Gain of MYC underlies recurrent trisomy of the MYC chromosome in acute promyelocytic leukemia

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Gain of chromosome 8 is the most common chromosomal gain in human acute myeloid leukemia (AML). It has been hypothesized that gain of the *MYC* protooncogene is of central importance in trisomy 8, but the experimental data to support this are limited and controversial. In a mouse model of promyelocytic leukemia in which the MRP8 promoter drives expression of the *PML–RARA* fusion gene in myeloid cells, a *Myc* allele is gained in approximately two-thirds of cases as a result of trisomy for mouse chromosome 15. We used this model to test the idea that MYC underlies acquisition of trisomy in AML. We used a retroviral vector to drive expression of wild-type, hypermorphic, or hypomorphic MYC in bone marrow that expressed the *PML–RARA* transgene. MYC retroviruses cooperated in myeloid leukemogenesis and suppressed gain of chromosome 15. When the *PML–RARA* transgene was expressed in a *Myc* haploinsufficient background, we observed selection for increased copies of the wild-type *Myc* allele concomitant with leukemic transformation. In addition, we found that human myeloid leukemias with trisomy 8 have increased MYC. These data show that gain of MYC can contribute to the pathogenic effect of the most common trisomy of human AML.

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Abbreviations used: +h8, trisomy of human chromosome 8; +m15, trisomy of mouse chromosome 15; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; MIG, mouse stem cell virus LTR-internal ribosomal entry site-GFP retroviral vector; PML, promyelocytic leukemia; RARα, retinoic acid receptor α; T-ALL, T cell acute lymphoblastic leukemia/lymphoma.

Acute myeloid leukemia (AML) is a disease with diverse genetic pathogenesis. More than 140 recurrent balanced chromosomal aberrations have been described, and the genes located at the chromosomal breakpoints have been identified for many of these aberrations. Additionally, >700 recurrent unbalanced aberrations have been associated with AML, but only a few of the responsible genes have been delineated (Le Beau and Larson, 2000). In the present study, we aimed to address the mechanism by which an unbalanced chromosomal gain might cooperate with the t(15;17) of acute promyelocytic leukemia (PML; APL; a subtype of AML) to accelerate leukemogenesis.

The t(15;17)-balanced chromosomal rearrangement juxtaposes the *PML* gene to the *retinoic acid receptor α (RARA)* gene, creating an

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aberrant PML-RARα fusion protein. PML-RARα inhibits gene expression and disrupts PML nuclear bodies (Hong et al., 1997; Grignani et al., 1998; Guidez et al., 1998; He et al., 1998; Lin et al., 1998). Although APL is associated with the accumulation of undifferentiated myeloid cells, PML-RARα must cooperate with additional genetic lesions to fully block neutrophil maturation and promote leukemia. In APL, secondary karyotypic lesions are seen in 38% of cases, with trisomy 8 being the most common (12% of cases; Le Beau et al., 2002). In fact, trisomy 8 is the most common unbalanced gain in AML in general (Grimwade et al., 1998).

In this study, we used a mouse model of APL in which the MRP8 promoter directs expression of the *PML-RARA* fusion gene in maturing myeloid progenitors, neutrophils, and monocytes. Although PML-RARα expression initially causes modest changes in neutrophil maturation, full progression to an APL-like disease requires additional mutations. We have previously shown that gain of mouse chromosome 15 (+m15) is the most common recurring abnormality (64% of cases) in our *PML-RARA* transgenic mice (Le Beau et al., 2002). This is consistent with the gain of chromosome 8 in human APL because m15 is syntenic to human bands 8q22-24.3. It has been difficult to identify genes that drive +h8/+m15. *MYC/Myc*, a candidate gene located in this region, has been implicated as a protooncogene in a wide array of human and mouse neoplasms and serves as a key regulator of cellular proliferation (Adhikary and Eilers, 2005). Small changes in MYC expression level have been shown to have significant phenotypic effects. For example, there is a correlation between *Myc* expression and growth of mice, where substantial differences in growth are seen with less than twofold difference in gene expression (Trumpp et al., 2001). Because *MYC* is required for normal hematopoietic differentiation (Trumpp et al., 2001; Wilson et al., 2004), gain of an additional allele of *MYC* might have significant effects on myelopoiesis.

It has been speculated that *MYC* contributes to trisomy 8 in AML; however, the importance of *MYC* copy number in AML pathogenesis is controversial. When overexpressed in mice, MYC can initiate the development of myeloid leukemia (Felsher and Bishop, 1999a; Luo et al., 2005); however, *MYC* transcripts were found to be decreased in AMLs with trisomy 8 relative to normal $CD34^+$ bone marrow cells (Virtaneva et al., 2001). Here, we show that *MYC* cooperates with PML-RARα in leukemic transformation and is an important driver of +15 in our APL mouse model. These data indicate a role for *MYC* gain in human myeloid neoplasia with trisomy 8.

RESULTS

MYC cooperates with PML-RARα to generate myeloid leukemia

We hypothesized that MYC is an important driver of chromosomal gain in APL and that it cooperates with PML-RARα to accelerate the development of leukemia. To assess this cooperativity, we transduced bone marrow cells from *PML-RARA* transgenic mice with MYC retrovirus (MSCV-MYC-IRES-GFP) and transplanted them into lethally irradiated histocompatible mice (resulting animals are referred to as PML-RARα + MYC mice). In parallel, we established control cohorts in which *PML-RARA* bone marrow was transduced with an empty mouse stem cell virus LTR-internal ribosomal entry site-GFP retroviral vector (MIG; MSCV-IRES-GFP) retrovirus (PML-RARα + MIG mice) and control FVB/n marrow was transduced with MYC retrovirus (control + MYC mice). Mice reconstituted with normal marrow cells transduced to express MYC became ill in a median of 90 d (Fig. 1 A). These control + MYC mice developed lymphoblastic disease, which presented as lymphomas involving the thymus with variable involvement of other tissues in seven mice and lymphomas of the orbit (site of injection of transplanted cells) in two mice. Representative pathology and an example of surface antigen expression are shown (Fig. 1, B and C). The disease was characterized by expression of T cell antigens, including CD90, CD3, variable CD4, and variable CD8. The blasts lacked cytoplasmic granules and were present in the thymus and other tissues. The time to illness appeared to be similar for recipients of PML-RARα + MYC cells (median time to illness 76 d, not statistically significantly decreased; Fig. 1 A), but the spectrum of disease was markedly different from that observed in the absence of PML-RARα. In contrast to the findings with MYC alone, eight of nine PML-RARα + MYC animals developed AML with numerous promyelocytes (hereafter referred to as APL). An example of surface antigen expression and representative pathology are shown (Fig. 1, C and D). The cells expressed variable levels of the Gr-1 and Mac-1 myeloid antigens and the immature markers CD117 (Kit) and CD34. Leukemic blasts frequently contained numerous primary granules, and there was uniform marked expansion of the spleen and infiltration of the liver. These leukemias were similar to those we have previously observed in *PML-RARA* mice (Brown et al., 1997). One recipient animal in this PML-RARα + MYC group developed lymphoblastic disease, and in one recipient a thymic lymphoblastic lymphoma was present concomitant with APL. Some recipients of *PML-RARA* bone marrow transduced with MIG became ill with long latency (median time to illness 274 d; Fig. 1 A), with findings similar to those previously observed in the absence of retroviral transduction (Brown et al., 1997). These results demonstrate that MYC accelerates the development of APL in *PML-RARA* mice.

MYC interacts with PML-RARα to impair myeloid maturation

To investigate the impact of the combination of PML-RARα and MYC on myelopoiesis, we reconstituted mice with control or *PML-RARA* transgenic bone marrow that had been transduced with control (MIG) or MYC retroviral vectors. 5 wk after transplantation, the cohorts were euthanized, tissues were collected for histopathology, and GFP^+ bone

marrow cells were analyzed by flow cytometric immunophenotyping and sorted for morphological examination. As compared with the other three groups, the combination of PML-RARα and MYC strongly inhibited the morphological maturation of myeloid cells (Fig. 2, A and B). The MRP8 *PML-RARA* transgene had been previously shown to decrease the expression of the Gr-1 myeloid differentiation antigen in preleukemic mice (Brown et al., 1997). We found that PML-RARα was also associated with an increase in cells expressing the immature CD34 marker. The combination of PML-RARα and MYC caused a statistically significant shift toward an immature immunophenotype compared with the effects of PML-RARα or MYC alone (Fig. 2, C and D). In short, morphological and flow cytometric analyses showed that PML-RARα and MYC cooperated to impair myeloid cell maturation.

The tissues of the recipient animals in the four groups were also examined 5 wk after transplantation. The bone marrow, spleens, and livers of the Control + Control, Control + MYC, and PML-RARα + Control groups were essentially normal (Fig. S1). In contrast, at 5 wk after transplantation abnormalities were already apparent in the PML-RARα + MYC recipients. In the spleens, there were not only areas of normal-appearing red pulp with mixed myeloid cell populations but also areas effaced by myeloid cells (Fig. S1; compare two insets of PML-RARα + MYC spleen). In the livers, modest spread of myeloid cells was apparent.

A strong cooperative interaction of PML-RARα and MYC was seen at 5 wk, but the APLs we observed in moribund animals after 8–13 wk may have reflected progression from an initiated state rather than the simple expansion of cells co-expressing PML-RARα and MYC. Morphological

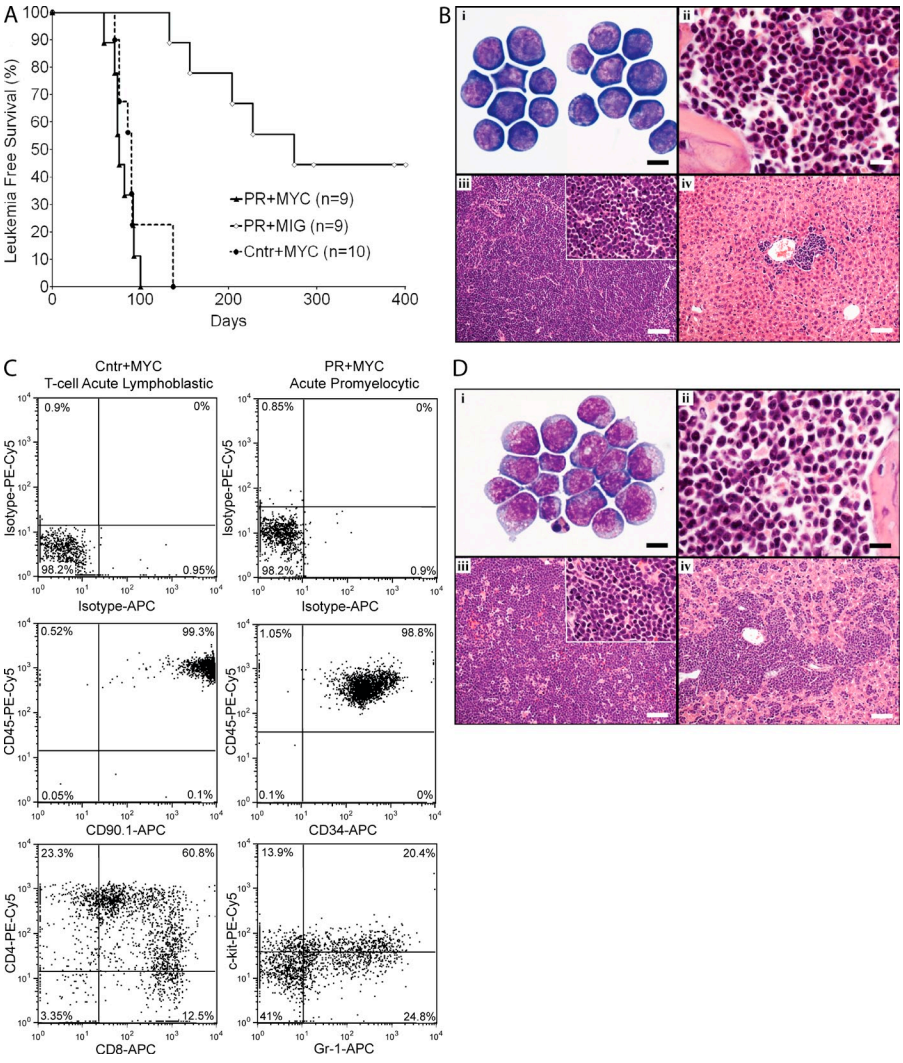

Figure 1. MYC cooperates with PML-RARα to induce AML. (A) Bone marrow of *PML-RARA* mice (PR) was transduced with a retrovirus encoding MYC and GFP (MYC) or control retrovirus encoding GFP only (MIG) and introduced into lethally irradiated recipient mice. Combined results from two independent experiments for each group are shown. Median time to AML of 8 PML-RARα + MYC mice was 75 d (1 additional mouse developed T cell acute lymphoblastic leukemia/lymphoma (T-ALL) at 92 d; overall median time to illness 76 d). With longer latencies, some PML-RARα + MIG control retrovirus mice developed leukemia (four mice developed AML, one mouse developed T-ALL, and four mice were euthanized without leukemia or lymphoma). Control FVB/n (Cntr) + MYC mice developed lymphomas with a median latency of 90 d (7 mice developed T-ALL, 2 mice developed lymphoblastic lymphoma in the orbit (injection site), and 1 mouse was euthanized without evidence of leukemia or lymphoma). Differences in leukemia-free survival: PR+MIG versus PR+MYC, $P < 0.0001$; PR+MYC versus Cntr+MYC, P = NS. (B) Pathology of Cntr + Myc mouse. Results representative of nine lymphomas are shown. (i) Cytology of lymphoblastic cells from the lymph node of mouse #609. Histology of (ii) bone marrow of mouse #34, (iii) thymus of mouse #33, and (iv) liver of mouse #32. (i) Wright's Giemsa stain. (ii-iv) H&E stain. Bars: (i) 8 µm; (ii) 12 µm; (iii and iv) 60 µm; (iii inset) 24 µm. (C) Immunophenotype of lymphoblastic lymphoma and APL. Splenocytes from a Control + MYC mouse (left; #609) and from a PML-RARα + MYC mouse (right; #608) were stained with the indicated antibodies. Plots are gated on GFP+ cells. Results representative of nine lymphomas and eight leukemias are shown. (D) Pathology of PML-RARα + MYC mouse. Results representative of eight leukemias are shown. (i) Cytology of leukemic cells from spleen of mouse #586. Pathology of (ii) bone marrow of mouse #478, (iii) spleen of mouse #478, and (iv) liver of mouse #478. (i) Wright's Giemsa stain. (ii-iv) H&E stain. Bars: (i) 8 µm; (ii) 12 µm; (iii and iv) 60 µm; (iii inset) 24 µm.

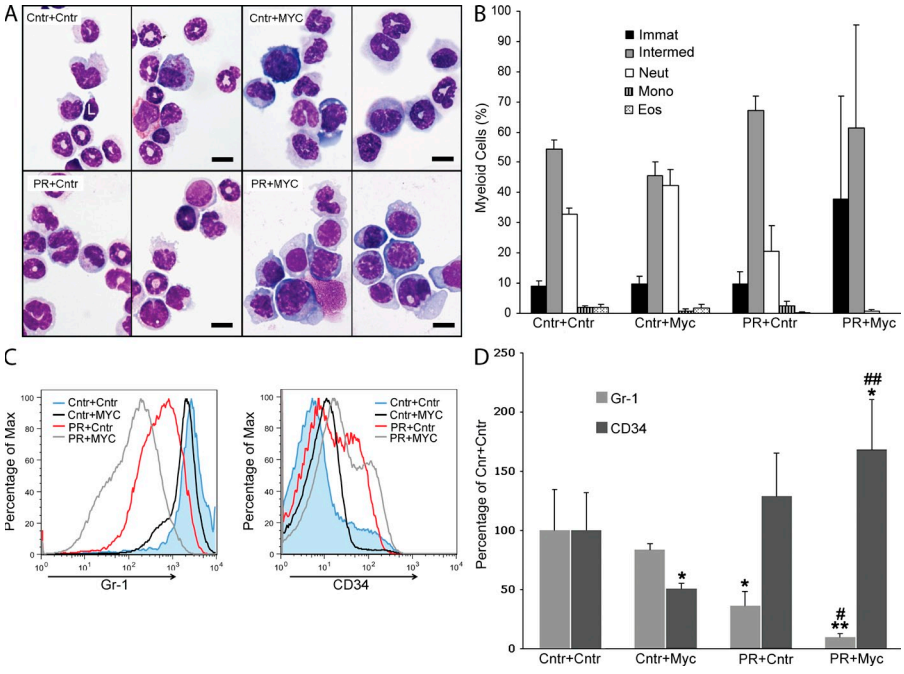

Figure 2. PML-RARα and MYC cooperate to alter myeloid maturation. (A) Control (Cntr) or *PML-RARA* (PR) transgenic bone marrow was transduced with MIG (Cntr) or MYC retroviruses, and lethally irradiated mice were reconstituted with this bone marrow. 5 wk after transplantation, mice were euthanized and GFP+ cells were sorted from bone marrow and stained with Wright's Giemsa stain. Results are representative of three to four animals per group. Bars, 8 µm. Each panel is comprised of two images of equal size, showing two different microscopic fields. L, Lymphocyte. (B) GFP+ bone marrow cells were stained as in A. 200 cell differential counts were performed. Percentages of cell types within the myeloid compartment are shown. Immat, Immature forms (blasts and promyelocytes); Intermed, myeloid intermediate forms; Neut, neutrophils; Mono, monocytes; Eos, eosinophils; *n*: Cntr+Cntr = 4; Cntr+MYC = 3; PR+Cntr = 4; PR+MYC = 3. Mice in each group were generated in single experiments. Means ± SD are shown. Differences in the percentages of intermediate forms and/or neutrophils were statistically significant for all comparisons except Cntr+Cntr versus Cntr+MYC. PR+MYC data for mature neutrophils differs from other three groups: PR+MYC versus Cntr+Cntr, $P < 0.0001$; PR+MYC versus Cntr+MYC, $P < 0.01$; PR+MYC versus PR+Cntr, $P < 0.02$. (C) Bone marrow cells from mice described in A were stained as described in Materials and methods. 34,000 GFP+ cells negative for lymphoid and erythroid antigens were analyzed for Gr-1 and CD34. Gr-1 and CD34 fluorescence histograms representative of three mice per group are shown. (D) Gr-1 and CD34 levels were analyzed in C. All values were normalized to Cntr+Cntr. Means ± SD are shown. $n = 3$ in each group. Mice in each group were generated in single experiments. *, $P < .05$; **, $P < .01$ for comparison to Cntr+Cntr; #, $P < 0.05$ PR+MYC versus PR+Cntr and $P < 0.01$ PR+MYC versus Cntr+MYC; ##, $P < 0.01$ PR+MYC versus Cntr+MYC.

observations provided support for this possibility. In fully developed APL (Fig. 1 D, i), the leukemic cells showed little evidence of maturation beyond the promyelocyte stage, with mostly oval or indented nuclei, open chromatin, and many cells with primary granules. In contrast, the PML-RARα + MYC cells at 5 wk (Fig. 2 A) showed a variable morphology, including more cells with differentiation beyond the promyelocyte stage. In fully developed APL, the bone marrow and spleen were effaced by immature cells with predominantly round to oval nuclei (Fig. 1 D, ii and iii), whereas the bone marrow and spleen of PML-RARα + MYC cells at 5 wk showed a more heterogeneous myeloid cell population (Fig. S1). Collectively, the morphological findings suggested that PML-RARα and MYC strongly initiate, but may not complete, the process of leukemogenesis.

MYC mutants provide further evidence of cooperation between increased MYC and PML-RARα in myeloid leukemogenesis

To further evaluate the role of MYC in APL pathogenesis, we introduced two mutant versions of MYC (MYC^{T58A} and $MYC^{\Delta MBII}$) into *PML-RARA* bone marrow and assessed the effect on leukemic transformation. MYC^{T58A}, exemplifying a hypermorphic allele, contains an alanine substitution at threonine 58 blocking phosphorylation that increases MYC protein stability (Sears et al., 2000). This mutation has also been reported to decrease MYC-induced apoptosis (Hemann et al., 2005). $MYC^{\Delta MBII}$, exemplifying a hypomorphic allele, has a deletion of aa 124–149 (MYC-box II), the deletion of which has been associated with loss of repression of some MYC target genes as well as decreased transforming activity (Stone et al., 1987; Freytag et al., 1990; Penn et al., 1990; Li et al., 1994; Herbst et al., 2005). Co-expression of PML-RARα with MYC^{T58A} cooperated to induce myeloid leukemias in recipient animals (Fig. 3 A). The leukemias were morphologically similar to those observed in mice co-expressing PML-RARα and wild-type MYC (Fig. S2 A). Median time to illness of 70 d for PML-RARα + MYC^{T58A} mice was slightly shorter than the 76-d median observed in mice expressing PML-RARα + MYC (Fig. 1 A; $P < 0.01$). Co-expression of PML-RARα with $MYC^{\Delta MBII}$ similarly showed cooperation in APL induction (Fig. 3 B and Fig. S2 B), with median time to illness of 92 d appearing modestly longer than seen with wild-type MYC (Fig. 1 A; $P = 0.05$). Hence, latency to disease correlated with MYC allele strength. Recipients of control bone marrow transduced with MYC^{T58A} developed myeloid leukemia and lymphoblastic disease with latencies (median 101 d) similar to those seen in control + MYC mice (Fig. 1 A; P = NS), but longer than for *PML-RARA* marrow transduced with MYC^{T58A} (Fig. 3 A; $P < 0.0001$), whereas a subset of recipients of control bone marrow transduced with $MYC^{\Delta MBII}$ developed lymphoblastic disease with

longer latency and incomplete penetrance (leukemia-free survival: PML-RARα + MYC$^{\Delta MBII}$ vs. control + MYC$^{\Delta MBII}$, $P < 0.0001$; control + MYC vs. control + MYC$^{\Delta MBII}$, $P = 0.0001$; Fig. 1 A and Fig. 3 B). The observation that the hypomorphic MYC$^{\Delta MBII}$ allele was only weakly oncogenic in the absence of PML-RARα, but induced APL with complete penetrance in the presence of PML-RARα, is noteworthy. This finding shows that a weakly transforming genetic change may nevertheless contribute to acute leukemia in the presence of cooperating genetic events.

Recipients of PML-RARα + MYC^{T58A} and PML-RARα + MYC$^{\Delta MBII}$ bone marrow, as well as recipients of control + MYC^{T58A} and control + MYC$^{\Delta MBII}$, were also studied at 5– 6 wk after transplantation. These alleles also cooperated with PML-RARα to impair myeloid maturation (Fig. S2, C and D, and unpublished data). Interestingly, splenic and liver pathology differed at 5 wk between the two alleles, with greater evidence of progression in recipients of PML-RARα + MYC^{T58A} than in recipients of PML-RARα + MYC$^{\Delta MBII}$; areas of myeloid expansion were seen in the PML-RARα + MYC^{T58A} spleens accompanied by myeloid infiltrates in their livers (Fig. S2 E).

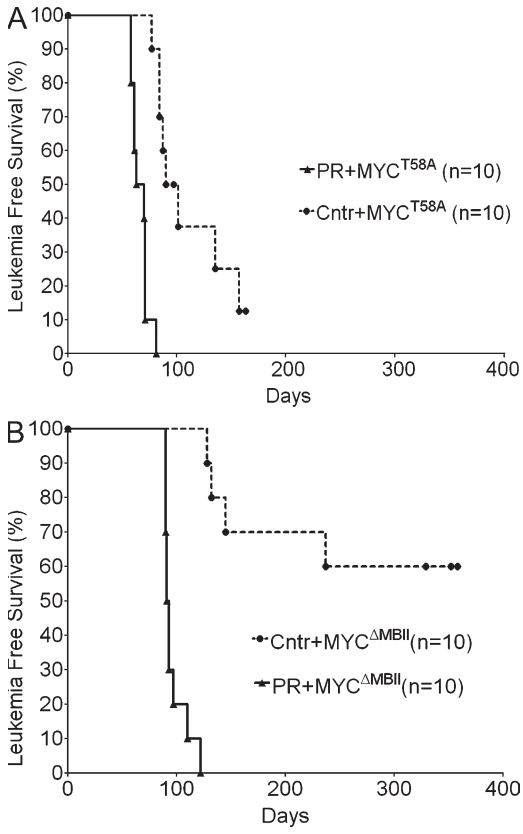

Figure 3. MYC mutants cooperate with PML-RARα to induce AML. (A) Bone marrow of *PML-RARA* (PR) or control FVB/n (Cntr) mice was transduced with a retrovirus encoding MYC^{T58A} and introduced into lethally irradiated recipient mice. Combined results from two independent experiments for each group are shown. Median time to APL of 10 PML-RARα + MYC^{T58A} mice was 70 d. Control FVB/n + MYC^{T58A} mice developed AML (5 mice), T-ALL (3 mice), or were euthanized without evidence of leukemia or lymphoma (2 mice). Median time to disease was 101 d. Difference in leukemia-free survival: $P < 0.0001$. (B) Bone marrow of *PML-RARA* (PR) or control FVB/n (Cntr) mice was transduced with a retrovirus encoding MYC with a deletion of the MBII domain (MYC$^{\Delta MBII}$) and introduced into lethally irradiated recipient mice. Combined results from two independent experiments for each group are shown. Median time to APL of 10 PML-RARα + MYC$^{\Delta MBII}$ mice was 92 d. Control FVB/n + MYC$^{\Delta MBII}$ mice developed T-ALL (four mice) or were euthanized without evidence of leukemia or lymphoma (six mice). Difference in leukemia-free survival: $P < 0.0001$.

Protein levels and studies of clonality also suggest interplay between MYC allele strength and myeloid transformation

We performed Western blotting for MYC using an antiserum that recognizes both human and mouse MYC protein (Chiariello et al., 2001; Teng et al., 2004). Representative data are shown in Fig. 4 A, and quantification of data from normal bone marrow and from leukemic bone marrow of PML-RARα + MYC^{T58A}, PML-RARα + MYC, and PML-RARα + MYC$^{\Delta MBII}$ mice is shown in Fig. 4 B. These data indicate that the retroviral constructs result in MYC overexpression at levels up to threefold of that present in normal marrow cells.

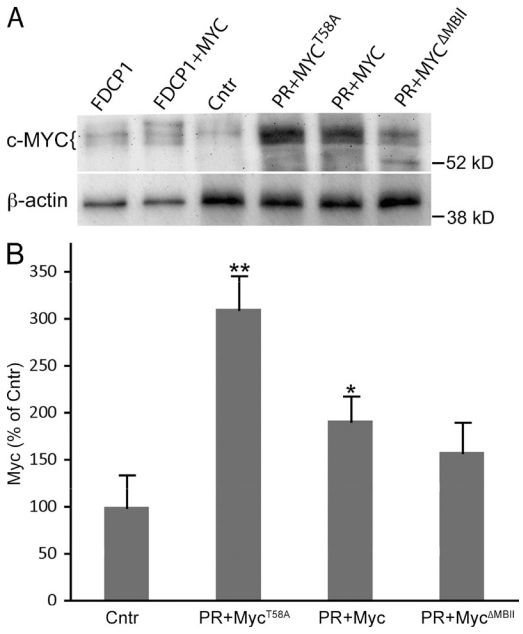

Figure 4. MYC protein levels in PML-RARα + MYC or MYC mutant leukemias. (A) Whole-cell lysates from normal bone marrow (Cntr) and PML-RARα + MYC, MYC^{T58A}, or MYC$^{\Delta MBII}$ leukemic cells were probed with anti-MYC. The same blot was stripped and reprobed with anti-β-actin antibody for loading control. Cell lysates of FDC-P1 cells and FDC-P1 transduced with MYC are also shown. (B) Optical density of MYC protein was normalized to β-actin and shown as the percentage of MYC level in normal bone marrow (Cntr). Means ± SD are shown. $n = 3$ in each group. Normal bone marrows were from three normal FVB/n mice. Leukemic samples were from nine independent APLs arising from the survival experiments shown in Figs. 1 A, 3 A, and 3 B. Data were obtained from two independent immunoblots; each sample was analyzed once. *, $P < .05$; **, $P < .01$ for comparison to normal Cntr bone marrow.

Two approaches were used to assess the clonality of leukemias arising in mice expressing any of the three MYC alleles: cytogenetic analysis, including Southern blotting for retroviral integration sites, and spectral karyotyping. Southern blotting was performed on PML-RARα + MYC, PML-RARα + MYC^{T58A}, and PML-RARα + MYC$^{\Delta MBII}$ leukemias and on bone marrow from PML-RARα + MYC mice 5 wk after transplantation, a retrovirally marked leukemia that arose in a PML-RARα + MIG mouse, and a retrovirally marked lymphoblastic lymphoma that arose in a Control + MYC mouse. Results are shown in Fig. 5. Retroviral integration patterns ranged from single-dominant bands in the PML-RARα + MIG (#6869) and Control + MYC (#34) mice to a pattern consistent with multiclonal integration (multiple low-intensity restriction fragments) in most PML-RARα + MYC mice at 5 wk (i.e., #547 and 548). In one PML-RARα + MYC mouse at 5 wk (#546), a dominant clone (or clones) was already present, and the fully developed leukemias showed integration patterns that were consistent with oligo- to monoclonal disease. These data revealed that recipients of PML-RARα + MYC bone marrow initially had multiple retrovirally marked clones in their marrow, and that one or a few clones arose within these mixed populations to achieve dominance by the time mice were outwardly ill. The low clonality observed by Southern blot suggested that the leukemias arose from only a few of the cells that expressed PML-RARα and MYC.

To further assess leukemia clonality and examine the impact of MYC expression on cytogenetic changes, spectral karyotyping was also performed. Tables S1–S3 present the results of cytogenetic studies in 8 PML-RARα + MYC leukemias, 4 PML-RARα + MYC^{T58A} leukemias, and 10 PML-RARα + MYC$^{\Delta MBII}$ leukemias. A striking finding is that a gain of chromosome 15 was observed in only 1 of the 22 leukemias with retroviral vector expression of MYC or a variant. This frequency of 5% is markedly less than the 60% frequency of trisomy 15 in PML-RARα leukemias that arose in the absence of MYC retroviruses (Table S4; $P < 0.00001$). Furthermore, the data observed in PML-RARα + MYC$^{\Delta MBII}$ leukemias indicated that the decrease in trisomy 15 was an effect of MYC expression: 80% of these leukemias showed clonal karyotypic abnormalities, but none showed the common gain of 15 seen when MYC was not introduced. These findings suggest that when MYC is overexpressed there is relief of selective pressure to gain chromosome 15, supporting our hypothesis that *Myc* contributes to this gain. The findings in the hypomorphic MYC$^{\Delta MBII}$ leukemias also demonstrate that allele strength influences the likelihood of karyotypic changes accompanying progression to leukemia.

Gain of Myc is selected for in a mouse model of APL

To further assess the importance of *Myc* gain, we generated *PML-RARA* mice that were haploinsufficient for *Myc* (*PR+Myc*$^{+/-}$) by crossing *PML-RARA* transgenic mice to mice that had the open reading frame of one *Myc* allele replaced with a *Pgk-hprt* minigene (Trumpp et al., 2001). Bone marrow was harvested from the resulting *PR+Myc*$^{+/-}$ mice and transplanted into lethally irradiated FVB/n recipients. The results were compared with mice transplanted with *PML-RARA* bone marrow expressing two wild-type *Myc* alleles (*PR+Myc*$^{+/+}$). Bone marrow haploinsufficient for *Myc* had decreased ability to contribute to long-term repopulation as compared with bone marrow with two copies of *Myc*. Peripheral blood granulocytes were assessed for CD45.1 (donor) and CD45.2 (recipient) 3 mo after lethal irradiation and reconstitution. Continued contribution to myeloid cells is a marker of persistence of transplanted cells within the stem cell compartment. Results were similar whether or not the *PML-RARA* transgene was present. Recipients of *Myc*$^{+/-}$ bone marrow cells ($n = 7$) showed a mean of 37% donor

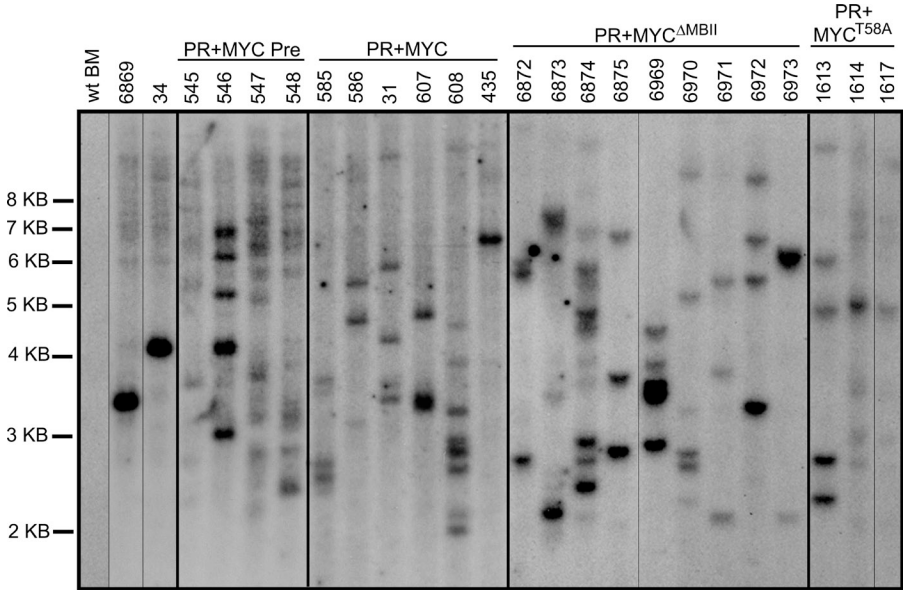

Figure 5. Southern blot of PML-RARα + MYC leukemias shows clonal retroviral integrations. Genomic DNA samples were digested with EcoRI, which cuts within the multicloning site of retroviral integrants, and the blot was probed with a probe hybridizing to GFP sequences. 6989, A GFP$^+$ leukemia that arose in a PML-RARα + MIG recipient mouse. 34, A GFP$^+$ lymphoblastic lymphoma that arose in a recipient of Control + MYC-transduced bone marrow. PR+MYC Pre, preleukemic bone marrow from PML-RARα + MYC mice 5 wk after transplantation. PR+MYC, PR+MYC$^{\Delta MBII}$, and PR+MYC^{T58A}, leukemias that arose from recipients of PML-RARα bone marrow transduced with various MYC alleles. Data in this figure were obtained in three independent Southern blots. Thick vertical lines separate groups of samples and indicate juxtapositions of lanes. Thin vertical lines also indicate juxtaposition of lanes.

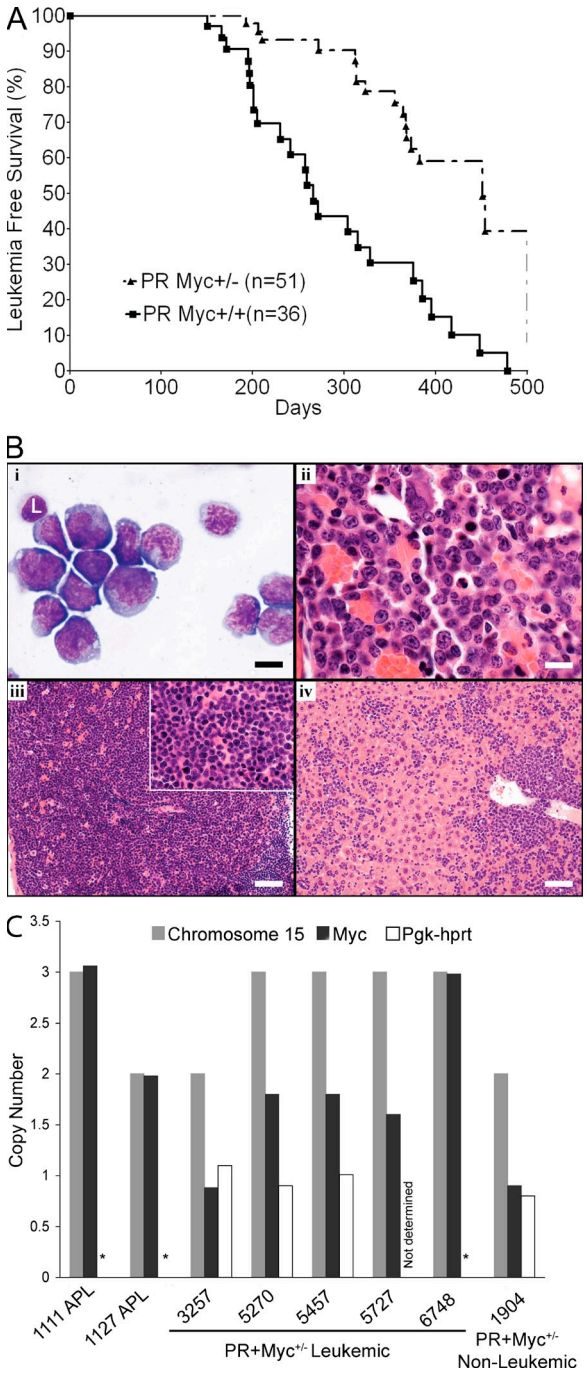

Figure 6. Haploinsufficiency for *Myc* delays leukemia development. (A) Bone marrows of *PML-RARA Myc*$^{+/+}$ or *PML-RARA Myc*$^{+/-}$ FVB/n mice were harvested and transplanted into lethally irradiated FVB/n recipient animals. Combined results from eight (*Myc*$^{+/+}$) or eleven (*Myc*$^{+/-}$) independent transplantation experiments are shown. Mice were followed for the development of leukemia. Nonleukemic deaths were censored at the time of death. 67% of *PML-RARA Myc*$^{+/+}$ deaths were from leukemia, whereas 31% of *PML-RARA Myc*$^{+/-}$ recipient mice died of leukemia. Median latency among leukemic animals was 258 d for *Myc*$^{+/+}$ and 339 d for *Myc*$^{+/-}$. P < 0.0001. (B) Morphology of leukemias arising in *PML-RARA Myc*$^{+/-}$ mice. Results representative of 16 leukemias are shown. (i) Cytology of leukemic cells from spleen of mouse #836. L, Lymphocyte. Histology of (ii) bone marrow of mouse #828, (iii) spleen of mouse #828, and (iv) liver of mouse #828. (i) Wright's Giemsa stain. (ii-iv) H&E stain. Bars: (i) 8 µm; (ii) 12 µm; (iii and iv) 60 µm; (iii inset) 24 µm. (C) Gain of chromosome 15 and the wild-type *Myc* allele in *PML-RARA Myc*$^{+/-}$ leukemias. The number of copies of chromosome 15 as determined by cytogenetic analysis is indicated for each sample; 2 previously characterized leukemias (#1111 and #1127), 5 *PR+Myc*$^{+/-}$ leukemias, and 1 nonleukemic *PR+Myc*$^{+/-}$ marrow. Copy numbers for the wild-type *Myc* and *Pgk-hprt* alleles are also given for the same samples. Samples were run in triplicate in one to eight independent experiments. *Pgk-hprt* copy number could not be determined for leukemia #5727 because of insufficient quantity of DNA. *, *Pgk-hprt* copy number values are 0. Results for leukemia #836 showing no gain of chromosome 15, but increased copy-number for the wild-type *Myc* allele, are not shown here, but are included in Table I and discussed in the text.

granulocytes at 3 mo as compared with a mean of 83% in recipients of *Myc*$^{+/+}$ bone marrow ($n = 5$; P < 0.001). In accord with the decreased repopulating ability of *Myc* haploinsufficient bone marrow, haploinsufficiency for *Myc* delayed the development of leukemia; median latency to disease was 339 d for *PR+Myc*$^{+/-}$ mice and 258 d for *PR+Myc*$^{+/+}$ (Fig. 6 A). Two thirds of *PR+Myc*$^{+/+}$ deaths were from leukemia, whereas only 31% of animals in the cohort haploinsufficient for *Myc* died from leukemia. The cytology and histopathology of leukemia arising from *PR+Myc*$^{+/-}$ bone marrow is shown in Fig. 6 B. These results indicate that mice transplanted with *PML-RARA* bone marrow haploinsufficient for *Myc* developed APL with decreased penetrance and increased latency.

To directly test our hypothesis that *Myc* is an important driver of +8/+15 in APL, we assessed whether there was a gain of chromosome 15 and *Myc* copy number in leukemias that arose from *PR+Myc*$^{+/-}$ mice. We performed quantitative PCR (Q-PCR) analysis on genomic DNA isolated from these leukemias to determine *Myc* and *Pgk-hprt* copy number and compared the results with cytogenetic analysis on the same samples. We first analyzed previously characterized murine leukemias 1111 and 1127 as controls for internal consistency between these methodologies. Leukemia 1111 contains an extra copy of chromosome 15, but leukemia 1127 does not. As expected, the number of wild-type *Myc* alleles was equal to the copy number of chromosome 15, and no *Pgk-hprt* (representing the null allele) was detected (Fig. 6 C).

We then analyzed six *PR+Myc*$^{+/-}$ leukemias by karyotyping and using Q-PCR to determine *Myc* and *Pgk-hprt* copy number. One *PR+Myc*$^{+/-}$ leukemia (#3257) showed neither gain of chromosome 15 nor gain of the wild-type *Myc* allele, whereas four leukemias analyzed with both techniques had +15 and showed gain of a *Myc* allele (Fig. 6 C and Table I). In one of these samples (#6748), all three copies of chromosome 15 appeared to have the wild-type *Myc* allele, suggesting that the *Myc*-null allele was replaced. Interestingly, analysis of one additional *PR+Myc*$^{+/-}$ leukemia that did not gain chromosome 15 showed four copies of the *Myc* allele and no *Pgk-hprt* allele by Q-PCR (#836; Table I; not

Table I. Q-PCR and karyotypic analyses of *PR+Myc*$^{+/-}$ mice

Mouse	Diagnosis	*Myc* alleles	*Pgk–hprt* alleles	Karyotype
3257	Leukemic	1	1	40,XY,der(14)t(6;14)(B1;E)[7]/42,XY,+6,+8[2]/42,XY,+X,+6[1]/41,XY,+12[1]/40,XY[1]
5270	Leukemic	2	1	42,X,-Y,+8,+10,**+15**[7]/42,idem,+1,del(1)(A2F),-11[1]/42,X,+X,-Y,+8,+10[1]/40,XY[1]
5457	Leukemic	2	1	44,XX,+6,+8,+10,**+15**[1]/43,idem,-X[9]/40,XX[1]
5727	Leukemic	2	Not determined	42,X,-X or -Y,+8,+10,**+15**[2]/43,idem,+7[7]48,idem,+2,+6,+7,+7,+10,+16[1]
6748	Leukemic	3	0	45,XY,+8,+10,+12,**+15**,+17[5]/46,idem,+14[4]/42,XY,t(2;12)(H1;F1),-8,+10,+13,+15[1]
836	Leukemic	4	0	40,XX,del(2)(DH3)[5]/41,idem,+8[4]/40,XX[1]
5287	Leukemic	2	Not determined	Not obtained
838	Leukemic	2	0	Not obtained
829	Leukemic	2	0	Not obtained
274	Leukemic	1	1	Not obtained
2652	Leukemic	1	1	Not obtained
1892	Leukemic	Not determined	Not determined	40,X,-Xor-Y,+der(18)t(1;18)(B;E3)[1]/44,idem,dup(4)(A2C4),der(5;11)(A1;A1),+8,+10,+12,**+15**,+16,+17,-18[9]
6675	Leukemic	Not determined	Not determined	43,XY,+7,+8,+10[10]
1904	Nonleukemic	1	1	40,XX[6]/40,XY[2]/41,XX,+15[1]/39,XX,del(1)(BD),-9,-19,+mar1[1]
643	Nonleukemic	1	1	Not obtained
6514	Nonleukemic	1	1	Not obtained
2651	Nonleukemic	1	1	Not obtained

Clonal gains of chromosome 15 are indicated in bold.

depicted in Fig. 6 C). Fluorescence in situ hybridization using a *Myc* probe demonstrated that only two *Myc* signals were present in this leukemia. This finding suggests the presence of a cytogenetically undetectable duplication event of the wild-type *Myc* allele on both copies of chromosome 15, or a local amplification event, resulting in three tandem copies of *Myc* on a single homologue.

We analyzed seven additional leukemias from the *PR+Myc*$^{+/-}$ cohort by either karyotyping or Q-PCR and found that four of them showed +15 or gain of a *Myc* allele (Table I). Four additional animals that died from causes unrelated to leukemia showed no increase in *Myc* copy number (Table I and one example shown in Fig. 6 C). Altogether, Q-PCR data and karyotypes indicated that there is selective pressure to gain *Myc* with transformation: although an increase in *Myc* gene-dosage was not obligatory, by one or both methods *Myc* copy-number was increased in 9–13 *PR+Myc*$^{+/-}$ leukemias analyzed. The wild-type *Myc* allele was gained in 8 of 11 samples studied by Q-PCR (in 6 leukemias two wild-type *Myc* copies were restored; in 2 leukemias wild-type *Myc* copy number was increased to 3 or 4), the *Myc*-null allele was lost in 4 of 9 cases for which Q-PCR data could be obtained, and we observed gain of mouse chromosome 15 in 5 of 8 samples studied karyotypically. In the three cases with +15 for which the identity of the gained chromosome could be definitively ascertained, Q-PCR results indicated gain of the chromosome encoding wild-type *Myc*. These results, in conjunction with our finding that +15 is suppressed when MYC is expressed by retroviral transfer, demonstrate that *Myc* is an important driver of +15 in APL.

Increased MYC is seen in human APL

Payton et al. (2009) performed gene expression of 14 human APL samples and of 5 samples of normal human promyelocytes using Affymetrix Human Genome U133 Plus 2.0 Arrays. Normalized signals for *MYC* transcript levels were obtained from Gene Expression Omnibus Dataset Series GSE12662. Mean *MYC* transcript levels (normalized within this study) were 2713 (SD, 1092; range, 1513–3911) for normal human promyelocytes and 31247 (SD, 21624; range, 5615–81602) for human APL ($P < 0.001$). This increase in MYC was seen in all samples: 11 cases with t(15;17) as the only karyotypic lesion, 2 cases with t(15;17) and +8, and 1 case with a complex karyotype (Fig. S3 A). Hence, increased *MYC* is a general feature of human APL. We further sought to ascertain whether APL with trisomy 8 had increased *MYC* levels relative to APL with only the t(15;17). Published literature and the GEO database were searched for available expression data on human APL with and without trisomy 8. Two additional datasets were identified (Ross et al., 2004; Verhaak et al., 2009) for which both karyotypic and expression data were available. However, small sample size (40 APLs with t(15;17) and 5 APLs with t(15;17) and +8) and variation within each group led to an inconclusive analysis.

MYC transcripts appear elevated in human AML with trisomy 8

In an effort to determine whether *MYC* levels are proportionally increased by gain of chromosome 8, we expanded our analysis to compare *MYC* transcript levels in normal karyotype AML as compared with AML with +8. For this purpose we used a large dataset from the Gene Expression Omnibus Dataset Series GSE6891 (Verhaak et al., 2009). In this dataset, *MYC* levels from 189 normal karyotype AMLs and 20 AMLs with +8 were available. Mean *MYC* levels in AML with +8 (normalized within this study) were 1256 (SD, 879; range, 139–2637), whereas for normal karyotype AML average levels were 866 (SD, 699; range, 82–3812). Hence, *MYC* levels with gain of a third chromosome encoding MYC averaged 45% higher than when only two MYC chromosomes were present. This value is close to what would be predicted if each chromosome were equally active. Even with the variability within each group, this difference reached statistical significance ($P = 0.03$; Fig. S3 B).

DISCUSSION

In this study, we demonstrate that MYC cooperates with PML-RARα to accelerate the development of myeloid leukemia and that gain of *Myc* is a driver mutation in gain of the chromosome on which MYC is encoded (i.e., *MYC/Myc* gain is selected for). One conceptual model of the genetic pathogenesis of AML holds that mutations of two classes cooperate to generate disease; i.e., mutations that enhance proliferation and/or survival but do not affect differentiation collaborate with mutations that impair differentiation and may expand progenitors (Graf and Beug, 1983; Beug et al., 1985; Gilliland and Tallman, 2002). An alternate conceptual model of leukemia holds that individual genetic changes do not fall neatly into two classes of mutation with linear relationships to cellular phenotypes (i.e., increased survival, enhanced proliferation, and arrested differentiation). Rather, in this view, it is the interaction of genetic changes that cumulatively generate the leukemic phenotype. Changes wrought by copy number increase appear more compatible with the alternate model, and our findings support this conception.

Our studies of *PR+Myc*$^{+/-}$, *PR+Myc*$^{+/+}$, *PML-RARA* + *MYC*$^{\Delta MBII}$, *PML-RARA* + *MYC*, and *PML-RARA* + *MYC*^{T58A} mice suggest that as MYC expression is increased, the latency to leukemia decreases (Table II). This indicates that as MYC levels increase either fewer additional events are needed to complete transformation or the likelihood of additional cooperating events increases, or both. Because in the same series of PML-RARα mice the karyotypic complexity of the leukemias was low when MYC and MYC^{T58A} were overexpressed (Table II), our data are most consistent with the hypothesis that at high levels of MYC fewer changes are needed. We further note that although in some settings deregulated MYC is associated with chromosomal instability, which may contribute to cancer development (McCormack et al., 1998; Felsher and Bishop, 1999b; Sargent et al., 1999; Barna et al., 2008), karyotypic complexity was inversely correlated with initial MYC level in our model. This finding supports the concept that the contribution of chromosomal instability to MYC-mediated transformation is context dependent (Wade and Wahl, 2006).

Although not definitive, our data suggest that the combination of PML-RARα and MYC is not sufficient to complete

Table II. Characteristics of leukemias initiated by the cooperation of PML-RARα and *MYC*

Characteristics	*PR+Myc*$^{+/-}$	*PR+Myc*$^{+/+}$	*PR+MYC*$^{\Delta MBII}$	*PR+MYC*	*PR+MYC*^{T58A}
Myeloid Leukemia (%)	31	67	100	89	100
Median Latency (d)	339	258	92	76	70
SKY analysis					
No. cases	8	15	10	8	4
Clonal abnormality (%)	8 (100)	14 (93)	8 (80)	1 (13)	1 (25)
Karyotype Complexity	Complex	Intermediate	Simple	Simple	Simple
Recurring clonal abnormalities (%)					
−2 or del(2)	1 (13)	1 (7)	0 (0)	0 (0)	0 (0)
+4 or dup(4)	1 (13)	2 (13)	0 (0)	0 (0)	0 (0)
+6	2 (25)	2 (13)	0 (0)	0 (0)	0 (0)
+7	2 (25)	1 (7)	0 (0)	0 (0)	0 (0)
+8	8 (100)	7 (47)	0 (0)	0 (0)	0 (0)
+10	6 (75)	5 (33)	5 (50)	0 (0)	0 (0)
+12	2 (25)	0 (0)	0 (0)	0 (0)	0 (0)
+14	1 (13)	4 (27)	0 (0)	0 (0)	0 (0)
+15	5 (63)	9 (60)	0 (0)	1 (13)	0 (0)
+16	1 (13)	5 (33)	0 (0)	0 (0)	0 (0)
+17	2 (25)	2 (13)	0 (0)	0 (0)	0 (0)
+18 or partial trisomy	1 (13)	2 (13)	0 (0)	0 (0)	0 (0)
−X/−Y	4 (50)	8 (53)	4 (40)	1 (13)	1 (25)

transformation, and therefore suggest that gain of h8/m15 is only one step on the path to APL. Our morphological studies and our examinations of clonality are consistent with the combination of PML-RARα + MYC acting as a powerful initiator of leukemia. Progression to mono- to oligoclonal APL is subsequently reflected as an arrest of differentiation at the promyelocyte stage and aggressive tissue dissemination. Retroviral insertional mutagenesis may have a role in cooperation in transformation and thereby selection for dominant clones. In leukemias arising in *Myc* haploinsufficient mice, increased karyotypic changes were apparent, and the recurrent gains of mouse chromosomes 8 and 10 and common loss of a sex chromosome could indicate selection for particular cooperative copy number changes in this context.

We found that three MYC alleles accelerated myeloid disease in the context of PML-RARα. The leukemias that arose in PML-RARα mice expressing any of the MYC alleles were all characterized by a predominantly promyelocytic morphology, consistent with the central role of the PML-RARα fusion in determining the differentiation state of the leukemia. The predominant immunophenotype of the leukemias included moderate expression of Gr-1 and expression of both CD117 (Kit) and CD34. There was some heterogeneity of immunophenotype, but antigen expression patterns did not correlate with the different MYC alleles (unpublished data).

Transduction of control FVB/n bone marrow with the same MYC retroviral vectors resulted mainly in the development of lymphoid disease, with some myeloid disease seen with the MYC^{T58A} allele. These results are consistent with previous studies demonstrating the ability of MYC to induce both lymphoid and myeloid neoplasms (Adams et al., 1985; Felsher and Bishop, 1999a; Hemann et al., 2005; Luo et al., 2005; Smith et al., 2006). Retroviral transduction studies of Hemann et al. (2005) and Luo et al. (2005) gave rise to pre–B cell lymphomas or AML, respectively. Differences in vector design used by Luo et al. (2005) that may explain the divergent results include murine species origin, inclusion of exon 1 translation start site, and expression levels. Our finding that the more highly expressed stable MYC allele, MYC^{T58A}, could initiate AML even in the absence of PMLRARα is compatible with the possibility that higher expression levels contribute to an AML phenotype.

Our studies of human APL and AML provide additional insight into the role of *MYC* gain and *MYC* levels in human myeloid leukemia. Interestingly, human APLs, whether or not they have trisomy 8, show increased *MYC* levels as compared with normal human promyelocytes. The leukemia stem cell of APL has been suggested to be within the promyelocytic population (Guibal et al., 2009; Wojiski et al., 2009), and hence increased *MYC* levels may be an integral part of transforming these normal precursors into self-renewing leukemic cells. Numerous genetic changes may impact *MYC* expression, including activation of FLT3 (Li et al., 2007), a common event in human APL (Kiyoi et al., 1997; Yokota et al., 1997; Kottaridis et al., 2001; Yamamoto et al., 2001; Schnittger et al., 2002). The identification of other changes that cause increased *MYC* in human leukemic promyelocytes awaits additional studies.

A previous study on *MYC* levels in human AMLs with +8 had noted decreased *MYC* in +8 AML as compared with normal human $CD34^+$ bone marrow cells, and had thereby implied that *MYC* might not be increased by gain of chromosome 8 (Virtaneva et al., 2001). However, the number of samples in this earlier study was low: 7 normal samples were compared with 10 normal karyotype AMLs and with 10 AMLs with trisomy 8. We examined a large publically available dataset (Verhaak et al., 2009) and observed that, on average, *MYC* levels were proportionately increased in the presence of trisomy 8. The large dataset permitted this finding to emerge despite the heterogeneity of *MYC* mRNA levels in human AML.

Although our data reveal a strong correlation between MYC dose and leukemic transformation, the notion that gain of the *MYC* protooncogene is of central importance in trisomy 8 was controversial. A study of AML with amplifications of 8q24 suggested that another gene located near *MYC* in this region, *TRIB1*, is the target of gene amplification. This suggestion was based on the finding that TRIB1 was overexpressed, whereas MYC RNA could not be detected (Storlazzi et al., 2006). Further substantiating its role, *Trib1* was identified as a common insertion site in leukemias induced by Hoxa9/Meis1 retroviruses (Jin et al., 2007), and both MYC and TRIB1 can be co-overexpressed in AML patients (Röthlisberger et al., 2007). Although the present study does not address the role of TRIB1 in APL, it is possible that MYC and TRIB1 cooperate in the disease process. *PVT1* is yet another nearby locus on human chromosome 8 that has been implicated in oncogenic transformation (Guan et al., 2007). Recent studies examined chromosome copy number changes at high resolution in a large spectrum of human cancer cell lines and tumor tissues including myeloid disorders (Beroukhim et al., 2010; Bignell et al., 2010). These studies revealed that the *MYC* containing chromosome region is among the most frequently gained chromosomal regions and that the *MYC* gene was specifically contained within the peak of regional gain. These data further support the notion that gain of *MYC* is important for pathogenic effects of gaining this portion of chromosome 8. Additional studies examined copy number alterations in APL and found that gains of distal 8q included *MYC* in all cases where this region was gained (Akagi, et al. 2009; Radtke, et al. 2009; Walter, et al. 2009). In these 3 studies, 12 of 68 APL samples (18%) showed increased *MYC* copy number as a result of trisomy 8 or focal gain. Interestingly, one of these studies did identify rare cases of non-APL AML in which a nearby, long-interspersed noncoding RNA at *CCDC26* was gained without *MYC*, implicating this locus in AML pathogenesis (Radtke, et al. 2009). Collectively, the data suggest that other changes caused by +h8/+m15, such as increased TRIB1, PVT1, or CCDC26, may also impact leukemogenesis and that there may be cooperative effects among *MYC* and nearby genes.

A role for additional genes further away from *MYC* on chromosome 8, including genes for which mouse chromosome 15 is not syntenic, is also possible.

Several lines of evidence have come together to support the hypothesis that modest changes in MYC level may influence malignant transformation. In addition to the current study, work by Murphy et al. (2008) demonstrated that a modest increase in MYC protein levels can increase development of lung adenocarcinomas and a single-nucleotide polymorphism associated with increased risk for human colon cancer shows a long-range interaction with the *MYC* locus and has been speculated to influence *MYC* expression (Pomerantz et al., 2009).

Clinically, our results suggest that agents that target increased MYC may be useful for the treatment of AML. Work with a dominant-negative MYC allele has shown the potential of MYC inhibition to prevent and reverse malignant transformation with reversible impacts on normal tissues (Soucek et al., 2008). Posttranscriptional control is an important mechanism for regulating protein levels of cellular MYC and the related protein N-MYC; phosphorylation can cause MYC to be degraded (Sears, 2004; Yaari et al., 2005). Inhibitors of phosphatidylinositol-3 kinase increase phosphorylation of N-Myc, and thereby cause protein degradation and tumor regression in a mouse model of N-Myc–driven neuroblastoma (Chesler et al., 2006). Similarly targeted anti-MYC therapies might prove useful in the treatment of AML.

MATERIALS AND METHODS

Plasmids. HA-tagged human MYC, MYC^{T58A}, and $MYC^{\Delta MBII}$ in MSCV-IRES-GFP have been previously described (Hemann et al., 2005; Herbst et al., 2005). All plasmids were sequence verified and sequences are available upon request.

Mice. Mice were bred and maintained at the University of California at San Francisco, and their care was in accordance with Institutional Animal Care and Use Committee guidelines. FVB/n mice were purchased from The Jackson Laboratory, and hMRP8d-*PML-RARA* mice have been previously described (Brown et al., 1997). *PR+Myc*$^{+/-}$ mice were generated by crossing our *PML-RARA* mice to mice that were heterozygous for the *Myc* allele *c-myc*$^{\Delta ORF/+}$ (FVB/n strain background; Trumpp et al., 2001). Mice were observed daily for signs of illness. When any abnormality was observed, mice were subjected to a brief physical examination. Blood was obtained on animals that showed signs of illness and also in selected animals to screen for unsuspected disease. Mice were sacrificed when moribund or when physical examination and blood cell counts indicated likely rapid progression of illness.

Cell culture. BOSC23 cells were maintained in DME supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Freshly harvested bone marrow cells were cultured in stem cell media containing Myelocult M5300 (StemCell Technologies) with 15% FBS, IL-3–conditioned media (Karasuyama and Melchers, 1988), 0.4 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 10 ng/ml rIL-3, 10 ng/ml IL-6, and 10 ng/ml stem cell factor.

Retroviral transduction and transplantation. BOSC23 cells were transfected with retroviral constructs as previously described (Pear et al., 1993). Retroviral supernatants were collected and used to transduce bone marrow cells (850,000 cells per well in 24-well plates) as previously described (Truong et al., 2003). Transduced cells were washed, counted with trypan blue, and injected into the retro-orbital sinus of lethally (9 Gy) irradiated recipient FVB/n mice or FVB/n CD45.2 congenic mice.

Pathological analysis. Blood was obtained from the retro-orbital sinus. White blood cell count, hemoglobin, and platelet count were measured with the Hemavet 950 cell counter (CDC Technologies). Blood smears and cytospins of cell suspensions prepared from bone marrow and spleen cells were stained with Wright's Giemsa stain. Tissues were initially fixed in a buffered formalin solution. Sternae were decalcified for 2–3 h before embedding (formic acid 11% and formaldehyde 85%). Paraffin-embedded sections were stained with hematoxylin & eosin (H&E). Photographs were taken on a Nikon Eclipse 80i microscope with a Nikon Digital Sight camera using NIS-Elements F2.30 software at a resolution of 2560 × 1920. Using Adobe Photoshop CS2, images were resized and set at a resolution of 300 pixels/inch, autocontrast was applied, and, in select cases, unsharp mask and/or variations:darken was used to improve image clarity.

DNA purification and Southern blot analysis. Cells from bone marrow and spleen were collected and lysed in DNA lysis solution (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 200 mM NaCl, 0.2% SDS, and 100 μg/ml proteinase K). Genomic DNA was isolated using isopropanol precipitation followed by 70% EtOH wash and resuspension in ddH20. After restriction digestion with EcoRI, DNA fragments were separated by electrophoresis and immobilized onto a Nitran membrane. A probe of GFP was isolated from a sequence-verified MSCV-IRES-GFP vector and labeled with radioactive α-dCTP using Rediprime II Random Prime Labeling System (GE Healthcare). DNA was hybridized with the radio-labeled probe in a solution (7% SDS, 0.5 M NaP, pH 7.2, 1 mM EDTA, and 1% BSA) at 60°C overnight, membrane was washed three times, and autoradiograph was performed.

Cytogenetic analysis. Cytogenetic analysis was performed on fresh or cryopreserved spleen cells obtained at the time of development of leukemia. Short-term (24 h) cultures were initiated by incubating 1.0×10^6 cells/ml in MyeloCult M5300 (StemCell Technologies) with 5 μg/ml hydrocortisone 21-hemisuccinate, 5% horse serum, 4% pokeweed mitogen spleen-conditioned medium, 100 ng/ml stem cell factor, and 6 ng/ml IL-3 or in MyeloCult M5300 with 15% fetal calf serum, 10 ng/ml stem cell factor, 10% IL-3 conditioned medium (Karasuyama and Melchers, 1988), and 10% IL-6 conditioned medium (Harris et al., 1992) at 37°C (5% CO_2/95% air, humidified atmosphere). Metaphase cell preparations and SKY analyses were performed as previously described (Le Beau et al., 2002).

Quantitative PCR analysis. Genomic DNA was isolated from spleen and/or bone marrow cells upon death of the animal. Quantitative PCR analyses were performed to determine gene copy number of the wild-type *Myc* gene, *Pgk-hprt* (representing the null allele), and *β2-microglobulin* (primer and probe sequences are available upon request). Copy number for *Myc* and *Pgk-hprt* was standardized to *β2-microglobulin* and compared with a reference curve generated using allelic ratios of *Myc* (ranging from 50–100% of alleles represented in the sample). Q-PCR values between 0.6 and 1.5 were scored as a copy number of 1, values between 1.6 and 2.5 were scored as 2 copies, and values between 2.6 and 3.5 were scored as 3 copies. When both spleen and bone marrow from the same animal were assessed, the values were averaged.

Cell lysates and immunoblotting. Cells from bone marrow and spleen were collected and lysed in RIPA lysis buffer (containing 25 mM Tris.HCl, Hepes, pH 7.6, 150 mM NaCl, 1% Nonidet-P40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Protein concentrations were determined using the Bio-Rad BCA protein assay kit (Bio-Rad Laboratories). Proteins were separated on 7.5% SDS–polyacrylamide gels and transferred to PVDF membranes (Millipore) and incubated with c-Myc antibody 1:500 (SC-764; Santa Cruz

Biotechnology, Inc.) or β-actin antibody 1:5,000 (mAbcam 8226; Abcam). An enhanced chemiluminescence blotting analysis system (GE Healthcare) was used to detect antigen–antibody complexes. Resolution, brightness, and contrast were adjusted for scanned image shown in Fig. 4 A. Density of each band was quantified using ImageJ software (National Institutes of Health), and MYC signals were normalized to β-actin for Fig. 4 B.

Cell staining, flow-cytometry, sorting, and analysis. For flow analyses of bone marrow, dead cells were excluded by staining with 7-AAD (20 μg/ml; Sigma-Aldrich), donor and recipient cells were separated by PE-conjugated anti–mouse 45.1 antibody (BD) staining, viral-transduced donor cells were identified by GFP expression, and lymphoid and erythroid lineage cells and Sca-1–positive stem and progenitor cells were recognized by stain with purified unconjugated antibodies to CD3, CD4, CD5, CD8, B220, Ter119, CD127, and HSC marker Sca-1 (eBioscience), followed by PE-Texas red–conjugated goat anti–rat antibody (Invitrogen). Maturation of myeloid cells was assessed by expression of Gr-1 (Pacific blue–conjugated anti–mouse Gr-1; eBioscience) and CD34 (biotinylated anti–mouse CD34; BioLegend). Flow-cytometric analysis and sorting were performed on a FACSAria II (BD) high speed digital sorter equipped with a 488 nm, 633 nm, and 407 nm lasers. Data were analyzed with FlowJo software version 8.3.3 for Mac (Tree Star, Inc.). To compare CD34 and Gr-1 expression in different samples to assess myeloid cell maturation, median fluorescence was drawn from 34,000 GFP^+ lymphoid and erythroid $lineage^-$ donor cells from each sample; representative histograms were based on 34,000 cells/sample. 50,000 GFP^+ myeloid cells were sorted as described above from each sample and stained with Wright's Giemsa stain (Thermo Fisher Scientific) per manufacturer's instructions. Differential cell counts (200 cells) were performed as previously described on blinded specimens (Kogan et al., 2002). For flow analyses of blood to assess relative reconstitution by transplanted cells, 50–100 μl peripheral blood was lysed with AKC lysis buffer before incubation with PE-conjugated anti–mouse CD45.1 antibody and biotin-conjugated anti–mouse CD45.2 antibody (BD) followed by addition of streptavidin-APC. Dead cells were excluded by 7-AAD. Flow-cytometric analysis was performed on a FACS LSRII (BD). Data were analyzed with FlowJo software (Tree Star, Inc.). For flow analyses of lineage of leukemic cells, cells were stained with antibodies to CD45, CD4, and CD117 (Kit) linked to PE-CY5 and CD90.1, CD34, CD8, and Gr-1 linked to biotin, followed by addition of streptavidin APC. Control antibodies included rat IgG2b-PE, rat IgG2a PE-CY5, rat IgG2a biotin, and mouse IgG2a biotin. Source for each antibody provided upon request. Flow cytometric analysis was performed on a FACSCalibur (BD) and data were analyzed with FlowJo software.

Human myeloid leukemia data. *MYC* expression data on human APLs were obtained from Gene Expression Omnibus Dataset Series GSE12662 (Payton et al., 2009) and GSE6891 (Verhaak et al., 2009), as well as from data available at http://www.stjuderesearch.org/data/AML1 (Ross et al., 2004). In addition, MYC expression data on 189 normal karyotype AMLs and 20 AMLs with +8 were obtained from GSE6891. Normalized levels that were not log transformed were used for analyses.

Statistical analyses. Comparisons were performed using Microsoft EXCEL, Student's *t* test, two-sided, unequal variance with one exception: comparison of *MYC* levels in human AMLs with normal karyotype and +8 addressed the hypothesis that going from 2 to 3 copies of MYC would increase expression levels and was therefore performed with Microsoft EXCEL, Student's *t* test, one-sided, unequal variance. Survival differences were assessed using Prism software, log-rank test. Differences shown for leukemia/lymphoma-free survival were similar if calculated based on total survival (unpublished data). χ^2 test in Microsoft EXCEL was used to assess difference in rate of gain of chromosome 15 in leukemias arising in the presence versus absence of MYC or MYC-variant retroviruses.

Online supplemental material. Supplemental Tables include cytogenetic data from PML-RARα + MYC, PML-RARα + MYC^{T58A}, PML-RARα + $MYC^{\Delta MBII}$ leukemias was well as from leukemias arising in *PML-RARA* transgenic mice (not transduced with retroviruses and with two copies of the wild-type Myc allele). Fig. S1 shows the histology of bone marrow, spleen, and liver 5 wk after transplantation of control or PML-RARα bone marrow transduced with MIG or MYC retroviruses indicating initial effects of combined PML-RARα + MYC. Fig. S2 shows that MYC^{T58A} and $MYC^{\Delta MBII}$ mutants also cooperated with PML-RARα to impair myeloid maturation and to initiate leukemogenesis. Fig. S3 shows that human *MYC* is more highly expressed in human APL than in normal promyelocytes and that *MYC* is increased in human AML with trisomy 8 as compared with AML with a normal karyotype. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091071/DC1.

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