Use of chromosome engineering to model a segmental deletion of chromosome band 7q22 found in myeloid malignancies

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Monosomy 7 and del(7q) are associated with adverse features in myeloid malignancies. A 2.5-Mb commonly deleted segment (CDS) of chromosome band 7q22 is implicated as harboring a myeloid tumor suppressor gene (TSG); however, molecular analysis of candidate TSGs has not uncovered loss of function. To determine whether haploinsufficiency for the 7q22 CDS contributes to myeloid leukemogenesis, we performed sequential gene targeting to flank a region of orthologous synteny on mouse chromosome band 5A3 with *loxP* sites. We then generated *Mx1-Cre*, 5A3^{fl} mutant mice and deleted the targeted interval in vivo. Although excision was inefficient, we confirmed somatic deletion of the *5A3* CDS in the hematopoietic stem cell compartment. *Mx1-Cre, 5A3^{fl}* mice show normal hematologic parameters and do not spontaneously develop myeloid malignancies. The *5A3^{fl}* deletion does not cooperate with oncogenic *Kras^{G12D}* expression, *Nf1* inactivation, or retroviral mutagenesis to accelerate leukemia development and did not modulate responsiveness to antileukemia drugs. These studies demonstrate that it is feasible to somatically delete a large chromosomal segment implicated in tumor suppression in hematopoietic cell populations in vivo; however, our data do not support the hypothesis that the 7q22/5A3 CDS interval contains a myeloid TSG. (*Blood*. 2010;115(22): 4524-4532)

Introduction

Loss of chromosome 7 and deletion of a segment of the long arm (monosomy 7 and del(7q)) are recurring cytogenetic abnormalities in de novo and therapy-induced myeloid malignancies that are associated with advanced age, antecedent myelodysplastic syndrome (MDS), and resistance to current treatments.[1] Based on precedents in other cancers, it is likely that loss of one or more 7q tumor suppressor genes (TSGs) contributes to leukemogenesis. To facilitate the identification of candidate myeloid TSGs, Le Beau et al delineated 2 commonly deleted segments (CDSs) in patients with myeloid disorders characterized by a del(7q), a proximal interval within band q22 that accounts for most cases, and a second CDS in bands q32-34.[2] Using an ordered set of yeast artificial chromosome clones as probes, these investigators then performed fluorescence in situ hybridization (FISH) experiments to further characterize leukemias with deletion breakpoints within 7q22 and implicated an approximately 2.5-Mb CDS as harboring a myeloid TSG. We and others have extensively characterized this CDS, identified and cloned multiple genes from the interval, analyzed leukemia samples for mutations in these candidate TSGs, and performed Taqman real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) assays to measure expression levels in normal and leukemic human bone marrows.[3-6] These studies did not uncover biallelic inactivation or epigenetic silencing of any candidate TSGs located within this CDS.[3-5] Thus, it was hypothesized that inactivation of a single allele (haploinsufficiency) of one or more TSGs located within the 2.5-Mb CDS might contribute to leukemogenesis.[4,5]

Recent technical advances such as high-throughput sequencing platforms and RNA interference (RNAi) provide powerful new tools for approaching the challenging problem of identifying haploinsufficient TSGs. Indeed, elegant RNAi-based studies by Ebert et al provided strong support for *RPS14* as a haploinsufficient disease gene in the 5q− syndrome subtype of MDS.[7] The use of "chromosome engineering" to delete large DNA segments in the mouse is another potent technique that can be harnessed to interrogate a region suspected of harboring a haploinsufficient TSG in vivo.[8,9] The essence of this strategy is to embed 2 complementary, but nonfunctional, fragments of a hypoxanthine phosphoribosyl transferase *(HPRT)* minigene cassette within *loxP*-containing targeting vectors that flank the region of interest. Cre recombination between the chromosomal *loxP* sites then joins the 2 *HPRT* fragments together and creates a functional *HPRT* minigene that can be used as a selectable genetic marker to identify embryonic stem (ES) clones with the desired recombination event. Advantages of chromosome engineering include the following: (1) it provides a viable, function-based alternative to traditional positional cloning strategies; (2) it represents an unbiased approach to identify segments that contain more than one TSG whose combined loss is required for the desired phenotype; and (3) it generates murine models of human disease that can be used to test new therapies and to study the biology of any embedded TSGs. Work that identified *CHD5* as a TSG within 1p36.3 illustrates the potential of this

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strategy for cloning human cancer genes.[10] Interestingly, many cancers with 1p36 loss show large deletions, and a recent study implicating *K1F1B*β as a second 1p36 TSG[11] raises the possibility that haploinsufficiency of several tumor suppressor genes contributes to some human cancers.

We developed mice harboring *loxP* sites that flank a 2-Mb DNA segment of mouse chromosome band *5A3* that is syntenic to the human 7q22 CDS found in myeloid malignancies and bred this strain with *Mx1-Cre* mice to induce the desired deletion in the hematopoietic compartment. Here we show that heterozygous *Mx1-Cre, 5A3$^{fl/+}$* and homozygous *Mx1-Cre, 5A3$^{fl/fl}$* compound mutant mice excise the *5A3* interval in a small percentage of bone marrow cells that includes some hematopoietic stem cells (HSCs). These mice display normal steady-state hematopoiesis and do not spontaneously develop MDS or acute myeloid leukemia (AML). Intercross experiments showed that coinheritance of the excised *5A3$^{fl/+}$* segment does not cooperate with either oncogenic *Kras* expression or *Nf1* inactivation in leukemogenesis. Similarly, induction of the *Mx1-Cre 5A3$^{fl/+}$* allele did not affect the latency and penetrance of leukemia in mice that were injected with the MOL4070LTR retrovirus.[12] In addition, loss of the *5A3* interval did not alter the response of MOL4070LTR-induced AMLs to antileukemia drugs. Together, these comprehensive studies suggest that the 7q22/*5A3* interval is unlikely to harbor a haploinsufficient myeloid TSG. Our data demonstrate the feasibility of deleting large DNA segments in the HSC compartment in vivo and as a late event in leukemic cell populations, which may prove useful for creating strains of mice to investigate other chromosomal deletions found in human hematologic malignancies.

Methods

Generation of *5A3^{fl}* mice

A 6.4-kb fragment containing part of introns 14 to 15, exon 15, and a portion of the 3′ untranslated region of the *Srpk2* gene was cloned in the *Not*I and *Spe*I sites in the pG12-WT-NP-IRES-GFP vector (kind gift from Dr Allan Bradley, Wellcome Trust Sanger Institute, Cambridge, United Kingdom) and linearized with *Eco47*III. E14 ES cells (129P2 strain) were electroporated with 25 μg of linearized *Srpk2* vector, and the clones were selected in puromycin (2 μg/mL) for 8 to 10 days. Correct gene targeting event was assessed by digesting ES cell DNA with *Hin*dIII and hybridizing Southern blots with a 1.3-kb probe containing sequences downstream of the genomic DNA used to construct the targeting vector. Three positive clones were expanded for a second round of targeting with a vector containing a 7.8-kb fragment of *Lrrc17* genomic DNA that includes parts of intron 1, intron 2, and exon 2 in the pL13-NP backbone (kind gift from Dr Allan Bradley). Singly targeted ES cells were electroporated with 25 μg of linearized vector, and clones were selected in G418 (250 μg/mL) for 8 to 10 days. The desired gene targeting event was assessed by digesting the DNA with *Bam*HI, followed by Southern blotting and hybridization with a 0.7-kb probe containing sequences downstream of the genomic DNA used to construct the targeting vector. Southern analysis was performed using Church-Gilbert solution at 60°C, and a final wash in 0.2× saline-sodium citrate solution, 0.1% sodium dodecyl sulfate.

Genotyping

Leukocyte DNA was isolated using the GFX genomic blood purification kit (Amersham), and tissue DNA was isolated as described.[13] Genotyping to assess inheritance of the *Mx1-Cre* transgene, presence of the correct *Lrrc17* integration flanking the targeted *5A3* interval, and somatic rearrangement to create the desired deletion was performed by PCR. The primers and assay conditions are given in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Analysis of gene expression

Total RNA was isolated from mouse bone marrow and frozen leukemia cells using the QIAGEN RNeasy Mini kit and treated with TURBO DNA-*free* kit (Ambion) according to the manufacturer's protocol. Total RNA (350 ng) was reverse transcribed using the High Capacity RNA-to-cDNA Master Mix according to the manufacturer's protocol (Applied Biosystems). The relative quantification of gene expression was performed by quantitative real-time PCR using the TaqMan Gene Expression Assays (Applied Biosystems). The primers, probes, and assay conditions are given in supplemental Methods.

Mouse strains and polyinosinic-polycytidilic acid treatment

Polyinosine-polycytidine (pIpC; Sigma or Amersham) was resuspended in phosphate-buffered saline at 10 mg/mL or 1 mg/mL. Mice received either a single dose of 100 μL of 10 mg/mL pIpC at 3 days or a single dose of 250 μL of 1 mg/mL pIpC at 3 weeks. For a high-dose regimen, mice received a dose of 250 μL of 1 mg/mL pIpC every other day 5 times. The breeding strategies for mouse strains are described in supplemental Methods. All mice involved in the research were housed in a specific pathogen–free facility at the University of California San Francisco, and all animal experiments were conducted under protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

Disease monitoring and isolation of hematopoietic cells

Hematopoietic cell collection and pathologic and flow cytometric analysis were carried out as previously described.[14]

MOL4070LTR virus and integration site cloning

A MOL4070LTR viral stock was generated as described elsewhere.[12] *5A3$^{+/fl}$* and *Mx1-Cre* mice were bred to generate *Mx1-Cre, 5A3$^{+/fl}$* pups on a F1 strain background, which were inoculated with 100 μL of MOL4070LTR virus intraperitoneally and monitored for signs of disease. MOL4070LTR integration sites were identified by performing PCR amplification (supplemental Methods) followed by either direct 454 sequencing or by cloning the products using a Zero Blunt cloning kit (Invitrogen), transforming TOP10 competent cells, and sequencing DNA minipreps isolated from bacterial cultures.

Adoptive transfer

For serial transplantation of recombined bone marrow, adult recipient mice were lethally irradiated with 9.5 Gy from a cesium source. AML transplant recipients were irradiated with 4.5 Gy. After irradiation, the cells were injected into the retro-orbital sinus of recipient mice.

Treatment with doxorubicin and Ara-C

Mice that were injected with AMLs were treated with intraperitoneal cytosine arabinoside (Ara-C; 100 mg/kg once daily for 5 days) and doxorubicin (3 mg/kg once daily for 3 days). The drugs were administered together.

Results

Generation of conditional *5A3$^{+/fl}$* mice

The human 7q22 CDS defined by Le Beau et al[2] is flanked by the *SRPK2* and *FBXL13* genes, includes 14 genes, and does not contain any microRNA or noncoding RNAs (Figure 1A). This interval corresponds to a single region of orthologous synteny on mouse chromosome band 5A3 (Figure 1A). We isolated λ phage clones containing *Srpk2* and *FbxL13* and used these to construct targeting vectors (Figure 1B). Thirteen doubly targeted ES cell clones that carry *loxP* insertions in *FbxL13* and *Srpk2* were generated through

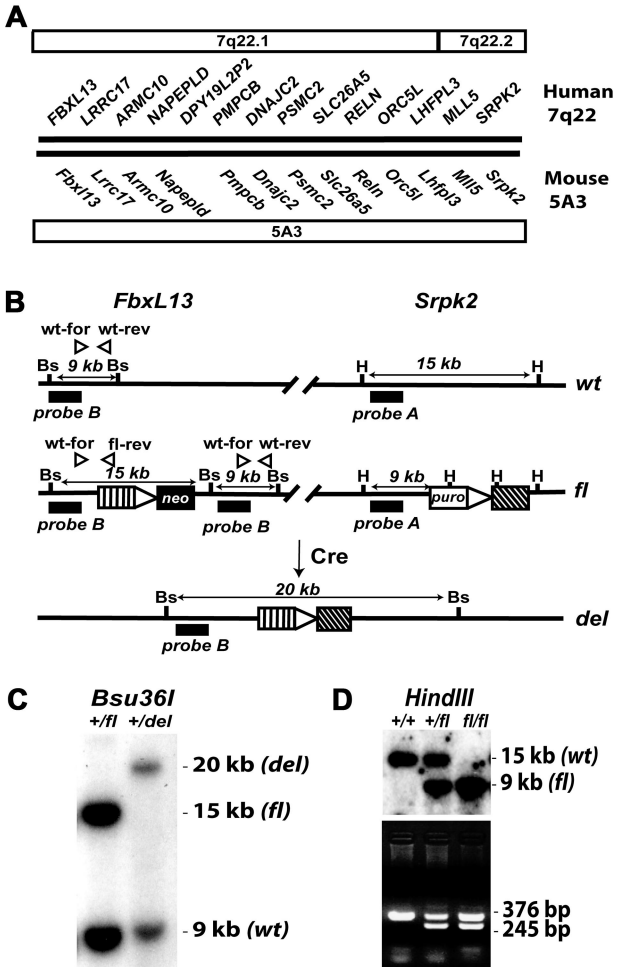

Figure 1. Comparative mapping and gene targeting of a region of mouse chromosome 5A3 syntenic to a commonly deleted segment of 7q22 identified in human myeloid malignancies. (A) Gene content and order in the human 7q22 CDS compared with that of a region in mouse chromosome 5A3 (based on the Ensembl database and not drawn to scale). (B) Overview of the gene targeting strategy. Targeting at *Fbxl13/Lrrc17* resulted in integration of a *loxP* site (triangle), a neomycin resistance cassette (neo), and the 5′ half of the *Hprt* minigene (hatched box) at the endogenous (wild-type, wt) *Fbxl13* locus, whereas gene targeting at *Srpk2* resulted in integration of a *loxP* site (triangle), a puromycin resistance cassette (puro), and the 3′ *Hprt-ires-gfp* (cross-hatched box) at the endogenous (wt) *Srpk2* locus. Expression of Cre recombinase results in *cis* recombination between the *loxP* sites contained in the 2 complementary cassettes and reconstitution of a functional *Hprt* cDNA. The parental cells are resistant to both G418 and puromycin and the recombined cells excise the drug resistance cassettes and are therefore sensitive to both antibiotics. The positions of primers for genotyping analysis (arrowheads) and probes used for Southern blots (solid boxes) are indicated. (C) Confirmation of the deletion of targeted *5A3* region in ES cells. Genomic DNA isolated from doubly targeted ES cells before and after the expression of Cre recombinase were digested with *Bsu36*I restriction enzyme (Bs), followed by hybridization with probe B (B). Whereas the 15-kb and 9-kb bands diagnostic of the targeted (*fl*) and wt (+) alleles, respectively, were observed in the doubly targeted ES cells before Cre recombination, a 20-kb band diagnostic of the deleted (*del*) allele was observed instead of the 15-kb floxed band after Cre expression. (D) Germline transmission of the latent mutant allele was assessed by Southern blotting (top). Tail DNA that is digested with *Hin*dIII (H) restriction enzyme and hybridized with probe A (B) shows a 15-kb fragment that corresponds to the *wt* allele and a 9-kb fragment derived from the *fl* allele. Confirmation of targeting at 5′ end using PCR genotyping (bottom). Primer fl-reverse (rev) is specific to the targeted allele. The wt allele results in a 376-bp fragment when amplified with wt-forward (for) and wt-rev primers, whereas the targeted allele gives a 245-bp fragment when amplified with wt-for and fl-rev primers. The targeted allele retained the wt amplification product due to duplication of the region of homology contained in the vector when using the insertional targeting strategy.

sequential rounds of gene targeting (supplemental Figure 1A-B), and these clones were electroporated with a vector encoding Cre recombinase and grown in media containing neomycin or puromycin. Four clones were sensitive to both drugs, indicating that targeting had occurred in the correct relative orientation in *cis* (ie, on the same chromosome 5 homolog). The desired Cre-mediated recombination event was confirmed by Southern blot analysis (Figure 1C and supplemental Figure 1C). We injected doubly targeted (unrecombined) ES clones into blastocysts to generate $5A3^{+/fl}$ mice. Germline transmission of the latent mutant allele was screened by coat color, and confirmed by Southern blot and PCR-based genotyping (Figure 1D). Heterozygous $5A3^{+/fl}$ and homozygous $5A3^{fl/fl}$ mice were born at the expected ratio, and were grossly normal and fertile. To induce excision of the *5A3* interval, we transduced bone marrow cells and mouse embryonic fibroblasts with Cre-expressing retroviral vectors in vitro. However, we observed Cre-mediated cellular toxicity, leading to markedly reduced cell proliferation and changes in cellular morphology.[15,16] Attempts to circumvent Cre-mediated cellular toxicity by transduction using adenoviral or self-excising retroviral vectors encoding Cre recombinase never achieved detectable levels of recombination (data not shown).

Recombination, survival, and hematologic parameters in *Mx1-Cre,* $5A3^{fl/+}$ and *Mx1-Cre,* $5A3^{fl/fl}$ mice

The interferon-inducible *Mx1-Cre* transgene is broadly expressed in hematopoietic cells and has been used extensively to develop mouse models of hematologic malignancies.[14,17-20] We mated $5A3^{fl/+}$ and *Mx1-Cre* mice, and administered a single dose of pIpC at 3 days of life to induce Cre recombinase expression. Compound *Mx1-Cre,* $5A3^{fl/+}$ and *Mx1-Cre,* $5A3^{fl/fl}$ mutant mice appeared normal. PCR analysis of genomic DNA isolated from blood leukocytes confirmed the desired recombination in mice that were tested between 8 weeks and 2 years of age (Figure 2A). Cohorts of *Mx1-Cre,* $5A3^{fl/+}$ (n = 35), *Mx1-Cre,* $5A3^{fl/fl}$ (n = 25), *Mx1-Cre*–negative $5A3^{fl/+}$ (n = 20), $5A3^{fl/fl}$ (n = 22), and wild-type (wt; n = 20) littermates were aged for 2 years to characterize any disease phenotypes. These groups of mice showed no significant differences in overall survival, causes of death, or blood counts (Figure 2B and data not shown). Southern blotting of bone marrow DNA was performed to assess the efficiency of *Mx1-Cre*–mediated recombination. Although the level of recombination was low in most *Mx1-Cre,* $5A3^{fl/+}$ and *Mx1-Cre,* $5A3^{fl/fl}$ mice (center and right lanes in Figure 2A), 3 (16%) of 19 *Mx1-Cre,* $5A3^{fl/+}$ mice showed high levels of recombined cells at death (see left lane in Figure 2A). Each of these *Mx1-Cre,* $5A3^{fl/+}$ mice was visibly sick, and pathologic analysis demonstrated centroblastic-type diffuse large B-cell lymphoma, histiocytic sarcoma, and possible infection in one animal each at 586 days, 755 days, and 568 days of age, respectively.

To determine whether increasing the percentage of recombined cells might modify the phenotype, we administered additional doses of pIpC (250 μg every other day for 5 doses) to 8-week-old *Mx1-Cre,* $5A3^{fl/+}$ and *Mx1-Cre,* $5A3^{fl/fl}$ mice. Southern blotting demonstrated higher levels of recombination in bone marrow and spleen 4 days after the last pIpC dose in comparison with mice that received a single dose of pIpC after birth (Figure 2C). We isolated bone marrow RNA 1 day after the final dose of pIpC and performed RT-PCR to assess the expression of 5 genes located within the *5A3* region. Although this analysis was complicated by the effects of sequential injection of pIpC on gene expression in hematopoietic cells and by incomplete excision of the *5A3* interval, the overall expression of all 5 genes after pIpC treatment was lower in *Mx1-Cre,* $5A3^{fl/+}$ mice (Figure 2D). However, this difference achieved statistical significance only for *Mll5* (Figure 2D). We aged 5 *Mx1-Cre,* $5A3^{fl/+}$ and 3 *Mx1-Cre,* $5A3^{fl/fl}$ mice that received

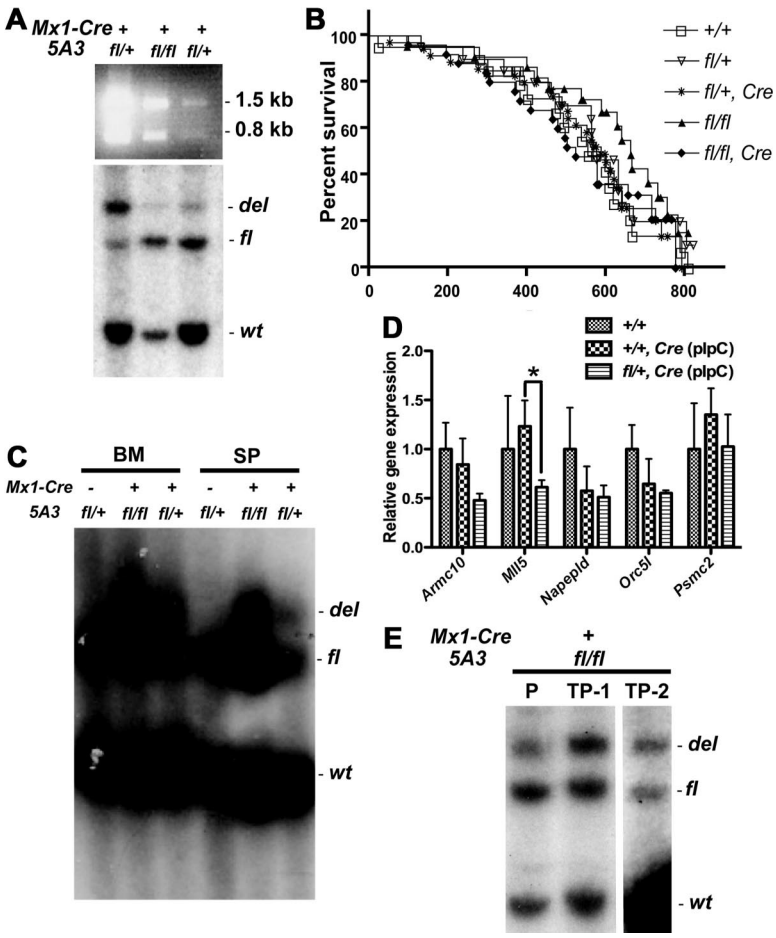

Figure 2. Recombination of the *5A3^{fl}* interval in vivo and survival of *Mx1-Cre, 5A3$^{fl/+}$* and *Mx1-Cre, 5A3$^{fl/fl}$* mice. (A) Analysis of the deleted allele in hematopoietic cells from *Mx1-Cre, 5A3$^{fl/+}$* and *Mx1-Cre,5A3$^{fl/fl}$* mice. (Top) PCR analysis of the deleted *(del)* allele in genomic DNA extracted from peripheral blood from mice of the indicated genotype at the time of killing. Because primers specific for the 5′ and 3′ *hprt* gene cassettes were used, the 2.3-kb PCR product is obtained only after *loxP* recombination. To confirm the specificity of the reaction, PCR products were digested with *Eco*RI, resulting in fragments of 1.5 kb and 0.8 kb. (Bottom) Southern blot analysis of genomic DNA isolated from the bone marrows (BMs) of the same mice as the top panel. The DNA was digested with the *Bsu36*I restriction enzyme and hybridized with probe B (Figure 1B). The wild-type (*wt*), floxed (*fl*), and deleted (*del*) alleles yield DNA fragments of 9, 15, and 20 kb, respectively. (B) Kaplan-Meier survival curve of *wt* (+/+; n = 20), *5A3$^{fl/+}$* (*fl/+;* n = 20), *Mx1-Cre, 5A3$^{fl/+}$* (*fl/+,Cre;* n = 35), *5A3$^{fl/fl}$* (*fl/fl;* n = 22), and *Mx1-Cre, 5A3$^{fl/fl}$* (*fl/fl, Cre;* n = 25) mice ($P = .499$). Mice that were killed without visible sign of disease were censored. (C) Southern blot analysis of genomic DNA from the bone marrows (BMs) and spleens (SPs) of *5A3$^{fl/+}$*, *Mx1-Cre*, *5A3$^{fl/fl}$*, and *Mx1-Cre*, *5A3$^{fl/+}$* mice 4 days after treatment with a high-dose pIpC regimen. The DNA was digested with *Bsu36*I and hybridized with probe B as in panel A. (D) Quantitative RT-PCR analysis of the expression of 5 genes within the *5A3* interval in the BM of *Mx1-Cre, 5A3$^{+/+}$* and *Mx1-Cre, 5A3$^{fl/+}$* mice after treatment with high-dose pIpC. Total RNA isolated from the BM of *Mx1-Cre, 5A3$^{+/+}$* (*+/+, Cre;* n = 3) and *Mx1-Cre, 5A3$^{fl/+}$* mice (*fl/+, Cre;* n = 3) was analyzed to assess expression of *Armc10*, *Mll5*, *Napepld*, *Orc5l*, and *Psmc2*. Expression levels were determined in triplicate, normalized to *Gapdh,* and calibrated to the levels found in untreated wt mouse bone marrow (+/+; n = 4). The graph shows means ± SD for each group. Statistical significance was determined using the Student *t* test (*$P \leq .05$). (E) Southern blot analysis of BM DNA from a *Mx1-Cre*, *5A3$^{fl/fl}$* mouse (P) that was killed 4 days after treatment with high-dose pIpC, and its transplant recipient (TP-1) killed 4 months later. TP-2 is the transplant recipient of bone marrow cells from TP-1, and killed 4 months after transplantation. The DNA was digested with *Bsu36*I and hybridized with probe B as in panel A. A vertical space has been inserted between the TP-1 and TP-2 lanes to indicate that they are from 2 separate experiments.

high-dose pIpC for 21 to 65 weeks. All remained free of disease and had normal blood counts at death.

The *5A3^{fl}* interval is deleted in HSCs

The observation that recombined cells persist in the blood of *Mx1-Cre, 5A3$^{fl/+}$* and *Mx1-Cre, 5A3$^{fl/fl}$* mice 2 years after a single injection of pIpC suggests that the *5A3* interval is excised in HSCs. Consistent with this idea, PCR analysis of DNA extracted from c-kit$^+$ Lin$^-$ Sca-1$^+$ (KLS) bone marrow cells from *Mx1-Cre, 5A3$^{fl/fl}$* mice that were treated with high-dose pIpC demonstrated recombination in this population (data not shown). To directly assess the long-term repopulating activity of recombined cells, we transplanted bone marrow from *Mx1-Cre, 5A3$^{fl/fl}$* mice into lethally irradiated wt recipients 4 days after high-dose pIpC treatment. These cells efficiently established donor-derived hematopoiesis that persisted for more than 4 months, which was characterized by the presence of *5A3*-excised hematopoietic cells as detected by Southern blot analysis of the bone marrow (Figure 2E). Injecting bone marrow cells from these mice into secondary recipients resulted in donor-derived hematopoiesis with the *5A3* deletion again detected by Southern blotting. The intensity of the recombined fragment in comparison with the unrecombined fragment, which was a reflection of the proportion of *5A3*-excised cells in the bone marrow, was relatively stable over serial transplantation (Figure 2E). Together, these functional data demonstrating that *5A3*-excised cells possess long-term repopulating potential and contribute to hematopoiesis in secondary recipients infer that some HSCs contain this deletion.

Effects of the *5A3* deletion in *Kras* and *Nf1* mutant mice

Activating a latent *Kras^{G12D}* oncogene in *Mx1-Cre, LSL-Kras^{G12D}* mice induces an aggressive myeloproliferative disorder (MPD) that models juvenile and chronic myelomonocytic leukemias.[18,21] This MPD is fully penetrant and results in death 70 to 90 days after a single injection of pIpC. Somatic inactivation of *Nf1* in *Mx1Cre, Nf1$^{fl/fl}$* mice causes a less aggressive monocytic MPD than *Mx1-Cre, LSL-Kras^{G12D}* mice.[14] Based on data showing that monosomy 7/del(7q) coexists with *NRAS, KRAS,* and *NF1* mutations in patients with de novo and therapy-induced myeloid malignancies,[22-26] we generated *Mx1-Cre, LSL-Kras^{G12D}, 5A3$^{fl/+}$* and *Mx1-Cre, Nf1$^{fl/fl}$, 5A3$^{fl/+}$* compound mutant mice to determine whether the *5A3* deletion alters the phenotype or latency of MPD. As shown in Figure 3A, all *Mx1-Cre, LSL-Kras^{G12D}* mice developed MPD with anemia as previously described after receiving a single dose of pIpC at weaning.[18,21] The survival of *Mx1-Cre, LSL-Kras^{G12D}, 5A3$^{fl/+}$* mice was similar to *Mx1-Cre, LSL-Kras^{G12D}* littermates (Figure 3B). Southern blot analysis revealed the rearranged *5A3* allele in bone marrow cells from compound mutant mice with MPD (Figure 3C). To determine whether the *5A3* interval was deleted in the cells that have activated the latent *Kras^{G12D}* allele, we assayed colony-forming unit granulocyte-macrophage (CFU-GM) growth in methylcellulose cultures in the absence of cytokines. Bone marrow cells from *Mx1-Cre, LSL-Kras^{G12D}* mice demonstrate growth factor–independent CFU-GM growth only after *Kras^{G12D}* expression is induced by Cre-mediated excision of the inhibitory *LSL* cassette[18,21] (and data not shown). As expected, *Mx1-Cre,*

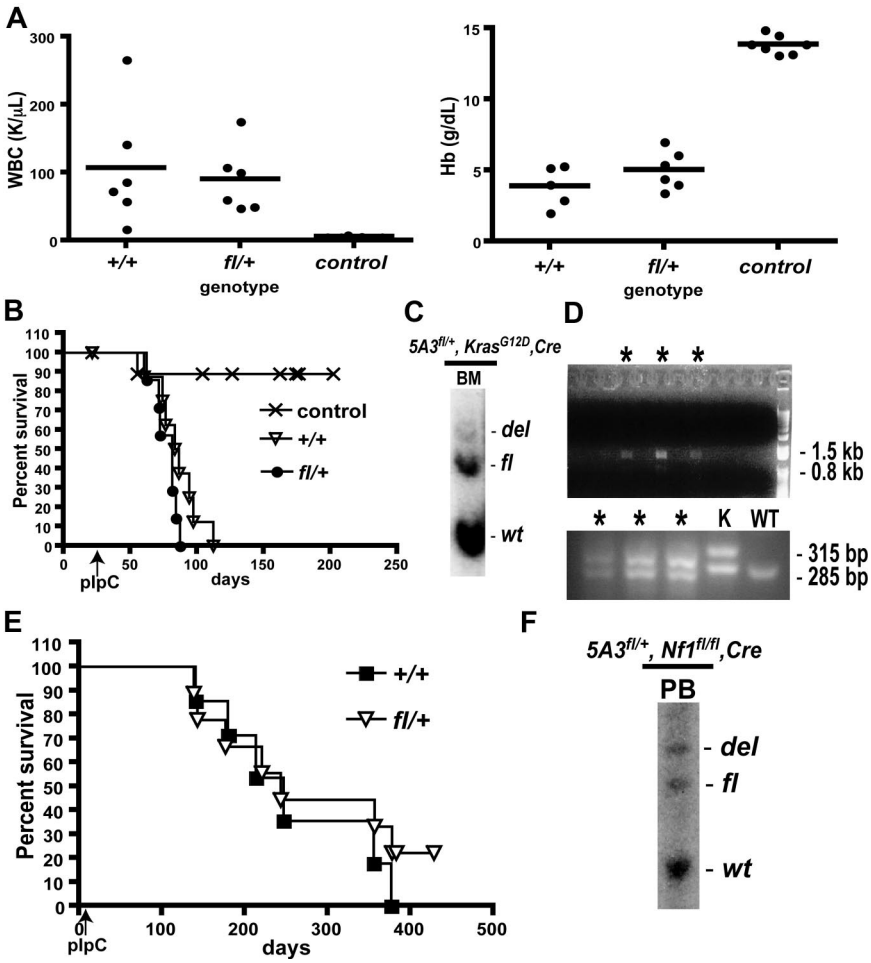

Figure 3. Analysis of *Mx1-Cre, LSL-Kras^{G12D}, 5A3$^{fl/+}$*, and *Mx1-Cre, Nf1$^{fl/fl}$, 5A3$^{fl/+}$* compound mutant mice. (A) Comparison of the white blood count (A left, $P = .692$) and hemoglobin (A right, $P = 0.120$) of *Mx1-Cre, LSL-Kras^{G12D}, 5A3$^{+/+}$ (+/+), Mx1-Cre, LSL-Kras^{G12D},5A3$^{fl/+}$ (fl/+),* and control *Mx1-Cre* negative littermates (control) at death after treatment with pIpC at weaning. (B) Kaplan-Meier survival curve of *Mx1-Cre*–negative (*control;* n = 9), *Mx1-Cre, LSL-Kras^{G12D}, 5A3$^{+/+}$* (+/+, n = 8), and *Mx1-Cre, LSL-Kras^{G12D}5A3$^{fl/+}$* (*fl/+*, n = 7) mice ($P = .136$). pIpC treatment at 21 days is indicated with arrow. (C) Southern blot analysis of genomic DNA from the bone marrow (BM) of a *Mx1-Cre, LSL-Kras^{G12D}, 5A3$^{fl/+}$* mouse showing the presence of the deleted allele. (D) *Kras^{G12D}* expression and deletion of the *5A3* region in the individual CFU-GM colonies. (Top) Bone marrow cells from a *Mx1-Cre, LSL-Kras^{G12D}5A3$^{fl/+}$* mouse were plated in methylcellulose medium without added cytokines. CFU-GM colonies that formed after 7 days were picked and lysed, and the presence of the deleted *5A3* allele was confirmed by PCR. Asterisks indicate the presence of the recombination PCR product. (Bottom) Cre-mediated recombination of the inhibitory *LSL* cassette 5′ of the *Kras^{G12D}* gene was verified in the same cells (*) by PCR. The 285-bp product is indicative of the wt *Kras* locus, whereas the 315-bp product is specific for excised *LSL-Kras^{G12D}* allele. The unrearranged *LSL-Kras^{G12D}* allele does not amplify. PCR conditions were confirmed with peripheral blood DNA from *Mx1-Cre, LSL-Kras^{G12D}5A3$^{fl/+}$* (K) and wt (WT) mice. (E) Kaplan-Meier survival curve of *Mx1-Cre, Nf1$^{fl/fl}$, 5A3$^{+/+}$* (+/+, n = 7) and *Mx1-Cre, Nf1$^{fl/fl}$, 5A3$^{fl/+}$* (*fl/+*, n = 9) mice. $P = .403$. (F) Southern blot of genomic DNA from the peripheral blood of a *Mx1-Cre, Nf1$^{fl/fl}$, 5A3$^{fl/+}$* mouse showing the presence of the deleted allele.

LSL-Kras^{G12D}, 5A3$^{fl/+}$ progenitors also formed "spontaneous" CFU-GM colonies in the absence of cytokines, and PCR analysis of DNA from individual colonies revealed recombination of both the *5A3* interval and *LSL* cassette in approximately 30% of them (Figure 3D).

Because the aggressive MPD in *Mx1-Cre, LSL-Kras^{G12D}* mice could have obscured modest effects of the *5A3* deletion, we generated *Mx1Cre, Nf1$^{fl/fl}$ 5A3$^{fl/+}$* mice. As shown in Figure 3E, the survival of *Mx1-Cre, Nf1$^{fl/fl}$, 5A3$^{fl/+}$* mice and of control *Mx1-Cre, Nf1$^{fl/fl}$* littermates that received a single dose of pIpC at 3 days was similar. In addition, *Mx1-Cre, Nf1$^{fl/fl}$, 5A3$^{fl/+}$* and *Mx1-Cre, Nf1$^{fl/fl}$* mice developed a similar MPD (data not shown).[14] Importantly, transformation to leukemia did not occur, even given the more indolent clinical course. We confirmed that cells with *5A3* deletion were present in the blood leukocytes of moribund *Mx1-Cre, 5A3$^{fl/+}$, Nf1$^{fl/fl}$* mice (Figure 3F). Together, our studies of *Kras* and *Nf1* mutant mice infer that *5A3* deletion neither confers a selective growth advantage nor alters the phenotype in mice that develop MPD due to hyperactive Ras signaling.

Retroviral insertional mutagenesis

Cytogenetic, molecular, and clinical studies of myeloid malignancies with monosomy 7/del(7q) strongly support a multistep model of leukemogenesis.[27,28] We used the MOL4070LTR retrovirus[12] to perform a forward genetic screen to assess the effects of the *5A3* deletion on the latency, penetrance, and phenotype of leukemia. *Mx1-Cre, 5A3$^{fl/+}$* and littermate control mice were injected with MOL4070LTR at 3 days of age, and this was followed by a single dose of pIpC at weaning. This design allowed us to investigate whether secondary loss of the *5A3* region confers a selective growth advantage and to screen any resulting leukemias for "second hit" integrations within the nontargeted *5A3* homolog. Mice were killed when they became moribund and hematopoietic tissues were collected for pathologic examination and molecular analysis. Although MOL4070LTR efficiently induced acute leukemia, the survival of *Mx1-Cre, 5A3$^{fl/+}$* mice was similar to that of control littermates (Figure 4A). Southern blot analysis of bone marrow from *Mx1-Cre, 5A3$^{fl/+}$* mice with leukemia revealed complete recombination of the *5A3* interval in 7 (23%) of 30 leukemias, partial recombination in 4 (13%), and no recombination in 19 (63%; Figure 4B). Serial transplantation confirmed that *5A3*-deleted leukemias could efficiently induce disease in sublethally irradiated secondary recipients (Figure 4B). Within the *Mx1-Cre, 5A3$^{fl/+}$* cohort, mice that developed leukemia with the loss of the *5A3* interval showed identical survival to mice in which the *5A3* deletion was not detected in the leukemic clone (data not shown). The 7 leukemias with complete loss of the *5A3* interval included 3 cases of acute myelomonocytic leukemia, 2 cases of AML without maturation, 1 case of MPD-like myeloid leukemia, and 1 case of T-cell acute lymphoblastic leukemia. This spectrum of disease is not significantly different from control littermates (data not shown).

We cloned retroviral integration sites from the 7 mice that developed *5A3*-deleted leukemias (supplemental Table 1). Importantly, none of these insertions mapped within the nontargeted *5A3* homolog. Integrations in or near the *Sox4* gene, which is a frequent

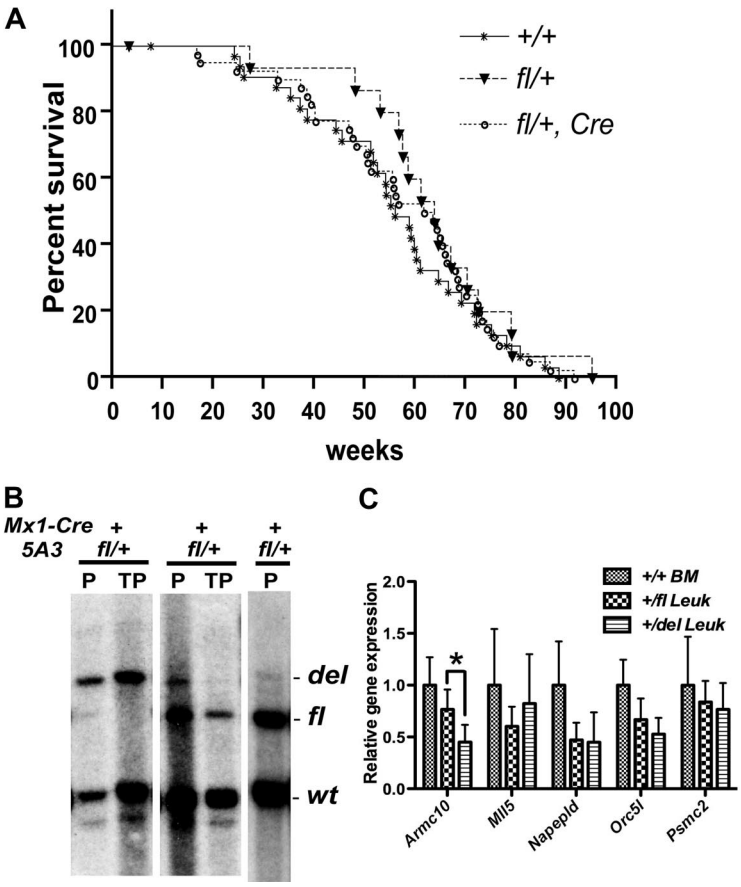

Figure 4. Retroviral mutagenesis with MOL4070LTR in *Mx1-Cre, 5A3$^{fl/+}$* mice. (A) Survival of *wt* mice (+/+; n = 32), control *5A3$^{fl/+}$* mice that did not inherit the *Mx1-Cre* transgene (*fl/+;* n = 15), and *Mx1-Cre, 5A3$^{fl/+}$* mice (*fl/+, Cre,* n = 30) treated with MOL4070LTR. In addition, we observed no difference in survival between *Mx1-Cre, 5A3$^{fl/+}$* mice with leukemia in which the latent *5A3* allele was recombined (n = 7) or not recombined (n = 23; data not shown). (B) Southern blot analysis of the bone marrow of 3 mice from the *Mx1-Cre, 5A3$^{fl/+}$* cohort that were diagnosed with acute myelomonocytic leukemia. The primary leukemia is designated P, and transplants are designated TP. The leukemia on the left (no. 40) demonstrated complete recombination, as indicated by the presence of a 20-kb band diagnostic of the *del* allele and the absence of the 15-kb band diagnostic of the *fl* allele. The leukemia in the center (no. 18) is partially recombined, as indicated by the presence of the *del* and *fl* bands at 20 kb and 15 kb, respectively. The leukemia on the right (no. 102) shows no evidence of the recombined *5A3* allele. (C) Quantitative RT-PCR analysis of the expression of 5 genes within the *5A3* interval in the unrecombined (*+/fl Leuk;* n = 4) and recombined (*+/del Leuk;* n = 7) leukemic cells from *Mx1-Cre, 5A3$^{fl/+}$* mice treated with MOL4070LTR. Total RNA was isolated from leukemic cells, and evaluated as described in Figure 2D. *Armc10* is the only gene that showed significantly reduced expression in *5A*3-excised leukemias (*).

common integration site in murine retroviral insertional mutagenesis screens,[29] was the only common integration site detected in *5A3*-deleted leukemias and occurred in 4 independent *5A3*-deleted AMLs (supplemental Table 2). By contrast, we did not detect *Sox4* integrations in any of the 16 unrecombined *5A3* leukemias (data not shown). However, quantitative RT-PCR analysis of *Sox4* expression did not reveal consistent changes in the *5A3*-deleted AMLs (supplemental Table 2). In summary, retroviral insertional mutagenesis demonstrates that the *5A3* deletion occurs in cells that can initiate acute leukemia in vivo. However, loss of the *5A3* interval had no significant effect on either the time to death or the phenotype of MOL4070LTR-initiated acute leukemias.

We also performed quantitative RT-PCR to compare the expression levels of 5 genes within the targeted *5A3* segment in AMLs that had either retained or lost the interval (Figure 4C). These studies revealed marked variability in gene expression in individual AMLs of both *5A3* genotypes, which could be due to the effects of cooperating mutations or the heterogeneous phenotypes of these leukemias.

Inducing the *5A3* deletion in established *Mx1-Cre, 5A3$^{fl/+}$* leukemias and treatment with chemotherapeutic agents

Clinical and epidemiologic data support the idea that monosomy 7 and del(7q) often occur as a cooperating event during leukemia progression.[22,25,27,30,31] The MOL4070LTR-induced *Mx1-Cre, 5A3$^{fl/+}$* AMLs in which the *5A3* allele was not recombined provided an opportunity to test the effects of inducing the *5A3* deletion in established leukemias. To test the system, we selected a *Mx1-Cre, 5A3$^{fl/+}$* leukemia with partial recombination (AML no. 18) and another in which the *5A3* interval is unrecombined (AML no. 102; Figure 4B center and right). We transplanted both leukemias into sublethally irradiated recipients and administered high-dose pIpC treatment beginning 10 days after transplantation (Figure 5A). As shown in Figure 5B, this protocol resulted in partial excision of the *5A3* region in both AMLs. However, serial transplantation of both leukemias, which are highly aggressive in secondary and tertiary recipients, did not result in outgrowth of recombined cells (data not shown).

Monosomy 7 and del(7q) are commonly observed in therapy-related leukemias and in other myeloid malignancies that are resistant to conventional chemotherapeutic regimens. To address whether loss of the *5A3* interval might modulate drug sensitivity in vivo, we identified clones of AMLs nos. 18 and 102 with approximately 50% recombined cells, transplanted them into sublethally irradiated mice, and treated these recipients with a regimen of doxorubicin and cytosine arabinoside that mimics AML induction therapy (Figure 5A).[32] All of the recipients with AML no. 18 transplant died 16 to 30 days later of progressive leukemia irrespective of treatment (Figure 5D). Southern blotting showed that *5A3* recombined cells were not selected during chemotherapy treatment, suggesting that this leukemia is resistant to doxorubicin and cytosine arabinoside regardless of the status of the *5A3* interval (Figure 5C). Treatment with doxorubicin and cytosine arabinoside in mice that received a transplant of AML no. 102 resulted in death due to severe leukopenia. Mice treated with the vehicle died of progressive leukemia in a similar time frame between 12 and 26 days (Figure 5E). These studies demonstrate that it is feasible to induce the *5A3* deletion as a late event in established AML clones and to administer and test the effects of therapeutic agents in leukemias that contain a mixture of unrecombined and recombined cells.

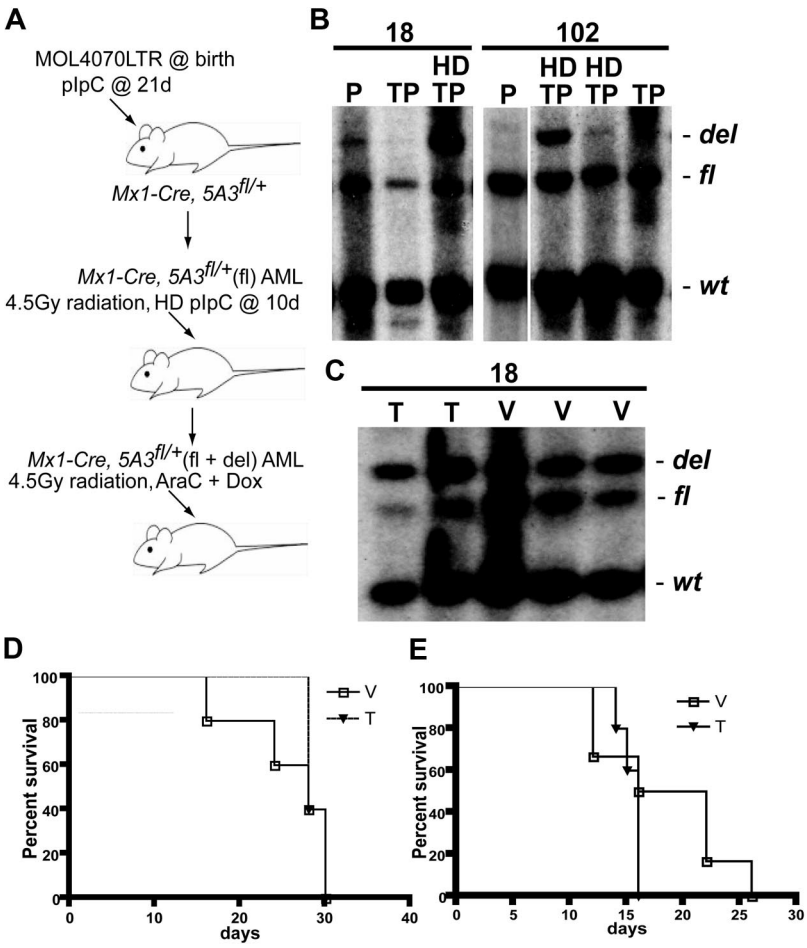

Figure 5. Generation of isogenic leukemia cells to test the effects of inducing the *5A3* deletion in established AMLs. (A) Overview of the experimental protocol used to induce the *5A3* deletion in established AMLs from MOL4070LTR treated *Mx1-Cre*, $5A3^{fl/+}$ mice. Primary leukemias were isolated from mice that had been injected with MOL4070LTR and pIpC, and transplanted into sublethally irradiated recipients. These recipients received a high-dose pIpC regimen beginning 10 days after transplantation, and leukemic cells were subsequently analyzed by Southern blotting to assess *5A3* recombination. Secondary leukemias that showed balanced ratio of recombined versus unrecombined cells were then reinjected into sublethally irradiated recipients that were treated with Ara-C and doxorubicin as described in "Treatment with doxorubicin and Ara-C" and monitored for survival. (B) Southern blot analysis of BM DNA from the primary mice nos. 18 and 102 (P) and recipients of these leukemias (TP). Note enrichment of the 20-kb recombined fragment in BM DNA from transplant recipients that were treated with high-dose pIpC (HD). (C) Southern blot analysis of BM DNA from the recipients of leukemia no. 18 after treatment with chemotherapy (T) or vehicle (V). (D) Kaplan-Meier survival curve of mice that received a transplant of a no. 18 leukemia that has 50% recombined and 50% unrecombined leukemic cells. (E) Kaplan-Meier survival curve of mice that received a transplant of a no. 102 leukemia that has 50% recombined and 50% unrecombined leukemic cell.

Discussion

Integrated cytogenetic and FISH studies of 81 well-characterized myeloid malignancies with 7q deletions provided a starting point for the chromosome engineering approach and subsequent analyses reported in this paper.[2] In that report, 7q22 was involved in approximately 80% of cases. Because almost all of the 7q22 deletions found in individual cases are relatively large, the proximal boundary of the 2.5-Mb CDS was defined by performing FISH on leukemias with deletions arising in 7q22 that extended toward the 7q telomere and the distal boundary was mapped by analyzing other cases with more centromeric interstitial deletions that terminated within 7q22.[2] To model loss of this interval in mice, we generated a "latent" chromosomal deletion by flanking a segment of orthologous synteny on chromosome *5A3* with *loxP* sites and used the *Mx1-Cre* transgene to excise this interval in vivo. This approach accurately models the pathogenesis of human hematologic cancers in which a small population of transformed cells achieves clonal dominance in vivo. We also used retroviral mutagenesis to establish leukemias without the desired deletion and then tested the impact of removing the *5A3* interval as a late event. Our data demonstrate that it is feasible to use this approach to create large somatic deletions in hematopoietic cells in vivo. Although inefficient, administering a single dose of pIpC to neonatal mice resulted in a population of *5A3*-deleted hematopoietic cells that was detectable for 2 years and persisted after serial transplantation. Moreover, multiple transplantable MOL4070LTR-induced AMLs contained the *5A3* deletion. These data provide strong evidence that the *5A3* deletion occurs in the HSC compartment and in leukemia-initiating cells.

$Mx1\text{-}Cre,\ 5A3^{fl/+}$ mice that were injected with pIpC did not spontaneously develop hematologic cancers, and the targeted *5A3* chromosome did not alter the phenotype or course of MPD in $Mx1\text{-}Cre,\ LSL\text{-}Kras^{G12D}$ or $Mx1\text{-}Cre,\ Nf1^{fl/fl}$ mice. Furthermore, retroviral mutagenesis caused acute leukemia with similar latency, penetrance, and phenotypic features in control and $Mx1\text{-}Cre,\ 5A3^{fl/+}$ mice. Finally, we found that the *5A3* deletion neither provides a clonal growth advantage in established AMLs nor alters responses to antileukemia drugs. The most straightforward interpretation of these extensive data is that the human 7q22/murine 5A3 CDS does not harbor a myeloid TSG. This idea is compatible with previous studies of myeloid malignancies with monosomy 7/del(7q), which revealed substantial heterogeneity and implicated the 7q22 CDS that we investigated as well as centromeric or telomeric DNA segments as potentially containing myeloid TSGs.[33-37] *SRPK2* and *EPO* bracket a minimal 4.6-Mb interval that is lost in some individual leukemias.[38] This minimal DNA segment includes the 7q22/5A3 CDS that we targeted and an additional approximately 2 Mb of DNA located between *FBXL13* and *EPO*. Interestingly, whereas the region of orthologous synteny in mouse *5A3* diverges beyond *Fbxl13,* most of the murine *Fbxl13-Epo* interval is located in a single segment of chromosome band 5G2 that could be targeted by chromosome engineering.

An alternative explanation for the absence of hematologic disease in $Mx1\text{-}Cre,\ 5A3^{fl/+}$ and $Mx1\text{-}Cre,\ 5A3^{fl/fl}$ mice is that the TSG has a weak effect, but cooperates with the loss of other chromosome 7 genes in leukemogenesis. *MLL5* is an intriguing

candidate TSG within the 7q22 CDS.[3] Three groups recently deleted the murine homolog and showed that homozygous *Mll5* inactivation reduces the numbers of phenotypic long-term repopulating cells in the bone marrow and markedly impairs HSC function under competitive repopulating conditions.[39-41] If *MLL5* plays a similar role in human HSC homeostasis, biallelic inactivation might reduce the fitness of potential leukemia-initiating cells. The observation that *MLL3,* a related gene located at chromosome band 7q34, is mutated in some human cancers raises the possibility that haploinsufficiency for *MLL5* and *MLL3* may interact in leukemogenesis.[42-44] A microdeletion located on chromosome band 7q21.3 that was recently implicated as containing a haploinsufficient myeloid TSG[45] might also cooperate with deletion of the 7q22 CDS. The idea that multiple 7q genes might contribute to leukemogenesis is consistent with recent studies of myeloid malignancies with 5q deletions, which suggest that haploinsufficiency for *CTNNA1*, *APC*, *EGR1*, *RPS14*, *miR-145*, and *miR-146a* cooperate to deregulate hematopoietic growth.[7,46-49]

Other potential explanations for the absence of myeloid disease in *Mx1-Cre, 5A3$^{fl/+}$* mice include the following: (1) radiation exposure, treatment with an alklyating agent, or transducing bone marrow cells with myeloid oncogenes might have uncovered a role of the *5A3* interval in leukemogenesis; (2) leukemic outgrowth could require efficient excision of the *5A3* interval in nonhematopoietic cells within the bone marrow microenvironment; and/or (3) a species-specific requirement for a 7q22 myeloid TSG that is confined to human hematopoietic cells.

Despite any impact of the *5A3* deletion on the incidence or phenotype of malignant hematologic disease, our data demonstrate that chromosome engineering can be used to create latent chromosomal deletions that can be induced in vivo in HSCs and leukemia-initiating cells. This allows investigators to bypass haplolethal developmental effects and to investigate chromosomal loss as a late event in leukemogenesis. However, excision of the 2-Mb targeted interval was inefficient in *Mx1-Cre, 5A3^{fl}* mice, and our efforts to delete this segment by transducing hematopoietic and mouse embryonic fibroblasts with a Cre-expressing retroviral vector were unsuccessful. It is uncertain whether the low rates of somatic recombination that we observed will hold for other genomic segments or whether this potential problem can be overcome by modifying the targeting constructs so that recombination leads to expression of a robust reporter. As we pursued the studies reported in this paper, it became obvious that the low rate of somatic recombination precluded ascertaining either subtle effects on hematopoietic growth and differentiation or the potential role of nonhematopoietic cells within the marrow microenvironment in promoting leukemic growth. After many attempts, we succeeded in generating mice with the *5A3* deletion in the germline. Homozygous deletion of the *5A3* interval is embryonically lethal, whereas mice with heterozygous deletion of the *5A3* interval are grossly normal and have blood counts similar to their wild-type littermates at age 3 months (data not shown). These preliminary observations are consistent with the results we obtained with the *Mx1-Cre, 5A3^{fl}* mouse model, and additional studies are in progress to fully address the role of this interval in hematopoietic growth control and leukemogenesis.

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Authorship

Contribution: J.C.Y.W., Y.Z., M.M.L.B., K.H.L., M.T.T., E.F., K.W., and P.A. conducted experiments and analyzed data; L.W. provided essential reagents; S.C.K. performed pathologic analysis; K.A. analyzed MOL4070LTR integration site data; J.C.Y.W., M.M.L.B., N.K., and K.S. designed research and analyzed data; and J.C.Y.W., N.K., and K.S. wrote the paper.

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