBF β and 1 μ g pCMV-cdk6 as indicated. After anti-Flag IP, the input (2.5%) and immunoprecipitates were analyzed by Western blotting with anti-CBF β antibody (upper panels) or anti-Cdk6 (lower panels). (B) 293T cells were cotransfected with 2 μ g pCMV-Flag-Runx1, 1 μ g pEF-CBF β , 0.5 μ g pRcCMV-Cyclin D3 and 2 μ g pCMV-Cdk6HA (lane 3), or 2 μ g pCMV-knCdk6HA (lane 4) in a 10 cm plate. Cells were lysed and lysates were precipitated with biotinylated Runx1-binding element oligonucleotides followed by analysis of these complexes using anti-Flag and anti-HA antibodies. (C) As in (B), but with 2 μ g pCMV-Cdk6HA, and 2 μ g pRcCMV-Cyclin D3HA (lane 2) or 2 μ g pRcCMV-Cyclin D3KEHA (lane 3), cells were transfected as indicated, and subjected to the oligonucleotide pulldown (DNAP) assay. Relative levels of DNA-binding Runx1 are indicated on the right. The relative expression level of lane 1 is designed 100%. (D) As in (B), but without pRcCMV-Cyclin D3, cells were transfected as indicated, and subjected to the DNAP assay. (E) Chromatin from mock and Cdk6-transfected LG cells was immunoprecipitated with anti-Runx1 polyclonal antibody and analyzed by PCR with primers that amplify a fragment of Csf1r intronic regulatory element, FIRE region and Mpo promoter.

kinase activity being dispensable for Runx1 inhibition. This was confirmed by transfection of a Cyclin D3 mutant (Cyclin D3KE) incapable of Cdk4/6 activation. Also in the presence of this, Cyclin D3 mutant inhibition of Runx1 DNA-binding was observed (Figure 4C). Indeed, Cdk6 blocked Runx1 DNA binding in the absence of cotransfected Cyclin D3 (Figure 4D). Finally, we analyzed the effect of ectopic Cdk6 expression on Runx1 binding to the M-CSF-R and MPO promoters in LG myeloid progenitor cells by chromatin immunoprecipitation (ChIP). In both cases, Cdk6 blocked promoter association of Runx1, as determined by ChIP with an anti-Runx1 polyclonal antibody, whereas PU.1 binding to these two promoters was unaffected in Cdk6-expressing, relative to control, LG cells (Figure 4E). This was not due to a decrease in Runx1 protein levels, which were the same in control and Cdk6-expressing LG cells (Supplementary Figure S3). These results indicated that the interaction between Cdk6 and Runt domain had inhibitory effects on the DNA binding of Runx1, and that this led to depletion of Runx1 from its target promoters.

Cdk6 blocks transcriptional synergy of Runx1 with C/EBP α

On differentiation-specific promoters, Runx1 functions in concert with lineage-specific transcription factors. In particular, on myeloid promoters, Runx1-binding sites are frequently found in conjunction with binding sites for PU.1 and C/EBP α (Zhang *et al*, 1996), and C/EBP α is critical for granulopoiesis (Zhang *et al*, 1997). Therefore, we examined whether Cdk6 interfered with the synergy between Runx1 and C/EBP α . As described, Runx1 cooperated with C/EBP α in activation of the M-CSF-R promoter (Figure 5A), and Cdk6 was able to block their cooperation. As we did not observe any direct effect of Cdk6 on C/EBP α -dependent activation of the same reporter gene, we speculated that Cdk6 interferes with the physical interaction between Runx1 and C/EBP α . To examine this possibility, we assessed the effect of Cdk6 on the interaction between Runx1 and C/EBP α by co-immunoprecipitation. When Flag-Runx1 and C/EBP α were coexpressed in 293T cells, C/EBP α was readily detected in the Flag-Runx1 immunoprecipitate (Figure 5B), and this association was disrupted by the simultaneous presence of Cdk6. These results show that Cdk6 can block the interaction of Runx1 with C/EBP α , thereby further repressing synergistic promoter activation.

Cdk6 inhibits granulocytic differentiation in a kinase-independent manner

The above results identified the inhibition of Runx1 DNA binding as a noncanonical kinase-independent function of Cdk6 (but not Cdk4). To further correlate Runx1 inhibition to the observed inhibition of granulocytic differentiation, we compared the ability of Cdk6, knCdk6 and Cdk4 to inhibit granulocytic differentiation of the LG cell line. LG cells stably transfected with expression vectors encoding Cdk6, knCdk6 or Cdk4 were induced to differentiate in the presence of G-CSF. While Cdk4 had no effect on G-CSF-induced LG cell differentiation (as measured by the emergence of cells with segmented nuclei), both Cdk6 and knCdk6 had a strong inhibitory effect (Figure 6A and B). When the DNA-binding activity of endogenous Runx1 in stably transfected LG cells was analyzed by oligonucleotide pull-down of nuclear ex-

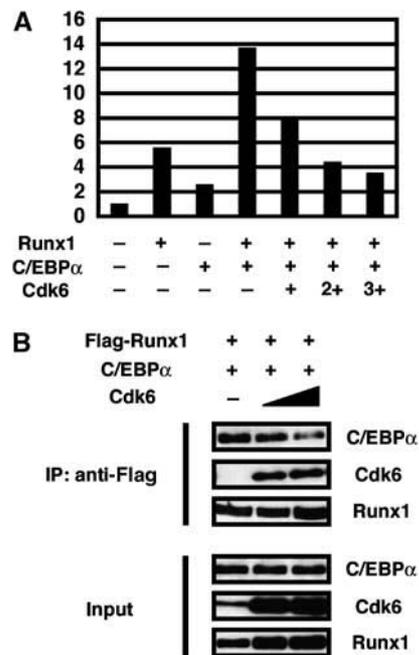


Figure 5 Cdk6 blocks transcriptional synergy of Runx1 with C/EBP α . (A) NIH3T3 cells were cotransfected with 200 ng of pM-CSFR-luc, 1 ng of pRL-TK, 80 ng of pEF-Runx1, 50 ng pEF-CBFB β (lanes 2, 4–7), 100 ng of pcDNA3-C/EBP α (lanes 4–7) and 50 ng (lanes 5), 100 ng (lanes 6) or 300 ng (lanes 7) of pCMV-cdk6 in a well of 12-well plates. The (–) sign indicates that an empty expression vehicle has been added instead of the corresponding expression plasmid. The means of two independent transfections are shown. (F) 293T cells were cotransfected with 1 μ g of pcDNA3-Flag-Runx1, 1 μ g of pcDNA3-C/EBP α and 1 μ g (lane 2) or 2 μ g (lane 3) of pCMV-Cdk6 in a 10 cm plate. Cell lysates of the transfected cells were immunoprecipitated with anti-Flag monoclonal antibody. Immunoprecipitates were analyzed by immunoblotting for the indicated proteins.

tracts, we observed strong inhibition by Cdk6, but only a mild effect of Cdk4 (Figure 6C, left panel). Also, knCdk6 inhibited endogenous Runx1 DNA binding (Figure 6C, right panel). In no case was the level of endogenous Runx1 affected.

These data were consistent with inhibition of Runx1 activity underlying the ability of Cdk6 to block progression granulocytic differentiation in a manner independent of its kinase activity. However, the possibility remained that the effects of Cdk6 were independent of Runx1 inhibition. To determine whether restoring Runx1 function would counteract the Cdk6-induced differentiation block, we introduced ectopically expressed Runx1 into Cdk6-expressing LG cells. This was able to partially reverse the differentiation block (Figure 6D), showing that Runx1 is indeed a critical target for Cdk6. These experiments involved introducing exogenous Cdk6 into differentiating cells. To determine whether endogenous Cdk6 regulated the progression from myeloblast to segmented granulocyte, we performed siRNA knockdown of Cdk6 in LG cells. Two independent siRNAs directed against Cdk6 mRNA were introduced into LG cells using retroviral transduction: α Cdk6.1 (which downregulated Cdk6 protein to undetectable levels) and α Cdk6.2 (which resulted in significant downregulation, but with residual Cdk6 still detectable). In both cases, Cdk4 expression was unaffected (Figure 7A). When switched from IL-3 to G-CSF, Cdk6-knockdown cells proceeded more rapidly towards morphological differentiation (Figure 7B and data not shown) and more cells upregu-

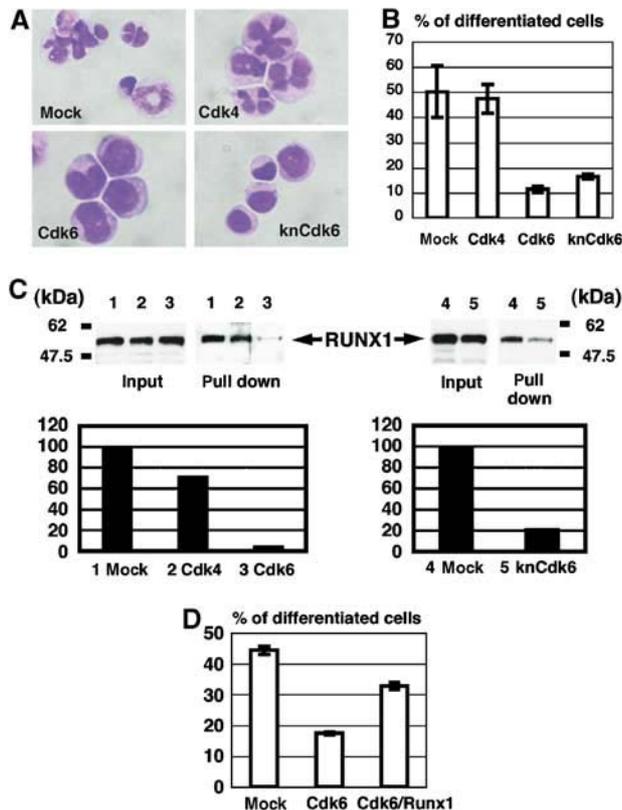


Figure 6 Cdk6 inhibits terminal granulopoiesis and Runx1 DNA binding. Control, Cdk4-, Cdk6- and knCdk6-transduced LG cells were cultured with G-CSF. (A) Cytopins were prepared from the culture at day 4, and stained with Giemsa solution. (B) Two hundred cells were counted per sample. An average proportion of segmented granulocytes from three independent studies are shown. (C) Nuclear extracts from the stable LG cells in (A) precipitated with biotinylated Runx-binding element oligonucleotides followed by analysis of these complexes using anti-Runx1 polyclonal antibody. Relative expression levels of DNA-binding Runx1 are indicated on the right. The relative expression level of control LG cells is designed 100%. (D) Cdk6-expressing LG cells in (A) were transduced with Runx1 and selected with Puromycin (1 μ g/ml) for 7 days. Subsequently, the selected cells were cultured with G-CSF, and the proportions of segmented granulocytes were monitored by Giemsa staining as shown in (A) and (B). Expression levels of Cdk6 and knCdk6 in stable LG cells were shown in Supplementary Figure S4.

lated Gr-1 (a marker for differentiated granulocytic cells; Figure 7C), demonstrating accelerated granulocyte differentiation in the absence of Cdk6. The α Cdk6.1 siRNA was most efficient in promoting accelerated differentiation, as would be expected from its capacity to more efficiently deplete cells of Cdk6 protein, but for both siRNAs the effect on differentiation was highly significant.

Cdk6 inhibits differentiation and enhances proliferation of normal myeloid progenitors

During granulocytic differentiation of bone marrow (BM), myeloblasts proliferation of myeloblasts and promyelocytes was observed, and it ceases during subsequent differentiation stages. To determine whether Cdk6 affected granulopoiesis *in vivo*, and whether this involved changing the balance between proliferation and differentiation, we carried out two experiments. First, we transduced Lin⁻Sca-1⁺c-Kit⁺ (LSK) BM cells of the CD45.2 allotype *in vitro* with pMSCV-based

expression vectors coexpressing Cdk6 and EGFP (Cdk6 vector) or expressing EGFP alone (control vector). These cells were transplanted back into irradiated CD45.1 recipients along with CD45.2 competitor BM. We observed no difference between Cdk6 and control transduced LSK cells with regards to repopulation of the hematopoietic system or lineage distribution (data not shown). Next, we sorted Cdk6 and control transduced CD45.2⁺EGFP⁺ cells from these mice and examined their lineage distribution in the BM. The distribution of granulocytic, eosinophil, erythroid, lymphoid and monocytic cells was similar in Cdk6 and control transduced populations (Figure 7D). However, when assessing granulocytic differentiation stages, we observed an increase of immature forms and decrease of mature segmented forms (Figure 7E), indicating that Cdk6 induces a left-shift in granulocytic differentiation during steady-state hematopoiesis *in vivo*. Finally, to investigate whether in normal BM myeloid cells Cdk6 downregulation in this way initiates the switch from the proliferative progenitor state to initiation of terminal differentiation, we isolated Lin⁻ cells from mouse BM and transduced them with the same MSCV-based retrovira expressing Cdk6 or Cdk4, or an EGFP-expressing control virus. EGFP⁺-transduced cells were isolated by cell sorting and serially replated in methylcellulose culture. After the third replating, no colony-forming cells (CFCs) were observed in the control cultures, whereas the level of CFCs was maintained in Cdk6- and knCdk6-transduced cultures; no effect was observed by transduction of Cdk4 (Figure 7F). These results showed that Cdk6 expression is sufficient to inhibit differentiation of BM granulocytic cells and enhance the limited proliferative capacity of primary BM myeloid progenitors.

Discussion

From the results presented we can arrive at two main conclusions. First, Cdk6-mediated inhibition of Runx1 constitutes a noncanonical kinase-independent Cdk function that is not shared with Cdk4. Secondly, Cdk6-Runx1 interaction combined with specific downregulation of Cdk6 in differentiating granulocytic cells provides a mechanism by which the activity of Runx1 in proliferating and differentiating cells may be differentially regulated, since Cdk6 blocks the association with differentiation-specific promoters.

Runx1-Cdk6 interaction inhibits Runx1 functions

We find that Cdk6 interacts with the Runx1 transcription factor in an interaction that requires the highly conserved Runx1 runt domain, and that this interaction leads to inhibition of Runx1 DNA binding, Runx1-mediated transcriptional activation. In addition, the direct interaction between Runx1 and C/EBP α was disrupted by Runx1-Cdk6 interaction, leading to loss of synergistic promoter activation. Cdk6 therefore regulates both Runx1 protein-DNA and protein-protein interactions. In contrast, no effect of Cdk6 on the ability of PU.1 or C/EBP α to activate target promoters was observed. Importantly, Cdk6-Runx1 interaction could be observed between endogenous Cdk6 and Runx1 proteins in myeloid progenitor cells (K562), and Cdk6 was found to modulate the DNA binding of Runx1 isolated from nuclei of Cdk6-expressing cells, demonstrating that the interaction is present

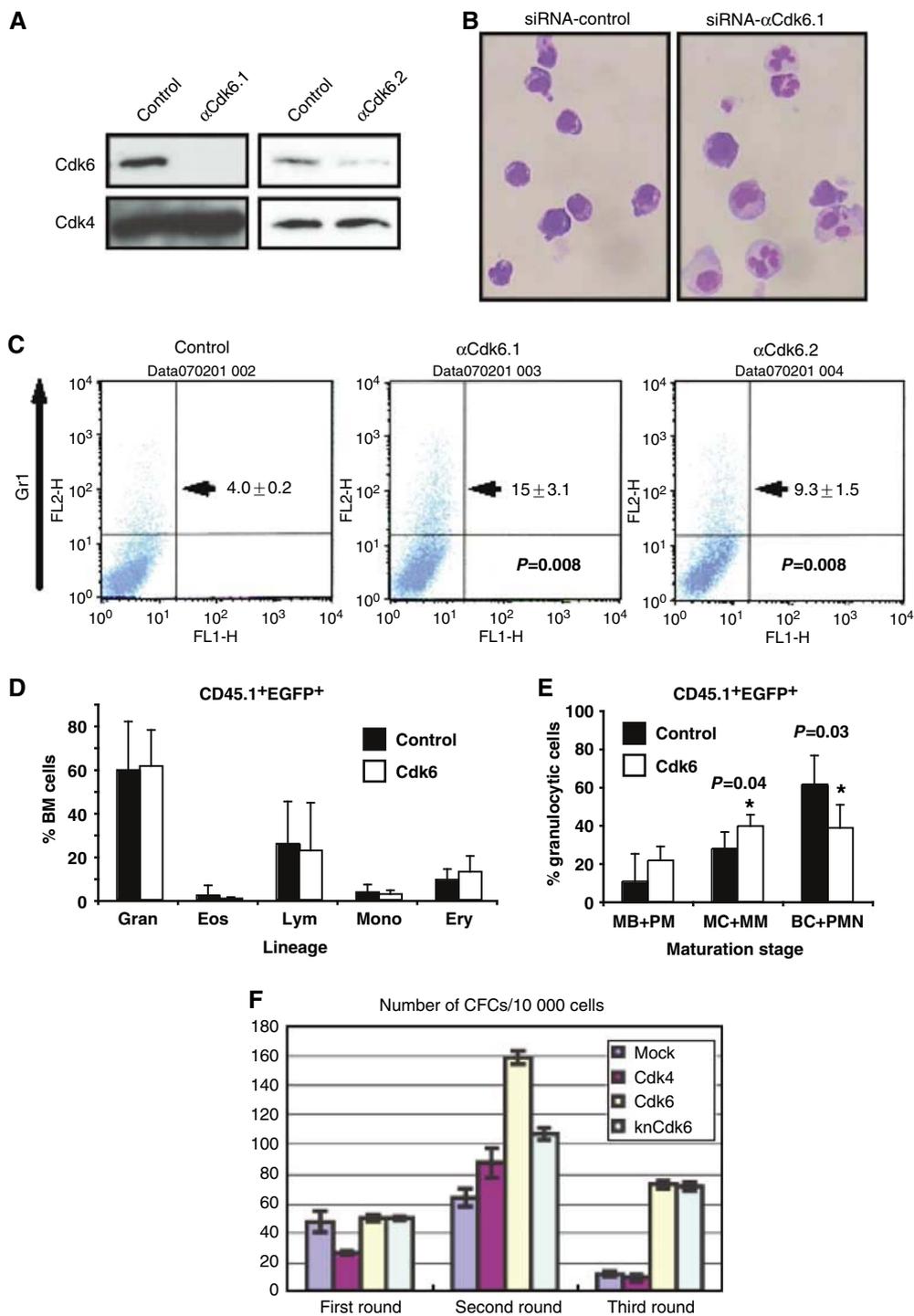


Figure 7 Cdk6 knockdown promotes granulopoiesis, and Cdk6 overexpression inhibits differentiation and enhances expansion of normal myeloid progenitors. **(A)** Western blot showing knockdown of Cdk6 expression in LG cells expressing a stable vector coding for a siRNA sequence against mouse Cdk6 and corresponding Cdk4 protein expression. **(B)** Giemsa staining of control and Cdk6-silenced LG cells after 1 day of G-CSF-induced differentiation. **(C)** Expression of granulocyte-specific (Gr-1) antigen was monitored by flow cytometry after 1 day of G-CSF-induced differentiation. The numbers indicate the average (\pm s.e.m.) percentage of Gr1⁺ populations ($n=3$). **(D)** Lineage distribution of CD45.1⁺EGFP⁺ cells sorted from mice transplanted with control and Cdk6-transduced LSK cells. Number of granulocytic cells (Gran), eosinophils (Eos), Lymphoid (Lym), monocytic (Mono) and nucleated erythroid (Ery) cells as % of total BM cells as indicated; error bars show standard deviation. **(E)** Distribution of CD45.2⁺EGFP⁺ cells on granulocytic maturation stages as % of total granulocytic cells. MB + PM: myeloblasts/promyelocytes; MC + MM: myelocytes + metamyelocytes; BC + PMN: band cells and polymorphonuclear neutrophils. Error bars show standard deviation. **(F)** 1×10^4 transduced Lin⁻ cells were plated in M3434 methylcellulose medium. Bulk cultures were harvested after 7–10 days in culture, and 1×10^4 cells were replated for each sample. Error bars show standard deviations.

and functionally relevant in Cdk6-expressing cells. Finally, the finding that ectopic Runx1 could reverse the Cdk6 differentiation block provides evidence that Runx1 is indeed a

relevant target for the observed effects of Cdk6. Cdk4 inhibited Runx1 weakly in transient transfection assays. However, we were unable to detect an interaction between endo-

genous Cdk4 and Runx1 in K562 cells, and DNA binding of Runx1 isolated from LG cells overexpressing Cdk4 was only weakly inhibited, indicating that only Cdk6 has sufficient affinity for Runx1 to affect its biological activity in a cellular context.

Cdk6-mediated inhibition of Runx1 did not require Cdk6 kinase activity: neither mutation of Cdk6, disabling its kinase activity, or of Cyclin D3, rendering it incapable of Cdk activation, prevented Runx1 inhibition by Cdk6. Indeed, in the absence of cotransfected Cyclin D3, Cdk6 was still capable of inhibiting Runx1 DNA binding. Interaction with or activation by a D-type cyclin therefore does not appear to be instrumental for the capacity of Cdk6 to inhibit Runx1, nor does Cyclin D3 interfere with this Cdk6 function.

Factors that specifically interact with Cdk4 or Cdk6 have previously been described. Sei-1 binds to Cdk4–Cyclin D1 complexes and prevents the association of the Cdk inhibitor p16^{INK4a} (Sugimoto *et al*, 1999). Fbxo7 associates specifically with Cdk6 to promote Cdk6–Cyclin D complex formation and cellular transformation (Laman *et al*, 2005). Both factors thus appear to enhance the canonical Cdk kinase function. The interaction between Cdk6 and Runx1 differ from these examples as here the Cdk acts as a modifier of the function of the interacting protein, and does so in a kinase-independent manner. This is reminiscent of the ability of Cdk4 to inhibit myogenesis by blocking MyoD function, also in a kinase-independent manner (Zhang *et al*, 1999). It is not clear whether this function is shared with Cdk6. It will be of interest to explore if other ‘out-of-the-box’ Cdk functions exist that participate in the coordination of cell proliferation and differentiation.

Cdk6 blocks granulocytic differentiation

The inhibition of Runx1-C/EBP α interaction may be of particular relevance to granulocytic differentiation, which is absolutely dependent on C/EBP α *in vivo* (Zhang *et al*, 1997). Cdk6 was downregulated in differentiating granulocytic cells, and the cellular effects of ectopic Cdk6 expression was inhibition of terminal granulocytic differentiation, decreased expression of differentiation-specific genes and increased proliferation of myeloid progenitor cells, indicating prolonged maintenance of cells in the proliferative myeloblast/promyelocyte compartment. Also in this case, the effect was specific to Cdk6 and not observed with Cdk4, and was independent of Cdk6 kinase activity.

Our data therefore provide evidence for a novel, noncanonical function for Cdk6, which is to block the ability of Runx proteins to participate in the execution of terminal differentiation programs, thereby maintaining progenitor proliferation and ultimately producing sufficient numbers of differentiated progeny. This notion is supported by the accelerated granulocytic differentiation seen upon siRNA-mediated knockdown of Cdk6, as well as the finding that ectopic expression of Cdk6 during steady-state hematopoiesis *in vivo* resulted in a left-shift of the granulocytic compartment, but no effect on HSC repopulation or formation of progenitors (K Anderson, C Nerlov and SEW Jacobsen, unpublished data), where endogenous Cdk6 is already present. Finally, loss of Cdk6 (but not of Cdk4) in the mouse has been observed to affect the production of terminally differentiated myeloid and erythroid cells, consistent with a specific func-

tion for Cdk6 in controlling terminal hematopoietic differentiation processes (Malumbres *et al*, 2004). These results suggest that accelerated differentiation in the absence of Cdk6, as observed in the knockdown experiments, under steady-state *in vivo* conditions result in fewer mature cells produced from each progenitor, and therefore reduced levels of myeloid cells. In contrast, constitutive Cdk6 expression impaired granulopoiesis *in vivo* due to accumulation of immature cells. The correct temporal regulation of Cdk6 expression is therefore critical to controlling the output of granulocytes.

Is Runx–Cdk6 interaction of general relevance?

Genetic evidence exists that Runx proteins are important for the differentiation of several cellular lineages. Thus, loss of Runx2 function leads to impairment of osteoblast differentiation (Komori and Kishimoto, 1998), and Runx3 plays an important role in the differentiation of TrkC-expressing DRG neurons (Inoue *et al*, 2002; Levanon *et al*, 2002). Runx1 is essential for the formation of the definitive hematopoietic system, as well as the differentiation of lymphoid cells and platelets. In the myeloid lineage, Runx1 is not required for terminal differentiation (Ichikawa *et al*, 2004). However, since transgenic rescue of the CBF β knockout in hematopoietic progenitors, but not in myeloid cells, leads to defective mono- and granulopoiesis (Miller *et al*, 2002), this is most likely due to compensation by Runx3 in myeloid cell types. While the Cdk6–Runx interaction may therefore be relevant to the regulated differentiation of multiple cell types, the available evidence suggests that it may be particularly important in osteoblasts and granulocytes. BMP-2-induced differentiation of the osteogenic MC3T3-E1 cell line involved the Smad-mediated downregulation of Cdk6, which, if ectopically expressed, inhibited terminal osteoblast differentiation (Ogasawara *et al*, 2004). This is accompanied by loss of Runx2 binding to the promoter of the gene encoding osteocalcin, a terminal differentiation marker. Runx2 is coexpressed with C/EBP β in osteoblasts, and these factors synergize in osteocalcin promoter activation (Gutierrez *et al*, 2002), a scenario analogous to that provided by Runx1 and C/EBP α in granulocytes. This is supported by the observation that overexpression of a transcriptionally inactive C/EBP β isoform inhibits bone differentiation and osteocalcin expression in transgenic mice (Harrison *et al*, 2005).

Finally, our results provide an explanation for the paradoxical ability of Runx proteins to act as both tumor suppressors and as oncoproteins: even though loss of Runx function in example acute myeloid leukemia (by mutation or translocation) may contribute to the characteristic differentiation block due to its requirement in the terminal differentiation program, in the presence of Cdk6 the differentiation function is impaired, and Runx-mediated activation of expression of cell cycle regulators (e.g. Cdks and/or cyclins) in collaboration with c-Myc may be predominant. Indeed, we find that in undifferentiated 32D myeloid progenitors, where Cdk6 is expressed, ectopic Runx1 increases Cdk4 and Cyclin D2 mRNA levels without promoting terminal differentiation (T Fujimoto and C Nerlov, unpublished data). As both these genes are known Runx1 targets, this indicates that Runx1 cell-cycle-promoting functions in progenitor cells are not impaired by the presence of Cdk6.

Materials and methods

Antibodies

Antibodies (Pharmingen, San Diego, CA) used for cell surface staining were E13-161.7 (Sca-1), 2B8 (c-Kit), RA3-6B2 (B220), RM4-5 (CD4), 53-6 (CD8), A7R34 (IL-7R α), M1/70 (CD11b, Mac-1), RB6-8C5 (Gr-1), A20 (CD45.1), 104 (CD45.2); streptavidin-TxR (Texas red) (Caltag, Burlingame, CA) was used to visualize biotin-conjugated primary antibodies.

Cell culture and transfections

32Dcl.3 and LG myeloblasts were grown in IMDM and RPMI1640, respectively, with 10% fetal bovine serum (FBS) and 1 ng/ml mouse IL-3 (Sigma). K562 cells were grown in RPMI1640 with 10% FBS. NIH3T3 fibroblasts, 293T cells and PLAT-E cells were grown in DMEM with 10% FBS. Cells were transfected using Fugene 6 (Roche). 32Dcl.3 cells and K562 cells were stably transfected by electroporation with a linearized pCMV-cdk6HA plasmid. Stable LG cells were generated by retroviral transduction. The cells were selected by growth in 800 μ g/ml (32D and LG cells) or 400 μ g/ml (K562 cells) of G418 for 10 days. Subsequently, bulk populations were subjected to differentiation induction or ChIP analysis. For the granulocytic differentiation of 32Dcl.3 and LG cells, cells were cultured with 30 ng/ml of G-CSF (R&D).

Isolation of fetal liver hematopoietic cells

Fetal liver cells were sorted essentially as described (Osawa *et al*, 1996).

RT-PCR analysis

Semiquantitative RT-PCR was carried out as described (Fujimoto *et al*, 2001) with *Taq* DNA polymerase (Promega). Primer sequences and amplification conditions are available from the authors on request.

Promoter assays and plasmids

pCMV-cdk4HA, pCMV-cdk6HA and pCMV-knCdk6HA (D163N mutant) constructs were obtained from Dr Ed Harlow and Dr Sander Van den Heuvel. pRcCMV-Cyclin D3 was obtained from Phil Hinds. pEF-Runx1 and pEF-CBF β constructs were obtained from Dr Motomi Osato. pVPI6, pAML1-VP16 and p(CBF)4TK-Luc constructs were obtained from Dr Alan Friedman. pM-CSFR-luc was obtained from Dr Dong Er-Zhang. Mouse Runx1 cDNA and PCR-generated deletion constructs were cloned into pFLAG-CMV-6c (Sigma). Luciferase activity-based promoter assays were performed in triplicate, using the Dual-Luciferase Reporter Assay System (Promega).

Immunoprecipitation and Western blotting

At 48 h after transfection, whole-cell lysates or K562 cell nuclear extracts were incubated with appropriate antibodies and bound to protein A-Sepharose beads (Amersham) for 5–6 h on ice in HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 mM DTT and 0.5% of NP-40). Protein complexes were separated on a SDS-polyacrylamide gel, and transferred to PVDF membranes (Hybond-P; Amersham). Proteins were detected using ECL (Amersham). M2 anti-Flag mAb (Sigma) was used for immunoprecipitation and for the detection of Flag-tagged proteins. N/A anti-Runx1 polyclonal antibody (Active Motif) was used for the immunoprecipitation of endogenous Runx1 protein. HA-tagged proteins were detected using HA-7 anti-HA monoclonal antibody (Sigma). Cdk6 and CBF β were detected using C-21 anti-cdk6 polyclonal antibody (sc-177; Santa Cruz) and E-20 anti-PEBP2 β polyclonal antibody (sc-17181; Santa Cruz), respectively.

Biotinylated DNA and ChIP

Biotinylated oligonucleotide precipitation was performed as described (Hata *et al*, 2000) in 293T cells. The sequences of the biotinylated probe was: 5'-GATCTAACAGGATGTGTTGACATTTA-3'. ChIP was performed as described (Luo *et al*, 1998) in LG cells with 2 μ g of anti-Runx1 polyclonal antibody (H65; Santa Cruz). Promoter sequences were detected by PCR. The following primers were used: Csf1r FIRE region: 5'-GAGGCTGTGAATCAGTTCTCAC-3', 5'-TCGCTTCTCTGAGCCTGCTG-3'; Mpo promoter: 5'-CCATCTTTAACCTGAACCTTCC-3', 5'-GCAACTTCTCTCTCTCTCCA-3'. Cycling parameters were 94°C/2 min, 34 cycles at 94°C/30 s, 58°C/30 s and 72°C/30 s.

siRNA knockdown

Anti-mouse Cdk6 siRNA was kindly provided by Dr Peter Sicinski. siRNA sequences were cloned into pMKO.1 retroviral vectors. For control, we used empty pMKO.1 retrovirus. LG cells were infected with pMKO.1 retroviruses and selected with puromycin (1 μ g/ml) for 4 days. Subsequently, cells were cultured with G-CSF (30 ng/ml) for 24 h. Differentiation of granulocytes was monitored by Giemsa staining and flow cytometry.

Analysis of transduced Lin⁻ BM cells

Lin⁻ cells were purified from BM cells of 2-month-old C57BL/6 mice. Low-density cells were isolated on Histopaque 1086 (Sigma) and stained with biotinylated anti-Gr-1, Mac-1, B220, CD4, CD8 and Ter119 monoclonal antibodies (Pharmingen). Lin⁺ cells were depleted with streptavidin-magnetic beads (M-450; Dynal Biotech). The human *cdk4* and *cdk6* cDNAs were subcloned into a site upstream of an IRES-EGFP construct in pMSCV-IRES-EGFP. Recombinant retrovira were produced by transient transfection of PLAT-E cells. Supernatants were concentrated 100 \times by centrifugation at (18 000 g/1 h). 5 \times 10⁵ Lin⁻ cells were incubated in IMDM, 10% FBS, 100 ng/ml mSCF, 10 ng/ml mL-3 and 10 ng/ml mL-6 (Sigma) for 24 h before transduction by centrifugation (990 g/90 min at 25°C) in the presence of protamine sulfate (4 μ g/ml; Sigma). The cells were incubated in the presence of SCF, IL-3 and IL-6 overnight at 37°C in 5% CO₂ and re-transduced. The following day transduced GFP⁺ cells were FACS purified and plated in methylcellulose medium (M3434; Stem Cell Technologies). Colonies were counted at day 7. To analyze the replating efficiency, bulk populations of colonies were harvested 7–10 days after plating. Cells were then replated in M3434.

Retroviral transduction of LSK cells and BM transplantation

C57BL/6 mice (CD45.2; Jackson Laboratories) were used as BM donors for purification of Lin⁻Sca-1⁺c-Kit⁺ (LSK) cells as previously described (Adolfsson *et al*, 2001). LSK cells were prestimulated in X-Vivo15 containing 1% BSA (both from Stem Cell Technologies), 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin (hereafter serum-free medium (SFM)) and supplemented with SCF (50 ng/ml; generously provided by Amgen), IL-3 (25 ng/ml; Immunex), IL-6 (50 ng/ml; Genetics Institute), FL (50 ng/ml; Immunex) and THPO (100 ng/ml; Genentech) for 48 h and subsequently transduced on retroviral (Takara Bio Inc.)-coated and virus (control or Cdk6 expressing) preloaded non-tissue culture treated 96-well plates in SFM with the same cytokine combination as above for another 48 h. Competitive reconstitution of lethally irradiated CD45.1 recipients was performed as previously described (Bryder *et al*, 2001), using 6000 transduced CD45.2 LSK cells, and 200,000 CD45.1 BM competitor cells. Peripheral blood multilineage reconstitution levels were analyzed by staining for CD45.2, B-cell (B220), T-cell (CD4 and CD8) and myeloid (Mac-1) cell surface antigens.

Cell morphology

10⁴ sorted CD45.1⁺GFP⁺ cells from the BM of killed mice previously transplanted with either control or Cdk6-transduced LSK cells were centrifuged onto glass slides, fixed and stained for 5 min in May-Grünwald stain, 20 min in Giemsa stain (Histolab, Gothenburg, Sweden), thereafter washed, dried and finally analyzed for lineage type by microscopy. Percentage of blasts, promyelocytes, myelocytes, metamyelocytes, band and segmented neutrophils, eosinophils, monocytes, erythroblasts, lymphocytes and plasma cells were counted for each individual mouse from control ($n = 5$) and Cdk6 group ($n = 5$).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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