Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1

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Transcription factors are believed to have a dominant role in acute myeloid leukemia (AML). This idea is supported by analysis of gene-knockout mice, which uncovered crucial roles of several transcription factors in normal hematopoiesis[1], and of individuals with leukemia, in whom transcription factors are frequently downregulated or mutated[2]. However, analysis of knockout animals has not shown a direct link between abrogated transcription factors and the pathogenesis of AML. *Sfpi1*, encoding the lineage-specific transcription factor PU.1, is indispensable for normal myeloid and lymphoid development[3,4]. We found that mice carrying hypomorphic *Sfpi1* alleles that reduce PU.1 expression to 20% of normal levels, unlike mice carrying homo- or heterozygous deletions of *Sfpi1*, developed AML. Unlike complete or 50% loss, 80% loss of PU.1 induced a precancerous state characterized by accumulation of an abnormal precursor pool retaining responsiveness to G-CSF with disruption of M- and GM-CSF pathways. Malignant transformation was associated with a high frequency of clonal chromosomal changes. Retroviral restoration of PU.1 expression rescued myeloid differentiation of mutant progenitors and AML blasts. These results suggest that tightly graded reduction, rather than complete loss, of a lineage-indispensable transcription factor can induce AML.

Figure 1 PU.1-knockdown mice have a graded reduction of PU.1 expression. (**a**) Map of *Sfpi1* (encoding PU.1) indicating the location of the URE in DNase I–hypersensitive (HS) Cluster 1. *Sfpi1* exons (filled boxes) and the direction of transcription (horizontal arrow) are symbolized. The targeting strategy is shown below. *FRT* sites are represented by open arrowheads and *lox*P sites by closed arrowheads. The orientations of the *neo* and *tk* cassettes are shown as open arrows. E, *Eco*RV restriction site; S, *Ssp*I restriction site. The location of the external probe A is indicated as a black horizontal bar. (**b**) Southern-blot analysis of *Eco*RV-*Ssp*I-digested mouse tail DNA. The DNA was hybridized with probe A, which recognizes a 9.7-kb wild-type fragment, a 4.7-kb *lox*P-targeted fragment and a 6.8-kb fragment indicating the knockdown allele (kd) that was generated after excision of the URE. (**c**) Northern blot showing downregulated *Sfpi1* mRNA in bone marrow of *Sfpi1*[kd/kd] mice compared to *Sfpi1*^{+/+} littermates. *Gapd* was used as a loading control. (**d**) Quantitative real-time RT-PCR analysis of bone marrow RNA samples showed that *Sfpi1* expression was reduced to 65% of wild-type level in heterozygotes (+/kd) and to 20% in preleukemic PU.1-knockdown (kd/kd) mice. *Sfpi1* expression was also reduced in bone marrow of a PU.1-knockdown mouse with AML. *Sfpi1* transcript numbers are shown as a percentage of 18S transcripts. (**e**) Western-blot analysis showing that PU.1 protein expression was five times lower in PU.1-knockdown mice than in wild-type controls. Total extracts equaling 10^6 cells from bone marrow (lanes 1–3) or fetal liver (lanes 4 and 5) were separated by SDS-PAGE, blotted onto a nitrocellulose membrane and probed with antibodies against PU.1 and actin. Lane 1, wild-type (+/+); lane 2, preleukemic PU.1-knockdown (kd/kd); lane 3, PU.1-knockdown with AML; lane 4, wild-type; lane 5, *Sfpi1* knockout (–/–). Shown on the right is the position of a 40-kDa marker.

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We previously identified a –14-kb upstream regulatory region (URE) in the *Sfpi1* locus[5]. Here we used a *cre-loxP*-based homologous recombination strategy in mice to replace the URE with a neomycin-resistance cassette (**Fig. 1a,b**). Germline deletion of the URE after breeding the conditional mutants to CMV-*cre* deleter mice[6] resulted in viable offspring at expected mendelian frequencies. Up to 8–10 weeks of age, these mice had no differences in size, behavior or reproductive ability compared with wild-type littermates. We found that PU.1 expression in the bone marrow of mice homozygous with respect to the ΔURE*neo* allele (referred to here as PU.1-knockdown mice) was reduced to 20% of wild-type levels (**Fig. 1c–e**). Thus, these mutants are an ideal *in vivo* model to study the consequences of low levels of PU.1.

At 2–3 months of age, PU.1-knockdown mice had more c-kit^{pos} precursor cells (2–2.5 times more) and neutrophils (Gr-1^{pos}) than wild-type littermates (**Fig. 2a,b,d**). The mutants also had moderately enlarged spleens (1.5–2 times bigger). Expansion of the progenitor cells and neutrophils was limited to bone marrow and spleen: peripheral white blood cell counts were normal (average of 11,300 leukocytes per l versus 9,800 leukocytes per l in wild-type littermates, $n = 4$ in each group; **Supplementary Table 1** online). Notably, 33 of 34 PU.1-knockdown mice rapidly became moribund or died at 3–8 months of age (**Fig. 2c**), after developing a fatal, aggressive neoplastic disease that strongly resembled human AML. Macroscopically, leukemic mice developed severe splenomegaly (weights: 660–1,600 mg versus 80–150 mg in wild-type littermates) and hepatomegaly (weights: 3.0–7.2 g versus 1–1.5 g in wild-type littermates). Flow cytometric examinations of moribund mutants showed a vast accumulation of immature myeloid cells (c-kit[pos] Mac-1^{low} Gr-1^{low}) in bone marrow and spleens (**Fig. 2a,b**). Both organs had grossly disrupted architectures and hypercellularity due to expansion of myeloblastic cells (30–75% blasts and
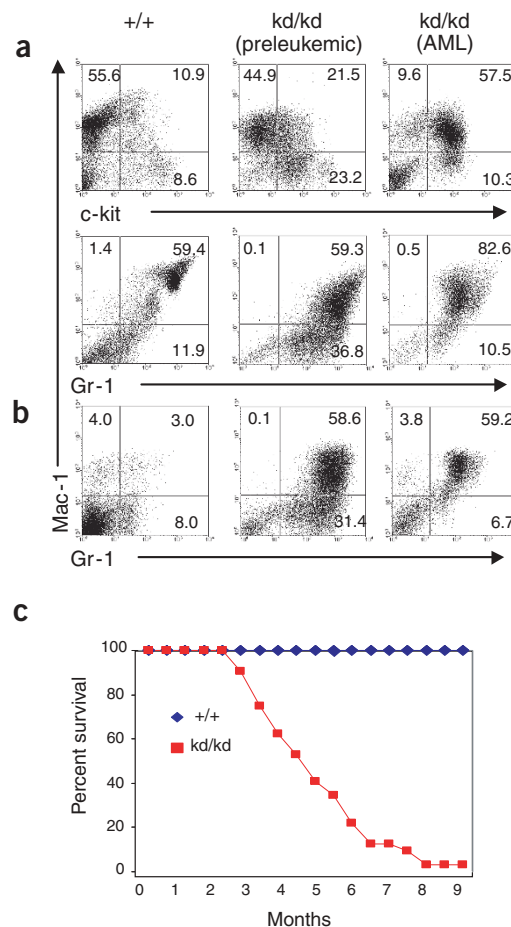
Figure 2 Development of rapidly fatal, transplantable AML. Flow cytometric analysis of single-cell suspensions from bone marrow (**a**) and spleen (**b**) of control and mutant mice before (preleukemic) and after (AML) the development of leukemia using indicated combinations of antibodies. Numbers in quadrants indicate the percentage of total cells. The results show a representative example of 4–6 mice per group. The presence of the c-kit antigen, as an early cell marker, on almost all Mac-1^{pos} Gr-1^{pos} myeloid cells indicates the immature phenotype of the neoplastic PU.1-knockdown (kd/kd) mice. (**c**) Cumulative survival of PU.1-knockdown (kd/kd) mice. $n = 20$ wild-type and 34 PU.1-knockdown mice. Mice were autopsied when visibly ill. No disease was observed in wild-type or heterozygous mice or in any mice before excision of the URE (which had the neomycin cassette incorporated in the locus). (**d**) Cytospin of spleen cells from a 3-month-old preleukemic PU.1-knockdown mouse showing the predominance of mature neutrophils (Wright-Giemsa stain, 400). Cytospins of spleen (**e**) and bone marrow (**f**) cells from a PU.1-knockdown mouse with AML. The accumulation of blasts and myeloid precursors comprised almost the entire primary hematopoietic organs (Wright-Giemsa stain, 400). (**g**) Blood smear showing large numbers of myeloblasts in the peripheral blood of a mutant after development of AML (Wright-Giemsa stain, 400). (**h**) Massive neoblastic infiltration of the liver. Note the accumulation of myeloblasts in the blood vessel in the lower right corner (hematoxylin and eosin, 100). (**i**) Peripheral lymph node densely populated with myeloid cells; most of the tumor cells stain positive for myeloperoxidase (100). (**j**) Invasion of leukemia cells into kidney tissue (hematoxylin and eosin, 200). (**k**) Neoplastic liver infiltration of a secondary recipient mouse after it was transplanted with cells from a leukemic PU.1-knockdown mouse (myeloperoxidase stain, 100). (**l**) Engraftment of transplanted donor cells from leukemic PU.1-knockdown mice in nonirradiated NOD/SCID recipient mice led to complete displacement of host primary hematopoietic tissue. Diagnostic Southern blot of *Eco*RV-*Ssp*I-digested genomic DNA from mouse tails (controls: lane 1, wild-type (+/+); lane 2, heterozygous (+/kd); lane 3, homozygous knockdown (kd/kd)) and various organs from one recipient mouse (lane 4, bone marrow; lane 5, kidney; lane 6, tail; lane 7, spleen) using probe A.
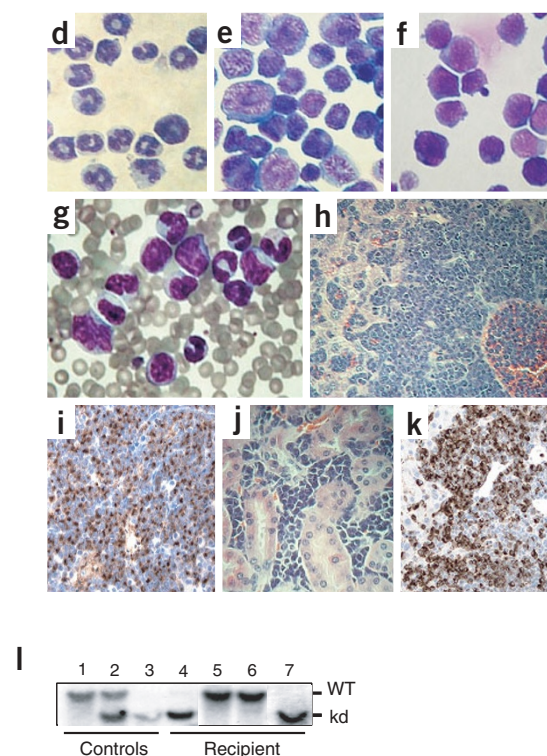
promyelocytes in bone marrow; **Fig. 2e,f**). In contrast, there were fewer B-lymphoid and erythroid cells (data not shown). We detected T-cell lymphomas that developed together with the myeloid leukemia in three PU.1-knockdown mice; in another two mice, lymphoma was the dominant disease (data not shown). In mice with AML, peripheral white-blood-cell counts were much higher (average of 75,200 leukocytes per l, $n = 4$) than in wild-type mice; normal components were replaced by >40% blasts and immature myeloid forms (**Fig. 2g** and **Supplementary Table 1** online). Hematocrits and platelets were within the normal ranges (data not shown). The aggressive malignant disease course was characterized by massive neoplastic organ infiltrations (**Fig. 2h–j**) with most invading cells staining positive for myeloperoxidase, indicative of granulocytic origin of tumor cells (**Fig. 2i,k**).

To further test leukemogenicity, we intravenously injected 10^6 splenocytes from three PU.1-knockdown mice with AML into 12 non-irradiated NOD/SCID recipients. All the mice that received transplants developed progressive leukemia leading to death within 3–12 weeks. They had the same disease symptoms as donors did, including high peripheral white-blood-cell counts, hepatosplenomegaly and myeloblastic invasions of hematopoietic and nonhematopoietic organs (**Fig. 2k** and data not shown). Donor origin of leukemic cells in recipients was confirmed by Southern blots (**Fig. 2l**). We also transplanted the leukemia into normal nonirradiated wild-type mice. Despite decreased engraftment due to histocompatibility barriers, 3 of 12 transplanted noncongenic hosts developed lethal AML with massive progression of donor cells within 6–12 weeks (data not shown).
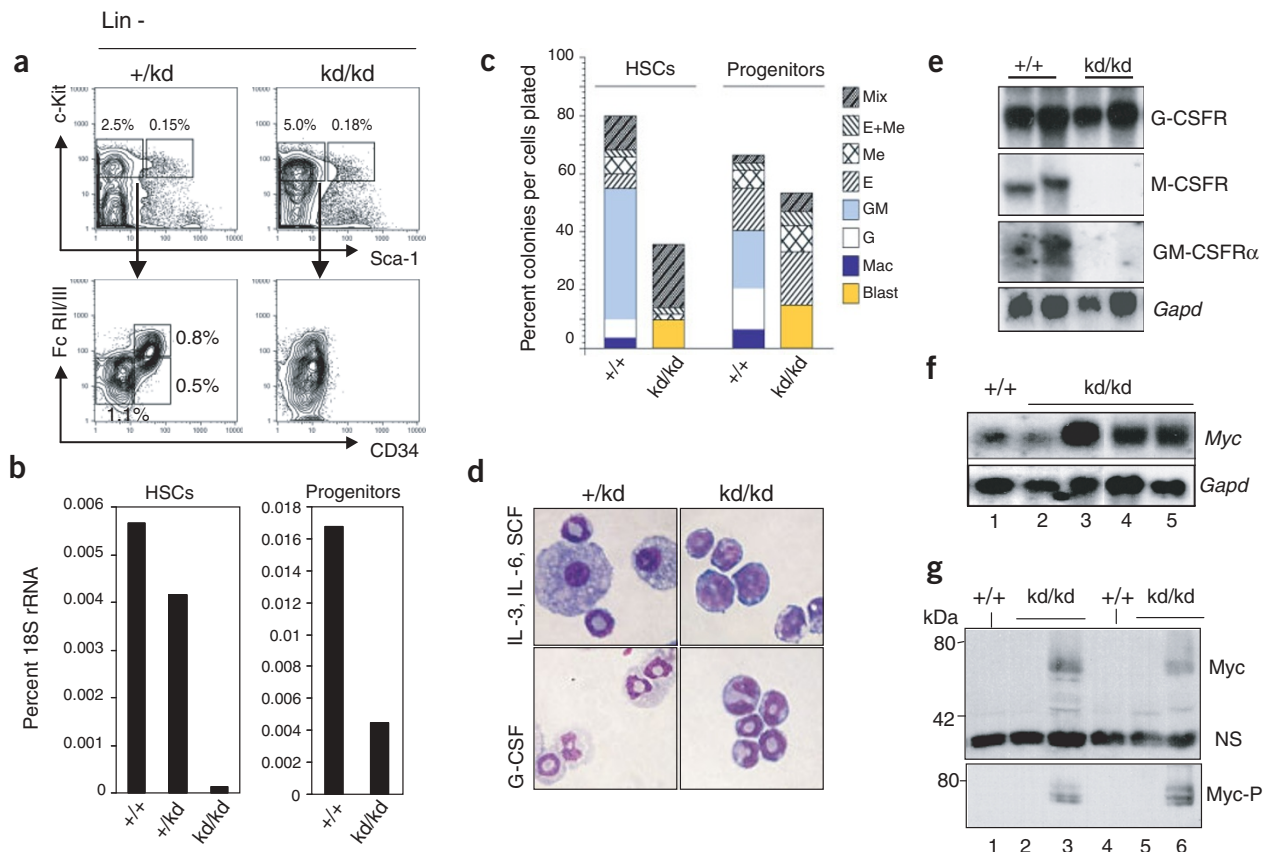
Figure 3 Reduced expression of PU.1 alters cytokine response of HSCs and myeloid progenitors. (**a**) Multiple color flow cytometry showing normal HSCs but accumulated progenitors in bone marrow of preleukemic PU.1-knockdown (kd/kd) mice. c-kit[pos] Sca-1^{pos} lin[neg] HSCs were phenotypically separated from c-kit^{pos} Sca-1^{neg} lin^{neg} progenitor cells, which were further split into common myeloid progenitors (c-kit^{pos} Sca-1^{neg} lin^{neg} FcgRII/III^{neg} CD34^{pos}), myeloid-erythroid progenitors (c-kit^{pos} Sca-1^{neg} lin^{neg} FcgRII/III^{neg} CD34^{neg}) and granulocyte-macrophage progenitors (c-kit^{pos} Sca-1^{neg} lin^{neg} FcgRII/III^{pos} CD34^{pos}). No differences were observed between wild-type and heterozygous (+/kd) mutant mice. (**b**) Multiplex of quantitative real-time RT-PCR using primers and probes specific for *Sfpi1* (encoding PU.1) and 18S rRNA as a control. *Sfpi1* (PU.1) transcript numbers are shown as percent of 18S transcripts in HSCs and progenitors. (**c**) Single cells from sorted HSCs and progenitors from wild-type and preleukemic PU.1-knockdown (kd/kd) bone marrow were cultured in the presence of 20 ng ml^{−1} of SCF, 10 ng ml^{−1} of TPO, 1 U ml^{−1} of EPO, 20 ng ml^{−1} of IL-3, 10 ng ml^{−1} of IL-11 and 10 ng ml^{−1} of GM-CSF. Colonies were counted and were identified by morphology and cytospins. The mixed populations from PU.1-knockdown mice contained normal erythroid and megakaryocytic cells in addition to myeloid blasts. (**d**) Normal myeloid differentiation induced with G-CSF, but not IL-3, IL-6 or SCF. Bone marrow cells from controls or preleukemic mutant mice were plated in methylcellulose containing either a mix of 10 ng ml^{−1} of IL-3, 10 ng ml^{−1} of IL-6 and 50 ng ml^{−1} of SCF or 10 ng ml^{−1} of G-CSF alone for 7 d. Individual colonies were picked and analyzed for cellular morphology. (**e**) Northern blot showing the absence of the mRNAs of the M-CSF receptor and the GM-CSF receptor α-chain, but not of the G-CSF receptor in preleukemic PU.1-knockdown mice. A total of 10 g of total RNA from bone marrow of 2-month-old mice was loaded per sample and probed as indicated. (**f**) *Myc* overexpression is associated with the leukemic phase in PU.1-knockdown mice. Expression levels of *Myc* in bone marrow were assessed by Northern blots. Lane 1, wild-type control; lane 2, preleukemic PU.1-knockdown mutant; lanes 3–5, PU.1-knockdown mice that developed AML. (**g**) Protein expression (upper panel) and phosphorylation (lower panel) of c-Myc in bone marrow (lanes 1–3) and spleen (lanes 4–6) extracts from wild-type (lanes 1 and 4) mice, a preleukemic PU.1-knockdown (kd/kd) mouse (lane 2) and PU.1-knockdown mice with AML (lanes 3, 5 and 6). Myc was overexpressed and phosphorylated (Myc-P) in most (examples in lanes 3 and 6), but not all (lane 5), tumors. NS, a nonspecific band that also served as a loading control.

Table 1 Reduced PU.1 expression alters cytokine response of bone marrow cells *in vitro*

Cytokine	Control	PU.1-knockdown	
		Preleukemic	AML
Medium alone	12.3 ± 4.3[a]	0.8 ± 0.4	313.8 ± 239.4[b]
IL-3, IL-6, SCF	71.1 ± 18.5	73.9 ± 20.8	134.0 ± 60.1
GM-CSF	94.7 ± 11.6	5.8 ± 4.7	245.3 ± 158.7
M-CSF	142.3 ± 12.8	0.75 ± 0.5	ND
G-CSF	27.3 ± 6.2	21.0 ± 3.5	599.5 ± 257.2

[a]Macrophage colonies. [b]Blastic colonies.

Duplicate aliquots of 2×10^4 bone marrow cells per mouse were plated in MethoCult containing 10 ng ml^{-1} of IL-3, 10 ng ml^{-1} of IL-6 and 50 ng ml^{-1} of SCF; 10 ng ml^{-1} of GM-CSF alone; 10 ng ml^{-1} of M-CSF alone; or 10 ng ml^{-1} of G-CSF alone. At least two independent experiments were done, each using 2–4 mice. Values are reported as mean ± s.d. Controls were either wild-type or heterozygous mice. ND, not done.

We found normal numbers of phenotypic hematopoietic stem cells (HSCs; c-kit^{pos} Sca-1^{pos} lin^{neg}) but an expanded progenitor compartment (c-kit^{pos} Sca-1^{neg} lin^{neg}) in preleukemic PU.1-knockdown mutants (**Fig. 3a**). Both HSCs and progenitors from PU.1-knockdown mice had reduced PU.1 expression (**Fig. 3b**). We could not separate mutant progenitors into distinct common myeloid progenitor, myeloid-erythroid progenitor and granulocyte-macrophage progenitor subpopulations[7], suggestive of an abnormal progenitor compartment (**Fig. 3a**). Therefore, we sorted the combined progenitors as well as the HSCs to test differentiation potential in single-cell clonogenic assays. Erythroid and megakaryocytic colonies were normal, but myeloid differentiation was blocked at a blast-like stage under the conditions we used (**Fig. 3c**).

Clonogenic assays using total bone marrow cells showed that mutant cells underwent profound changes in response to myeloid cytokines (**Table 1**). Cells from PU.1-knockdown mice with AML produced large numbers of colonies, even in the absence of any cytokines, further demonstrating their transformed, growth factor–independent nature. Cells from preleukemic PU.1-knockdown mice almost completely lacked M- and GM-CSF response but produced normal colony numbers with G-CSF or a mixture of IL-3, IL-6 and SCF. Differentiation of preleukemic mutant cells in the presence of IL-3, IL-6 and SCF was again blocked at a myeloblast-like stage; G-CSF alone induced generation of neutrophils (**Fig. 3d**), providing an explanation for the enhanced granulopoiesis observed *in vivo*. To examine whether the altered cytokine response of cells from PU.1-knockdown mice could be a consequence of differential growth factor receptor expression, we carried out northern-blot analysis (**Fig. 3e**). Expression of M- and GM-CSF receptors was substantially reduced in PU.1-knockdown

Table 2 Cytogenetic analysis of PU.1-initiated leukemias

Mouse	Tissue	Karyotype
F101	Spleen	41,XX,+15[2]/40,XX[8]
F105*	Bone marrow	40,XX[8]/40,X,der(X)t(X;16)(F3;C2)[1]/40,X,der(X)t(X;4)(F1;C4)[1]
	Spleen	40,XX[10]
F279	Spleen	41,XY,+15[10]
F331	Spleen	39,X,-Y[6]/40,idem,+15[2]/41,XY,+15[2]
F332	Spleen	40,XY,del(3)(BF1)[10]
F333*	Bone marrow	40,XY[10]
F431	Spleen	41,XY,+15[2]/40,XY[8]

*Absence of chromosome 15 trisomy in these samples was confirmed by FISH.

At least ten metaphase cells were analyzed per leukemia.

mice, but G-CSF receptor was expressed normally. These findings were unexpected, because PU.1-knockout mice express markedly less RNA of all three receptors[8] and do not respond to M-, GM- or G-CSF[9,10]. Haploinsufficient expression of PU.1 in heterozygous *Sfpi1*$^{+/-}$ mice is not sufficient to induce defects in cytokine receptor expression and response[9,10]. Thus, a narrow window of reduced (to ~20%) PU.1 expression seems to create the dosage that selectively preserves specific growth factor signaling and survival while leading to the disruption of the response to other cytokines.

To identify cooperative changes that contribute to leukemogenesis in PU.1-knockdown mice, we carried out spectral karyotyping (SKY) analysis and fluorescence *in situ* hybridization (FISH). We found simple clonal chromosomal abnormalities in five of seven mice with AML (**Table 2**). A gain of chromosome 15 was observed in four cases (**Supplementary Figs. 1** and **2** and **Supplementary Table 2** online), and one case showed an interstitial deletion of chromosome 3. Trisomy 15 is the most common abnormality in mouse T-cell lymphomas, as well as some mouse models of myeloid leukemia (reviewed in ref. 11). Trisomy 8 is the most common abnormality in human AML and is homologous to mouse chromosome 15. The smallest region gained is mouse 15D2-3 or human 8q24, containing the *Myc* proto-oncogene, which is often overexpressed in human tumors. Notably, expression and activation of c-Myc was elevated in six of eight PU.1-knockdown mice with AML, suggestive of a functional role for overexpression of c-Myc in PU.1-initiated neoplastic transformation (**Fig. 3f,g**).

To determine whether the reduction in PU.1 expression was directly responsible for the PU.1-knockdown phenotype, we restored PU.1 expression in mutant cells using a bicistronic retrovirus expressing PU.1 and green fluorescent protein (GFP). PU.1 expression by this retroviral construct correlates with GFP expression[12], as confirmed by western blotting (data not shown). We transduced bone marrow from preleukemic PU.1-knockdown mice and separated GFP-positive (high PU.1 expression) from GFP-negative (hypomorphic PU.1 expression) cells. We assessed the myeloid differentiation potential of both fractions in the presence of IL-3, IL-6 and SCF by analyzing cell-surface marker expression (**Fig. 4a**) and cellular morphology (data not shown). The GFP-positive progenitors differentiated into mature macrophages (Gr-1^{neg} Mac-1^{pos}) and neutrophils (Gr-1^{high} Mac-1^{high}), similar to wild-type controls. In contrast, the GFP-negative population gave rise to immature granulocytic cells (Gr-1^{pos} Mac-1$^{neg-low}$).

We next examined whether restoration of PU.1 expression could rescue the response of preleukemic PU.1-knockdown progenitor cells to GM- and M-CSF (**Fig. 4b,c;** shown for GM-CSF conditions). Control cells infected with control or PU.1-expressing retrovirus formed comparable numbers of colonies in GM- or M-CSF, whereas mutant cells produced colonies only after transduction with the retrovirus expressing PU.1 and not with the control virus. Most notably, whereas transduction with control virus of bone marrow from PU.1-knockdown mice with AML led to cells that were arrested at a myeloblastic stage in IL-3, restoration of PU.1 expression in leukemic cells induced dispersed colonies of mature macrophages and granulocytes (**Fig. 4d**). Thus, reintroduction of PU.1 was sufficient to restore normal myeloid differentiation of leukemic PU.1-knockdown cells. These data indicate that downregulated expression of PU.1 is directly responsible for the disorder of the PU.1-knockdown mice and suggest that introduction of PU.1 into blasts of individuals with AML could be a therapeutic option to restore myeloid differentiation.

Our results show that reduced PU.1 expression induces AML in mice with high frequency and short latency. The importance of PU.1 concentrations on lineage fate decisions during normal hematopoiesis has been noted before[12]. Haploinsufficiency of PU.1 triggered granulocyte

Figure 4 PU.1 retroviral rescue of myeloid differentiation in PU.1-knockdown mice. (**a**) Bone marrow cells from preleukemic PU.1-knockdown (kd/kd) mice were transduced by a retrovirus expressing bicistronic PU.1*ires*GFP. Cells were then sorted by fluorescence-activated cell sorting for GFP expression, split into GFP-positive and GFP-negative fractions, cultured in IL-3, IL-6 and SCF for 7 d and analyzed for myeloid cell surface markers by flow cytometry. Wild-type control cells were mock-transduced and kept under same culturing conditions. (**b**) Bone marrow cells from control and preleukemic PU.1-knockdown mice were infected with retroviruses expressing either GFP alone or PU.1*ires*GFP. Clonogenic assays were done in the presence of GM-CSF. Numbers of mutant colonies after PU.1 restoration were comparable to those in control mice. (**c**) PU.1-knockdown colonies transfected with the PU.1 virus were GFP-positive and produced mature macrophages and granulocytes comparable to those of wild-type controls (see **Fig. 3d**). (**d**) Bone marrow of a PU.1-knockdown mouse suffering from AML was infected with the GFP control virus or with the PU.1*ires*GFP virus. GFP-positive cells were sorted by fluorescence-activated cell sorting, plated in methylcellulose containing 10 ng ml^{-1} of IL-3 and analyzed after 7 d. Colony shape is shown in left panels as phase-contrast images, and cellular morphology is shown in right panels by Wright-Giemsa stains. Colony numbers of control and PU.1-infected cells were equal.
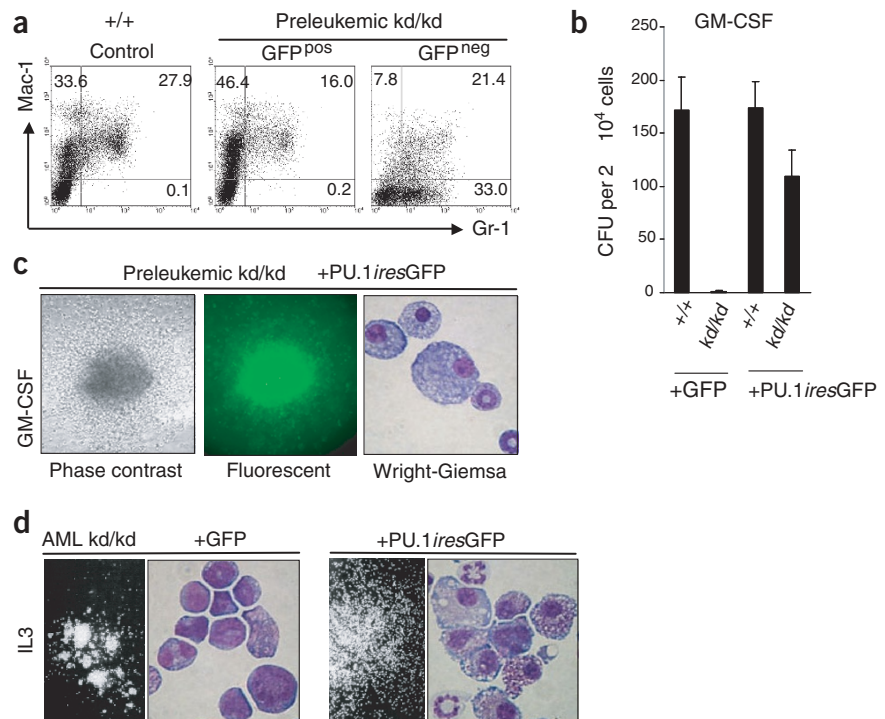
production in a G-CSF-null background[13], in agreement with our observation of accelerated granulopoiesis in the presence of hypomorphic levels of PU.1. Like mice deficient in other transcription factors implicated in human AML[1,14–17], however, neither nonconditional nor conditional deletion of the gene encoding PU.1 induces leukemia[3,4,18]. Because haploinsufficient PU.1 expression also does not induce a spontaneous malignancy[19], a narrow window of reduced PU.1 expression seems to be required for AML development. In addition, humans with AML have mutations in genes encoding transcription factors that lead to reduced function but generally not complete loss of function[2,20–24]. Taken together, these data suggest that retaining residual transcription factor function can be crucial for AML development.

The mechanism behind the development of neoplasia in PU.1-knockdown mice probably includes the failure of the mutant cells to express sufficient levels of essential growth factor receptors. Whereas the complete absence of PU.1 globally abrogates growth factor signaling, however, reduction of PU.1 expression to 20% of normal levels preserves specific cytokine response (*e.g.*, G-CSF and perhaps others) as an essential requirement for cell survival. This may be an important step in the development of a leukemic stem cell[25]. Malignant transformation was accompanied by chromosomal abnormalities with recurring gain of chromosome 15, containing the gene *Myc*. *Myc* is frequently overexpressed in human neoplasia[26] and induces tumorigenesis in mice[27,28]. Thus, reduced expression of PU.1 promotes recurring chromosomal changes, creating a secondary event that results in the subsequent outgrowth of leukemic cell clones.

In conclusion, we showed that graded reduction of physiological PU.1 expression below a critical level can induce AML. Therefore, it might be possible to change cancerous cell fate considerably with relatively small modulations that increase lineage-determining transcription factors above this critical level.

METHODS

Generation of the targeting construct. We subcloned a 3.3-kb *Bam*HI-*Kpn*I genomic fragment containing the proximal part of the –14-kb URE plus the downstream homology into the *Bam*HI-*Kpn*I sites of pPNT-*lox*P*neo*. To add the distal part of the URE, we released a 1.7-kb *Sal*I-*Bam*HI fragment from a pBluescript plasmid containing the entire URE as a 3.4-kb *Hind*III fragment and inserted it into the *Sal*I-*Bam*HI sites of the targeting construct. This causes the original neomycin gene and the proximal *lox*P site to drop out of the construct. We then released a *lox*P site from pPNT-*lox*P*neo* with *Xba*I and *Sma*I and inserted it into the *Nhe*I site at the very 3′ end of the URE by blunt-end ligation. To obtain the upstream homology, we amplified a 2.2-kb fragment by PCR from a genomic mouse *Sfpi1* P1 clone[5] using primers with *Not*I or *Xho*I site overhangs. We then subcloned this fragment into the *Not*I-*Xho*I sites of pBluescript. We released a *FRT*-PGK*neo*-*FRT* resistance cassette from pK-11 (ref. 29) and subcloned it into *Sac*I-*Sal*I sites of pBluescript. After inserting an *Xho*I site adaptor into the *Not*I site of the plasmid, we released *FRT*-PGK*neo*-*FRT* by *Sal*I and *Xho*I and subcloned it into the *Xho*I site of the pBluescript that already contained the upstream homology. We released another *lox*P site from pPNT-*lox*P*neo* using *Sal*I-*Xho*I and inserted it into the *Xho*I site downstream of *FRT*-PGK*neo*-*FRT*. Finally, we subcloned the entire fragment containing the upstream homology, the *FRT*-flanked PGK*neo* and the *lox*P site into *Not*I-*Sal*I sites of pPNT that already contained the URE-*lox*P-downstream homology fragments. This procedure created the final targeting construct with the elements in the following order: 2.2-kb upstream homology, *FRT*-PGK*neo*-*FRT*-*lox*P, 3.4-kb URE-*lox*P, 1.7-kb downstream homology, PGK*HSK* thymidine kinase gene. We linearized this construct with *Not*I and used it to transfect embryonic stem cells.

Generation of mutant mice. We electroporated 30 g of linearized targeting construct into R1 embryonic stem cells and grew them under double selection on feeder layers treated with mitomycin C. We digested DNA of individual double-resistant clones with *Ssp*I and *Eco*RV and probed them with the external probe A and the neomycin cDNA as an internal probe. We identified homologous recombinant clones by a 4.7-kb band representing –14-kb URE^{loxP}+*neo* in

addition to the 9.7-kb wild-type band in Southern blots using probe A. We generated chimeric mice by standard injection procedures, bred chimeric males to C57/Bl6 females and determined germline transmission by agouti color and Southern blots from tail genomic DNA. To remove the URE, we bred conditional mutants to CMV-*cre* deleter mice[6]. We identified heterozygous offspring with the proper deletions by Southern blotting. Incorporation of the neomycin cassette into the *Sfpi1* locus (–14-kb URE^loxP+neo) did not cause changes in expression of PU.1 or any other phenotypic changes before deletion of the URE. Furthermore, Southern-blot assays of cells derived from mice with AML had a pattern similar to that shown in **Figure 1**, indicating that the structure of the URE was not altered after transformation (data not shown). Mouse experiments were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (Protocol # 115-2001).

Northern-blot assays. We isolated RNA from bone marrow cells using Trizol Reagent (Molecular Research Center) according to the manufacturer's protocol. We detected *Sfpi1* (PU.1) RNA with a labeled 431-bp mouse *Sfpi1* 5′ cDNA fragment, *Myc* RNA with a probe containing exons 2 and 3 of the mouse gene and *Gapd* RNA with a 1.3-kb *Pst*I fragment of the rat cDNA. Reduction of PU.1 expression in Northern (**Fig. 1c**) blots was determined using Imagequant densitometry software (Amersham).

Histologic and immunohistologic methods. We carried out hematoxylin and eosin staining or immunohistochemical analysis for myeloperoxidase on 5-µm thick formalin-fixed, paraffin-embedded tissue sections. We removed the paraffin from the slides, pretreated them with 1.0 mM EDTA, pH 8.0 (Zymed), in a steam pressure cooker (Decloaking Chamber, BioCare Medical) following the manufacturer's instructions and washed them in distilled water. All further steps were done at room temperature in a hydrated chamber. We pretreated slides with Peroxidase Block (DAKO USA) for 5 min to quench endogenous peroxidase activity and then with a 1:5 dilution of goat serum in 50 mM Tris-Cl, pH 7.4, for 20 min to block nonspecific binding sites. We applied primary rabbit antibody to myeloperoxidase (DAKO) at a 1:2,500 dilution in 50 mM Tris-Cl, pH 7.4, with 3% goat serum for 1 h. We washed slides in 50 mM Tris-Cl, pH 7.4, and applied goat antibody to rabbit conjugated with horseradish peroxidase (Envision detection kit, DAKO) for 30 min. After further washing, we developed the immunoperoxidase staining using a DAB chromogen kit (DAKO) following the manufacturer's instructions and counterstained with hematoxylin. Blood films and cytospins were fixed with methanol and stained with Wright-Giemsa staining solutions.

Flow cytometry. We analyzed single-cell suspensions from spleens and bone marrow by flow cytometry using unlabeled antibodies or antibodies conjugated to phycoerythrin, fluorescein isothiocyanate, Texas red or allophycocyanin (all from Pharmigen) against the following cell-surface molecules: Mac-1/CD11b (M1/70), CD3 (KT31.1), CD4 (GK1.5), CD8 (53-6.7), B220 (6B2), Gr-1 (8C5), TER119, CD19 (1D3), IgM (R6-60.2), IL-7Ra chain (A7R34), FcRII/IIIg (2.4G2), CD34 (RAM34), Sca-1 (E13-161-7) and c-Kit (2B8). We analyzed total bone marrow and spleen samples on a FACSscan cytometer (Becton Dickinson) according to standard protocols. Gates on viable cells were set according to the exclusion of propidium iodide staining. Flow cytometric analysis and sorting methods for HSCs and myeloid precursors using a double laser (488 nm/350 nm Enterprise II + 647nm Spectrum) high-speed cell sorter (Moflo-MLS, Cytomation) were previously described[7].

Colony-forming assays. We carried out *in vitro* clonogenic assays in Methocult M3234 (Stem Cell Technologies) supplemented with cytokines as indicated. We scored cells at 4–7 d. For myeloid precursor and HSC single-cell clonogenic assays, we used an automatic cell deposition unit system to sort cells into 96-well plates (Becton Dickinson). We cultured these highly purified cell populations in Methocult H4100 (Stem cell Technologies) supplemented with 20% fetal bovine serum, 1% bovine serum albumin, 2 mM L-glutamine and 50 mM 2-mercaptoethanol. We purchased recombinant human IL-6 and mouse M-CSF, G-CSF and GM-CSF from Stem Cell Technologies and mouse SCF, IL-3, IL-11 and TPO and human erythropoietin from R&D Systems.

Western-blot assays. We extracted total cell lysates as described[30]. We resolved proteins by SDS-PAGE and electrotransferred them to a nitrocellulose membrane (Bio-Rad Laboratories). We used polyclonal rabbit antibody to PU.1 (Santa Cruz), polyclonal rabbit antibody to c-Myc (Santa Cruz), polyclonal rabbit antibody to phosphorylated c-Myc (Thr58/Ser62; Cell Signaling) and monoclonal mouse antibody to actin (Sigma). We detected immunoreactive proteins using HPRT-conjugated antibodies to rabbit or mouse (Santa Cruz) and the ECL system (Amersham). Reduction of PU.1 expression in Western blots was determined using Imagequant densitometry software (Amersham).

Real-time PCR. We extracted RNA with Trizol, treated it with DNase I, reverse-transcribed it and then amplified it using an AbiPrism 7700 Sequence Detector (Applied Biosystems) with the following parameters: 48 °C (30 min), 95 °C (10 min) followed by 40 cycles of 95 °C (15 s), 60 °C (1 min). Primer and probe sequences are available on request.

SKY analysis. We carried out cytogenetic and SKY analysis on spleen or bone marrow cells as described previously[11].

Retroviral transduction of mouse bone marrow cells. We transduced bone marrow cells with MIG and MIG-PU.1 retroviruses as described[9]. We cultured cells in IL-3, IL-6 and SCF or IL-3 alone for 2 d and then infected them by resuspension in high-titer cell-free retroviral supernatants for 4 h in the presence of 24 µg ml^−1 of polybrene. We washed infected cells three times in complete medium and then cultured them for 2 d in complete medium containing IL-3, IL-6 and SCF or IL-3 alone to allow retroviral integration and expression. We washed infected cells three times before plating them in methylcellulose to assess colony-forming potential.

Note: Supplementary information is available on the Nature Genetics website.

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