

Sequential Gene Targeting to Make Chimeric Tumor Models with *De Novo* Chromosomal Abnormalities

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Abstract

The discovery of chromosomal translocations in leukemia/lymphoma and sarcomas presaged a widespread discovery in epithelial tumors. With the advent of new-generation whole-genome sequencing, many consistent chromosomal abnormalities have been described together with putative driver and passenger mutations. The multiple genetic changes required in mouse models to assess the interrelationship of abnormalities and other mutations are severe limitations. Here, we show that sequential gene targeting of embryonic stem cells can be used to yield progenitor cells to generate chimeric offspring carrying all the genetic changes needed for cell-specific cancer. Illustrating the technology, we show that *MLL-ENL* fusion is sufficient for lethal leukocytosis and proof of genome integrity comes from germline transmission of the sequentially targeted alleles. This accelerated technology leads to a reduction in mouse numbers (contributing significantly to the 3Rs), allows fluorescence tagging of cancer-initiating cells, and provides a flexible platform for interrogating the interaction of chromosomal abnormalities with mutations. *Cancer Res*; 74(5); 1588–97. ©2014 AACR.

Introduction

Chromosomal translocations are hallmarks of all types of human cancers (1) and it seems likely that these arise in the cancer-initiating cells. The most common outcome of chromosomal translocation is the creation of a fusion gene, whereas the activation of oncogenes can also occur via translocation to either antibody genes or T-cell receptor genes, generally in lymphoid tumors. Gene fusion can also result from trisomy of chromosomes, for example, *MLL* self-fusion (2), and from intrachromosomal events such as those found in prostate cancer fusions (3). The range of specific tumors in which chromosomal translocations and gene fusions have been

described is ever increasing and new data from cancer genome sequencing projects are illuminating many candidate translocations that may have pathogenic consequences for tumor subtypes (1) or even idiopathic changes that may be biologically significant (4). Furthermore, the range of genes involved in chromosomal translocations is large and the resulting biologic consequences include alteration of signaling, protein complex formation, protein transport, and transcriptional regulation.

Modeling these multiple types of event in mice is a critical technical development for cancer biology and cancer therapeutics. Ideally mouse cancer models should encapsulate the main features of the corresponding human cancer, in particular, the cancer-initiating event that contributes to the overt phenotype of the cancer. Chromosomal translocations are thought to occur in the cancer-initiating cells but are not sufficient, in most cases, for progression to the full-blown, frank neoplasia. Therefore, modeling of the single-cell origin of cancer necessitates a method that can create or emulate a chromosomal translocation in specific cells of target tissues. Crucially, a rapid method is required for producing specific fusion genes in an animal model to address the issue of biologic relevance of the many known and emerging translocations.

These abnormal chromosomes can be reproduced in embryonic stem (ES) cells in culture (5, 6) and recapitulated in mice using *Cre-loxP* recombination and gene-targeted ES cells (7, 8). The latter are referred to as translocator mice (9). In translocator mice, translocations occur *de novo* in ontogeny and the restricted efficiency of the recombination gives single-cell origin of the tumors, truly reflecting the situation of clonal origin of cancer in man. However, the time taken for the establishment of tumor cohorts in the translocator mice is considerable due to the need for three independently

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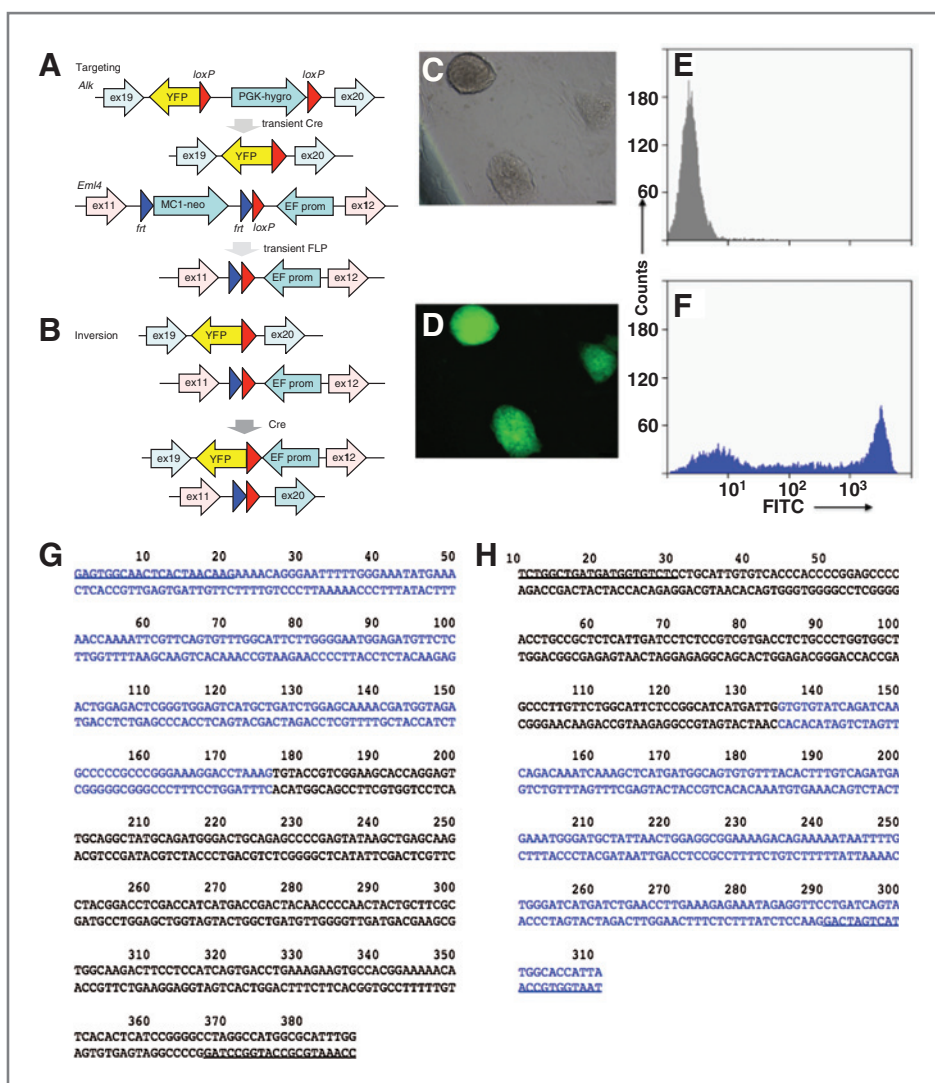
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Figure 1. Chromosome engineering to activate GFP following gene fusion. Gene targeting was used to introduce a YFP cDNA lacking a transcriptional promoter between exons 19 and 20 of *Alk* (A) or a transcriptional promoter (*EF-1*) lacking a reading frame between exons 11 and 12 of *Eml4* (B). The genomic coordinates of the targeting events were at MouseGRCm38 chromosome 17:83451327-83451328 for *Eml4* and chromosome 17:71898373-71898374 for *Alk*. After removal of the selection genes, bounded by *loxP* or *frt* sites, by transient expression of Cre or FLP, inversion was induced using Cre and selection of fluorescent cells due to amalgamation of the EF-1 promoter and YFP. Fluorescent cells are shown in C and D (by light and fluorescence microscopy, respectively). The cells were also verified by flow cytometry comparing noninverted (E) with inverted cells (F). mRNA was prepared from cells expressing YFP and RT-PCR carried out with *Eml4* exon 11 plus *Alk* exon 20 primers or *Alk* exon 19 plus *Eml4* exon 12 primers (RT-PCRs were performed with cDNA prepared from YFP-expressing ES cells and using annealing temperature that ranged from 68°C to 54°C over 14 cycles, with a final 19 cycles annealing at 54°C, each with 30 second extension time). The RT-PCR products were cloned and the sequences shown in G and H where the blue nucleotides are *Eml4* and black are *Alk* (primer sequences are underlined). FITC, fluorescein isothiocyanate.



segregating alleles (two carrying a *loxP* site in the relevant genes and one carrying a *Cre* gene, typically a knock-in allele; ref. 9). To expedite the process, we have exploited a conclusion drawn from the first oncogene fusion knock-in, where we observed that chimeric mice containing ES cells with the *Mil-ALK* knock-in fusion gene developed leukemia at the same rate and with the same phenotype as germline transmitting mice (10). Accordingly, we have now utilized sequential gene targeting of ES cells to generate donor cells with the appropriate complex genotype for creating translocator mice. We show that these ES cells, which have been subjected to three targeting events, can be used to generate high percentage chimeric mice that can develop tumors with specific, engineered chromosomal translocations, thus avoiding the necessity for complex breeding programs. Germline transmission of all three alleles can be achieved. We have verified the method by creating a translocation that generates an *Mil-Enl* gene fusion and characterizing the ensuing myeloid leukemia in an experimental cohort of chimeric mice.

Materials and Methods

EML4-ALK-targeted ES cells

The *ALK*-targeting cassette (Top, Fig. 1A) was made in a series of steps. First, the blunted XbaI-HindIII fragment containing the thymidine kinase selection cassette from pMC1-tk was cloned into the blunted SacII site pMG2 (11) to generate pMG2-tk (clone A). The *PGK-hygro-pA* gene fragment was amplified from pHA58-hygro using oligonucleotides with flanked by XhoI-*loxP* sequence, and cloned into the blunted SalI site of pMG2-tk to generate pMG2-*loxP*-PGK-hygro-*loxP*-tk (clone B). The yellow fluorescent gene (YFP) cDNA plus polyA signal was amplified from pEYFP-N1 (Clontech) using oligonucleotides with several unique restriction enzyme sites, and cloned into the blunted HindIII and BamHI sites of pMG2-*loxP*-PGK-hygro-*loxP*-tk (clone C). The mouse *Alk* genomic DNA 4.5-kb fragment with a SalI site containing exon 16, 17, 18, and 19 and 2.8-kb fragment with SalI-NotI sites containing exons 20, 21, and 22 was amplified from mouse 129sv genomic DNA using specific primers, and cloned into the XhoI site of

pMG2-loxP-PGK-hygro-loxP-tk as left arm and Sall-NotI sites of as right arm (clone D). The *Alk*-targeting construct with correct homologous arms orientation was confirmed by DNA sequencing. The targeting construct was digested with SceI for linearization before transfection ES cells.

The *Eml4*-targeting cassette (bottom half, Fig. 1A) was made in a series of steps. The *neomycin* selection gene flanked with *frt* sequences was extracted from pC2A-*frt*-neo-*frt* (11) by digestion with BglII, and cloned into the BamHI site of pMG2-tk to generate pMG2-*frt*-neo-*frt*-tk (clone E). The construct was digested and blunted with HindIII-BamHI and self-ligated to destroy several unique restriction enzyme sites (clone F). The human *EF1- α* promoter gene was amplified from pEF/myc/cyto (Invitrogen) using specific primers with *loxP* sites, and cloned into the SpeI-NotI sites of clone F to generate pMG2-*frt*-neo-*frt*-loxP-*EF1a*-tk (clone G). The mouse *Eml4* genomic DNA 3.1-kb fragment with Sall-ClaI sites containing exon 9, 10, and 11 and 2.8-kb fragment with NotI sites containing exon 12 was amplified from mouse 129sv genomic DNA using specific primers, and cloned into the Sall-ClaI sites of pMG2-*frt*-neo-*frt*-loxP-*EF1a*-tk as left arm and NotI site of as right arm (clone H). The *Eml4*-targeting construct with correct homologous arms was confirmed by DNA sequencing. The targeting construct was digested with SceI for linearization before transfection into ES cells.

Gene targeting was carried out (12) sequentially with *Eml4* and *Alk* using multiresistant feeders DR4 (13). ES clones were transfected with the *Alk*-targeting vector and selection was carried out at 150 μ g/mL hygromycin. The targeted ES clones were confirmed by filter hybridization (9, 14) and transiently transfected with PGK-Cre to delete the floxed *hygromycin* gene (Fig. 1A). The deleted clones were confirmed by filter hybridization and hygromycin sensitivity. One clone was subsequently transfected with the *Eml4*-targeting vector and clones selected at 300 μ g/mL G418. The selected clones were transiently transfected with pEFBOS-FLPo to remove the neomycin gene flanked by *frt* sites (Fig. 1A).

Inverted ES clones were selected by picking individual YFP+ colonies under fluorescence microscopy. One YFP+ clone was sorted by flow cytometry to further purify the population, where 500 cells of the clone were sorted into one well of a 6-well plate. This YFP+ sorted population was grown on a feeder cell layer in parallel with the noninverted parent clone, as the YFP-negative control. Once at the appropriate density, both clones were trypsinized and made into a single-cell suspension. To create a homogeneous population of ES cells and remove contaminating feeder cells, the entire cell suspensions were separately plated on tissue culture-treated plates and incubated at 37°C, 5% CO₂, in growth medium for approximately 40 minutes. The larger feeder cells were sedimented and attached to the dish. The nonattached ES cells were then pelleted and resuspended in growth medium for fluorescence-activated cell sorting (FACS) analysis. The live ES cell populations were gated on their forward and side scatter characteristics, avoiding autofluorescent dead cells and other debris. The inverted clone was analyzed on the fluorescein isothiocyanate channel for the percentage and intensity of YFP+ cells (Fig. 1F) compared with the noninverted (YFP-) parent clone (Fig. 1E).

MLL-Enl-Lmo2Cre-targeted ES cells

The *MLL*-targeting cassette (allele F1, Fig. 2A) was made in a series of steps. An *frt* site was cloned, using oligonucleotides, into the BamHI site of pMG2 (11) to generate pMG2FRT2 (clone I). A blunted NotI-BamHI fragment containing the SV40 polyA sequence was inserted into the blunted ClaI site of pHA58Hygro to make SVpAHygro-4 (clone J). An EcoRV fragment of clone B was cloned into the SmaI site of pMG2FRT2, producing pMG2FRT-SVHygro6 (clone K). A *loxP* site was inserted in-frame into the *Flpe* gene using PCR, generating pminiRSET-FLPeLOX1 (clone L). The 3' portion of *Flpe*, together with the *loxP* sequence, was excised as an EcoRV-HindIII (blunted) fragment of clone D and inserted into the EcoRV site of clone C to yield pMG2-FRT-SVHyg-CFLPeLox3 (clone M). A *luciferase-Venus* fusion gene was generated by cloning *luciferase* (without a stop codon) into the BamHI (blunted)-SmaI sites of pBspt, and subsequently inserting *Venus* [SmaI SpeI (blunted) fragment] into the SmaI-EcoRV sites. A SpeI-HindIII fragment containing the *luciferase-Venus* fusion was blunted into the NotI site of clone E to produce G2-Translocator-Cassette 1 (clone N). A 5.5-kb EcoRI genomic fragment of *MLL* was subcloned to generate pMG1-MLL5.5RI-1 (clone O; ref. 7). An EcoRV-Sce-I fragment of clone F was cloned into the blunted BglII site of clone G to construct MLL-G2-Translocator-10 (clone P). Finally, an XbaI fragment containing the thymidine kinase selection cassette (MC1-Tk) was blunted into the NotI site of clone H to produce the completed targeting vector MLL-G2-Translocator-Tk1 (Supplementary Fig. S1A).

The *Enl*-targeting cassette (allele F2; Supplementary Fig. S1B) was also made in a series of steps. The *EF1- α* promoter was cloned as a blunted HindIII-EcoRI fragment into the SmaI site of pMG2 to generate pMG2Bospro-3 (clone Q), and into the blunted EcoRI site of pMG2FRT (clone A) to produce pMG2FRT-BOS1 (clone R). A SpeI-EcoRV fragment of clone J was blunted into the Sall site of clone Q yielding pMG2BOSpro+FRTBOSpro2 (clone S). The PGK-puro selection cassette was cloned as a blunted Sall fragment into the EcoRV site of clone S to generate pMG2BOSpro-PURO-FRTBOSpro7 (clone T). A BamHI-XhoI fragment of clone L, containing the 5' portion of *Flpe*, together with the *loxP* site, was blunted into the NotI site of clone T to construct G2-Translocator-Cassette 2 (clone U). The entire insert was excised from clone M using Sce-I and inserted into the blunted SphI site of an *Enl* genomic clone to produce ENLK9+G2-Translocator-Cassette 2-3 (clone V). A further 1 kb of *Enl* genomic sequence was subcloned into pMG1 (11) as a BamHI-KpnI fragment (pMG1-ENLBK1, clone W). A KpnI fragment of clone N was subcloned into clone W to construct ENL-G2-Translocator-1 (clone X). Finally, the MC1-thymidine kinase cassette (XbaI fragment) was blunted into the NotI site of clone X to generate the completed targeting vector ENL-G2-TranslocatorTk9 (Supplementary Fig. S1B).

Gene targeting with *MLL* and *Enl* was carried out in the recipient ES cell line with Cre knock-in of the *Lmo2* gene (CCB cells, 129 origin) outlined in Supplementary Fig. S2). Both targeting vectors were linearized using Sce-I for ES cell targeting and selection was carried out at 150 μ g/mL hygromycin

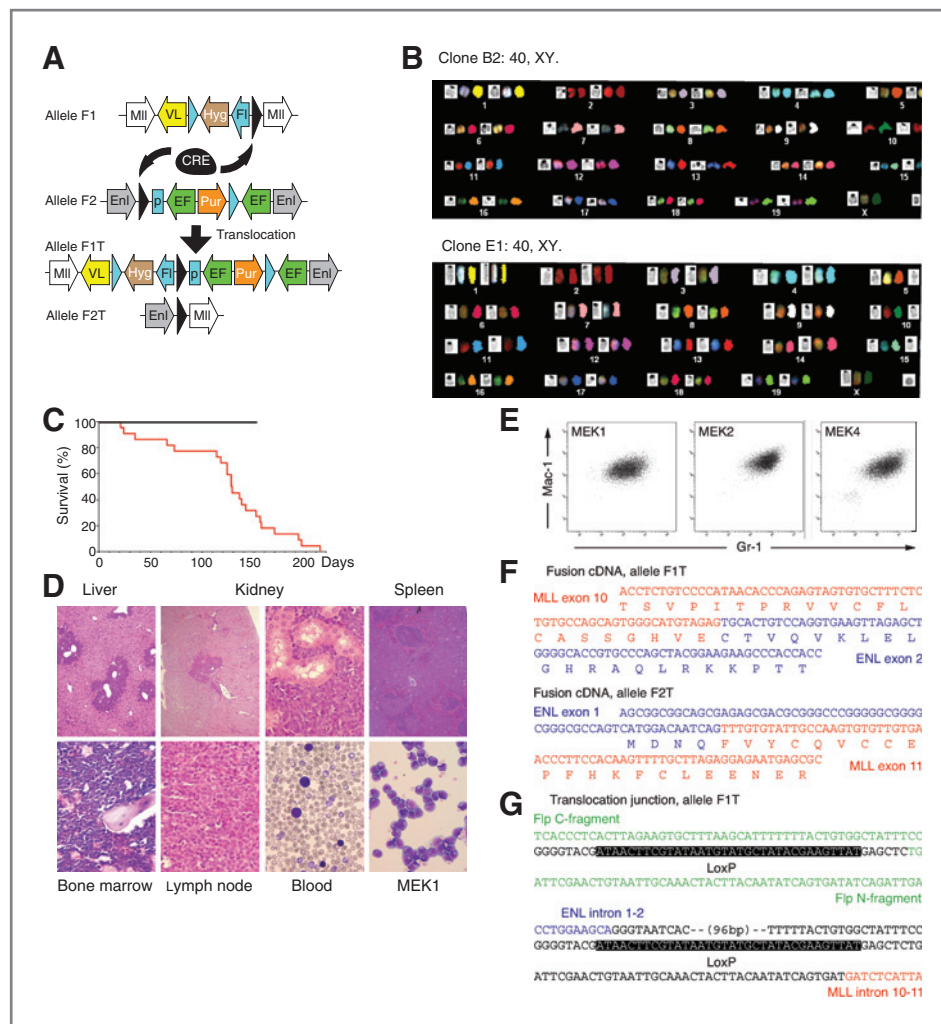


Figure 2. Fast throughput *Mll-Enl* translocations cause leukemias in chimeric mice. Sequential gene targeting of ES cells was carried out to produce triple-targeted clones with a knock in of *Cre* into the coding region of *Lmo2*, together with translocator alleles of *Mll* and *Enl* (depicted in A as alleles F1 and F2, respectively) at the locations previously described (7, 9). Two independent triple-targeted clones were selected and normal karyotype confirmed by SKY-FISH (B). These ES clones were injected into blastocyst and resultant *Mll-Enl* translocator chimeras rapidly developed disease. A Kaplan–Meier plot of survival of *Mll-Enl* translocator mice is shown in C (red line, survival of *Mll-Enl* translocator chimeras; black line, wild-type controls). D, hematoxylin and eosin-stained sections of tissues from a diseased chimeric mouse (top four panels and bottom left two). A blood smear and cytospin from one of the cultured tumor cells (designated MEK1) are shown bottom right. The sections are shown at $\times 20$ (liver, kidney), $\times 200$ (kidney, bone marrow), $\times 40$ (spleen), and $\times 400$ (blood and cytospin) magnification. E, FACS analysis of myeloid marker expression in three chimera tumors (designated MEK1, 2, and 4). F, cDNA was prepared from spleen cell RNA and the sequence of the junctions in fusion mRNAs expressed from both *Mll-Enl* and *Enl-Mll* alleles was determined. G, the sequences of the genomic translocation junctions from both *Mll-Enl* and *Enl-Mll* alleles were obtained from genomic DNA of cultured tumor cells. VL, venus-luciferase fusion; FI and P represent the split FLP recombinase; hyg, hygromycin; pur, puromycin; EF, EF1 α transcriptional promoter.

(*MLL-G2-Translocator-Tk1* confers hygromycin resistance) or 2 $\mu\text{g}/\text{mL}$ puromycin (*ENL-G2-Translocator-Tk9* confers puromycin resistance). First-round transfection was conducted with *MLL-G2-Translocator-Tk1* and targeted ES clones detected by filter hybridization (9, 14). A double-targeted ES clone (designated *Lmo2-Cre; Mll-loxP*) was retargeted with *ENL-G2-Translocator-Tk9* to obtain triple-targeted clones (i.e., *Lmo2-Cre; Mll-loxP; Enl-loxP*) were selected for generation of chimeras by injection into C57BL6 blastocysts. Chimerism was judged by coat color. The karyotypic stability of the triple-targeted ES cells was verified by growth in feeder-free condi-

tions and metaphase chromosomes prepared. A total of 2×10^6 cells in 5 mL were treated with 50 μL demecolcine 10 $\mu\text{g}/\text{mL}$ (Sigma) for 3 hours in 37 $^\circ\text{C}$, spun to recover cells, and resuspended in 5 mL fresh 0.075 mol/L KCl. These were incubated at room temperature for 10 minutes, spun, and pellet gradually resuspended in 5 mL of fix (fresh 3:1 methanol:acetic acid). This step was repeated twice, and metaphases stored in EtOH at 4 $^\circ\text{C}$. These chromosomes were subjected to SKY-FISH. Briefly, metaphase chromosomes were dropped on pretreated slides and hybridized according to the manufacturer's protocol (Applied Spectral Imaging). Images (20 metaphases) were acquired with an SD300 Spectra Cube (Applied Spectral

Imaging) mounted on a Zeiss Axioplan microscope using a custom-designed optical filter (SKY-1; Chroma Technology).

Analysis of neoplasias in chimeric mouse leukemias

Signs of endpoints were monitored closely and mice were sacrificed when this became apparent by slowness, hunched gait, ruffled fur, and loss of vitality. Post-mortem was carried out and samples taken from affected tissues for histology (fixed and sectioned after wax embedding), nucleic acid preparation, FACS, or tissue culture. Cytospins were prepared from these cultured cells and stained with KaryoMax Giemsa Stain for microscopy. Flow cytometry was performed on splenic cells from individual mice using CD markers for identification of B cells (CD19), T cells (Thy1; CD4; CD8), and myeloid cells (CD11b; Mac1). Cell cultures were established from chimeric mouse tumors. Spleen cells from afflicted mice were prepared as single-cell suspensions with 1×10^6 cells cultured in 2 mL of growth medium. The spleen cell growth medium comprised RPMI+Glutamax (Gibco, Invitrogen) 10% FBS (GE Healthcare), supplemented with 10 U/mL (equal to 2 ng/mL) granulocyte macrophage colony-stimulating factor (GM-CSF; Sigma) and 5% WEHI231 supernatant [to provide interleukin (IL)-3 source], 200 μ g/mL gentamycin (Sigma), and $1 \times$ penicillin-streptomycin (Gibco, Invitrogen). WEHI231 supernatant was prepared by growing the cells in DMEM+Glutamax (Gibco, Invitrogen) supplemented with 10% FBS (PAA) and $1 \times$ penicillin-streptomycin (Gibco, Invitrogen), to the end of log phase, then holding them at stationary phase for 2 days to secrete IL-3 into the medium. Cells were pelleted out by centrifugation and the cleared supernatant purified through a 0.22 μ mol/L syringe filter.

Translocation junctions were sequenced from PCR fragments amplified from genomic DNA isolated from cell cultures. PCR of translocation and fusion cDNA junctions was performed with Phusion High-Fidelity DNA Polymerase (New England Biolabs) using standard reaction conditions recommended by the manufacturer. A DNA fragment spanning the translocation junction on allele 1 was amplified using the primers FlpLox-F (5'-AAGAGAGCCACATTCATGAG) and FlpLox-R (5'-TCCA-CAATTGATGAAAGTAGC). Cycling parameters were: 98°C 30 seconds; 30 cycles of 98°C 10 seconds, 59°C 30 seconds, 72°C 14 seconds; and 72°C 5 minutes. The translocation junction on allele 2 was amplified using the primers ENLIntron1-2-F (5'-GACCATCAAGTCTCAGTGATG) and MLLIntron10-11-R (5'-AAATACTCAGGGGCTGGAGAG) for the following cycles: 98°C 30 seconds; 30 cycles of 98°C 10 seconds, 66°C 30 seconds, 72°C 15 seconds; and 72°C 5 minutes. PCR products were gel purified and sequenced with the same primers used initially to amplify each fragment.

Total RNA was isolated from cultured cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Contaminating genomic DNA was removed by addition of DNaseI, Amplification Grade (Invitrogen). cDNA was synthesized from total RNA samples using Superscript First-Strand Synthesis System (Invitrogen) and oligo(dT) primers, following the manufacturer's protocol. The MLL-ENL fusion cDNA was amplified using the primers MLLExon10-F (5'-TGGGAGATGG-GAGGCTTAGG) and ENLExon2R (5'-CGCTTGGGTTTAGG-

GAAGCTG) and the following cycling parameters: 98°C 30 seconds; 30 cycles of 98°C 10 seconds, 67°C 30 seconds, 72°C 15 seconds; and 72°C 5 minutes. The reciprocal ENL-MLL fusion cDNA was amplified using the primers ENLExon1-F (5'-CCCCGACAGACAATGAGAGC) and MLLExon11-R (5'-GATGCTGTCTTCCACACACATG). In this case, a Touchdown PCR protocol was used: 98°C 30 seconds; 11 cycles of 98°C 10 seconds, n° C 30 seconds, and 72°C 15 seconds (where n = annealing temperature, declining 1°C per cycle, from 70°C to 60°C); 24 cycles of 98°C 10 seconds, 60°C 30 seconds, 72°C 15 seconds; and 72°C 5 minutes. PCR products were gel purified and sequenced with the same primers used initially to amplify each fragment.

Results

Tumor evolution can be studied in mouse preclinical models where human cancers are intransigent due to the lack of material from the initiating stages. To develop methods that will allow recapitulation of the single-cell origin of human cancers, we have used gene targeting of ES cells to manipulate chromosomal translocation surrogates (9, 11). In addition, we required a method for detection of premalignant cells. We first tested the ability to activate gene expression (i.e., in this case, the *YFP*) by chromosome engineering to fuse a gene-less promoter with a promoter-less *YFP* gene following Cre-*loxP* recombination. ES cells were made in which the elongation factor *EF1- α* promoter was introduced into the *Eml4* gene on chromosome 17 (between exons 11 and 12, corresponding to human *EML4* exons 13 and 14) and the *YFP*-coding sequence, with a poly-adenylation site but no promoter, on the same chromosome 17 between exons 19 and 20 of *Alk* (corresponding to human chromosome 2, short arm, 20 and 21 exons; Fig. 1A). These engineered ES cells do not express YFP because the *EF1- α* promoter and the *YFP* gene are far apart. As a procedure to determine those targeted clones with the two events on the same chromosome (because interchromosomal translocation are infrequent; refs. 5, 6, 9), we used the production of YFP, following transient Cre-mediated intrachromosomal rearrangement, to judge which clones had undergone dual intrachromosomal gene targeting.

The ES were sequentially engineered to add the promoter-less YFP (removal of floxed PGK-hygromycin cassette was by transient Cre expression; Fig. 1A) and subsequently to add the gene-less promoter (removal of the *frt*-flanked MC1-neomycin cassette was by transient FLP expression; Fig. 1A). The double-targeted ES cells were transiently transfected with Cre recombinase (Fig. 1B) to achieve the inversion event bring the *EF1 α* promoter and the YFP cDNA together. Fluorescent clones could be detected microscopy showing normal ES morphology (Fig. 1C and D). One clone was picked and expanded prior for sorting YFP+ cells by flow cytometry. Figure 1 E and F shows flow cytometry of the clone before Cre-mediated inversion (E) and after inversion following purification and expansion (F). Thus recombination of the gene-less promoter and a promoter-less gene in engineered mouse chromosomes allows detection of the specific cells that have undergone a site-specific recombination. The inversion event that brings together the

EF1- α promoter and the *YFP* cDNA occurs within the newly created fusion intron of *Eml4* and *Alk*. We have also analyzed the *Eml4-*Alk** and the *Alk-Eml4* fusion mRNA production by cloning reverse transcriptase PCR (RT-PCR) products. Figure 1G and H, respectively, shows the *Eml4-*Alk** and the *Alk-Eml4* fusion products, observed in cloned RT-PCR products.

Our previous work has shown that *de novo* chromosomal translocations could be induced in mice using *Cre-loxP* recombination (9). We wanted to evaluate the robustness of using serially targeted ES cells as donor cells for blastocyst injection to make chimeric mice for tumor studies. Translocations that generate an *Mll-Enl* fusion gene induce tumors with either a lymphoid or myeloid lineage in infants and children (15, 16). We have previously used translocator mice to model *Mll-Enl* translocations, in which *Cre* (expressed from an *Lmo2* knock-in allele) mediated the site-specific fusion of *Mll* and *Enl*. Malignancies of myeloid cells arose rapidly after chromosomal translocation in these mice (9). Although this system recapitulated the *MLL-ENL* chromosomal translocations in man, the timeframe of the whole process is very long because three independently inherited, targeted alleles are needed before a "translocator" mouse is made. Clearly there is a need to simplify and accelerate this process. Given our original observation that chimeric *Mll-Af9* knock-in mice developed leukemia at the same rate as germline carriers (10), we used the *Mll-Enl* system to test a new fast-throughput methodology based on sequential targeting of ES cells and use of chimeras for tumor etiology studies.

We designed targeting plasmids for homologous recombination with the *Mll* and *Enl* genes previously (9) and used a similar design as shown as allele F1 and F2 (Fig. 2A and in detail in Supplementary Fig. S1). For simplicity and speed, we used 129sv genomic DNA to PCR amplify the homologous arms in our vectors (each arm being about 2 kb) to establish a transferrable and facile approach. These were targeted into an ES cell line derived from the 129sv strain harboring a knock-in of *Cre* into the *Lmo2* gene on chromosome 2 (diagrammatically shown in Supplementary Fig. S2; ref. 9). Two independent, triple-targeted ES cell clones were isolated and their karyotypes were verified (40, XY) using SKY-FISH (Fig. 2B). These clones were injected into recipient blastocysts for production of a cohort of chimeras to monitor the possible development of leukemia. Disease became apparent in mice as early as 30 days after birth and eventually all mice from both recipient lines developed neoplasia (Fig. 2C). The rate of tumor incidence in these chimeric mice was close to that seen in our original model that relied on germline transmission and interbreeding [Supplementary Fig. S3 compares the leukemia onset in the chimerism (A) with the originally published data for germline carriers of the three alleles; ref. 9]. The chimera cohort used in this study was specifically generated for the study and all had high level ($\geq 80\%$ based on coat color). Supplementary Figure S3C details the degree of chimerism in this cohort. In our original study of using chimeras for tumor studies (10), all chimeras, even ones as low as 5% chimerism, developed neoplasias and in some cases at time points that preceded approximately 100% chimeras.

Post-mortem examination of the neoplastic chimeric mice showed characteristics similar to the first-generation translocators. Symptoms included splenomegaly, thymic enlargement, livers and kidneys with nodular, pale areas of tumor infiltration, and lymphadenopathy. Tissue sections revealed tumor nodules composed of medium sized blast cells in spleen, liver and kidney, and prominent sinusoidal infiltration within the liver and spleen (Fig. 2D). The bone marrow was diffusely effaced by a monomorphous population of medium sized blast cells with little residual normal hemopoiesis. Blast cells were present in the peripheral blood and comprised up to 25% of the total white cell count. Lymph nodes showed lymphadenitis displaying diffuse replacement by neoplastic blast cells. Cell lines (designated MEK1-5) were established from splenic cells and showed morphologic evidence of myeloid differentiation in the form of promyelocytic and myelocytic granule formation (Fig. 2D, bottom right shows MEK1 cells). FACS analysis demonstrated that the abnormal cells are all Mac-1+; Gr-1+ myeloid cells (Fig. 2E shows MEK1, 2, and MEK5 cells, see below). Both reciprocal chromosomal fusion genes were expressed in the tumors and the sequence of RT-PCR products has the correct open reading frames for both *Mll-Enl* and *Enl-Mll* (Fig. 2F). Finally, site-specific recombination at the *loxP* sites was confirmed by sequencing both reciprocal chromosomal breakpoints from PCR products of tumor DNA (Fig. 2G).

The fast-throughput chimeric system is dependent on the expression of *Cre* recombinase, as ES cells carrying targeted *Mll* and *Enl* alleles but lacking *Cre* did not develop into tumors (Fig. 2C). Five cell cultures were established from independent tumors and SKY-FISH analysis of metaphase chromosomes from these confirmed that they all carry the expected *Cre-lox*-mediated reciprocal translocation *t* (Fig. 3; refs. 9, 17). Otherwise, they have a normal chromosome number (40) and no other detectable abnormalities, with the exception of an additional, acquired *t* (11, 14) in MEK1 and a duplicated Y chromosome in MEK3.

As an approach to expand on the feasibility of incorporating additional features at the junctions of chromosomal translocations, we added a spilt *FLP* gene at the two sides of the *Mll* and *Enl* chromosomes (Fig. 2A, allele F1 and F2 and Supplementary Fig. S4), each part being adjacent to the *loxP* site to reconstitute *FLP* following chromosomal translocation. In pilot experiments, transiently transfecting CHO cells with the two targeted alleles and a vector-expressing *Cre*, we were able to PCR amplify a product that was made from the linkage of *EF1* promoter with luciferase (Supplementary Fig. S4) that results from interchange between the two vectors in trans. However, we were unable to find evidence of *FLP* activity in the chimeric mice via venus-luciferase activity, presumably because the low efficiency of the *FLP* recombinase had been additionally compromised by the additional 16 amino acids encoded by the *loxP* site. The level of mRNA production from the untargeted *Mll* gene and the *Mll-Enl* translocation fusion gene was compared using quantitative PCR (qPCR). These data show that the mRNA levels for both genes were very similar (Supplementary Fig. S5 shows the qPCR data and verification of PCR product identity). The presence of complex DNA elements introduced

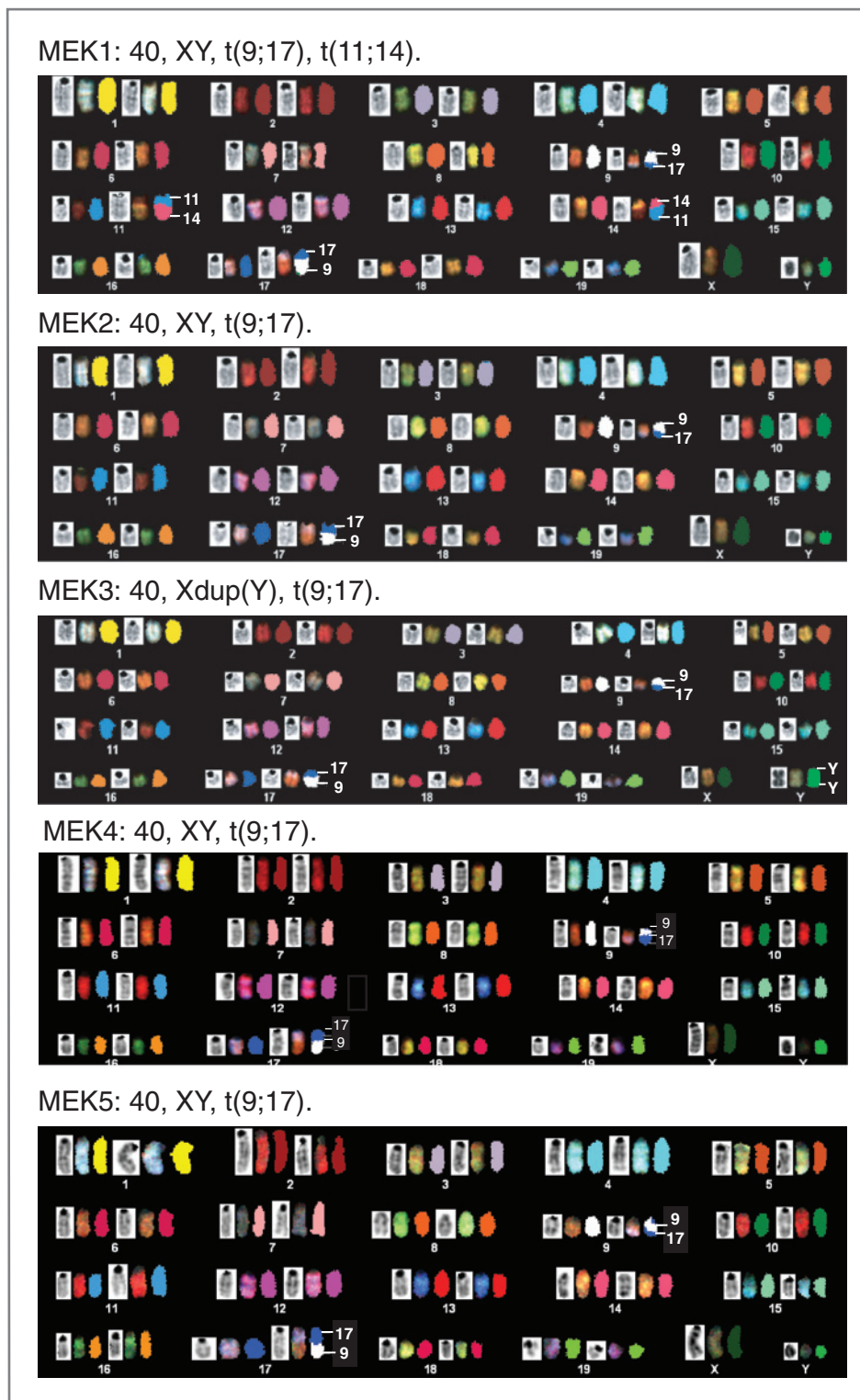


Figure 3. Karyotypes of tumors in *Mll-Enl* translocator chimeric mice. The presence of the reciprocal chromosomal translocations in tumors from the chimeras was confirmed by SKY-FISH. Five independent karyotypes were obtained in tumor-bearing mice designated MEK1-5. *Mll* is located on chromosome 9 and *Enl* on chromosome 17.

by gene targeting in the intron at the fusion junction has no detectable effect on mRNA levels of *Mll-Enl* fusion.

The tumors arising in the chimeras described here appear rapidly suggestive that the MLL-ENL is sufficient for myeloid proliferation but not frank leukemia. Transplantation of tumor

cells from these mice into immunodeficient recipients failed to produce secondary tumors. *In vitro* growth analysis of spleen cells isolated from five tumor-bearing chimeric mice showed growth factor dependence because GM-CSF was needed for proliferation (data for one line are shown in Fig. 4A). This

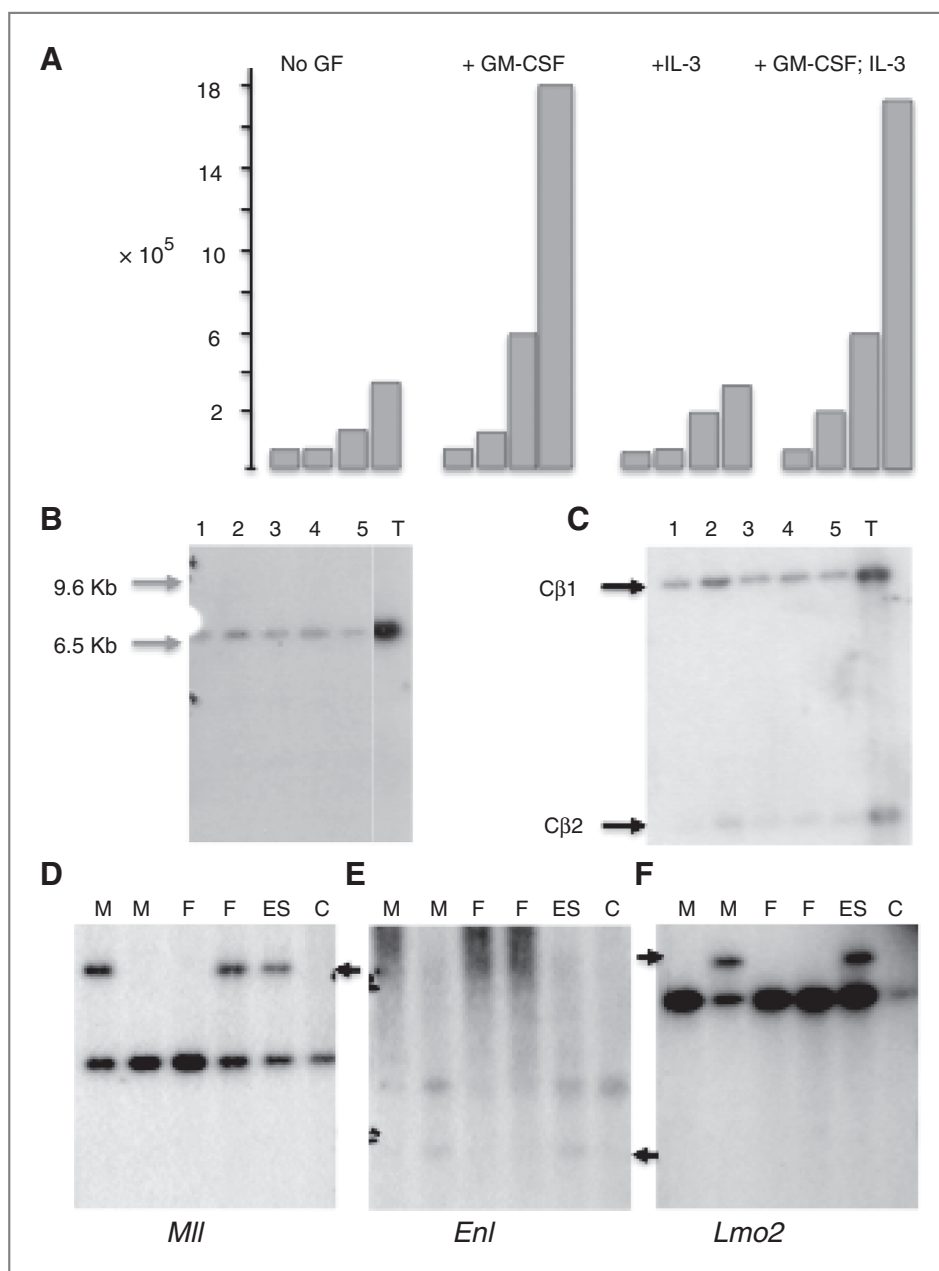


Figure 4. MLL-ENL tumors in chimeric mice are growth factor dependent. Spleen cells were obtained from tumor-bearing chimeras and cell cultures were established. Single-cell suspensions were grown in medium with or without the growth factors GM-CSF and IL-3 as indicated. A total of 10^4 cells were seeded on day 1 and cell numbers were counted for 4 subsequent days. A representative set of data for one (of the five) cell lines are shown (A). The possible lymphoid progenitor origin of the MLL-ENL translocator tumors was investigated by analysis of immunoglobulin heavy chain and T-cell receptor β chain gene organization in genomic DNA prepared from the cell cultures from B and C. Southern filters were hybridized with probes for a heavy chain enhancer intron (B) or C β 1 (C). Size markers are indicated by arrows on the left. Bands corresponding to C β 1 and C β 2 are indicated by arrows on the right. Numbers 1 to 5 represent cell lines. T is control tail biopsy DNA. As an indicator of pluripotency of the multiply targeted ES cells, some of the chimeric mice were bred with wild-type mice and the germline transmission of targeted alleles determined in their pups using Southern hybridization. The Southern filters of tail biopsy DNA were hybridized using probes detecting the inserted *loxP* site in *Mll* (D), the inserted *loxP* site in *Enl* (E), or the *Lmo2-Cre* knock-in F (9). Male (M) and female (F) represent the four offspring of a chimeric mouse. Lanes marked ES contain DNA from triple-targeted ES cells and C represents control, nontargeted DNA. Arrows, the targeted alleles detected with each probe. All three targeted alleles were transmitted, with one male mouse carrier of the *Enl*- and *Lmo2*-targeted alleles.

indicates that the myeloproliferation found in these mice is not frank leukemia and that secondary mutations are required to build on the presence of the translocated chromosomes for the

overt cancer state. Moreover, the myeloid lineage of the tumors shown by the histology is underscored by the lack of evidence for either immunoglobulin heavy chain or T-cell receptor- β

chain gene rearrangements in genomic DNA isolated from the established cell lines (Fig. 4B and C).

Finally, maintenance of pluripotency for the sequentially targeted ES cells was confirmed by breeding chimeras with wild-type mice to provide evidence of germline transmission of all the three targeted alleles (Fig. 4D–F shows Southern filter hybridization detected carrier alleles from one litter of a 90% coat color chimera). Our data demonstrate the efficacy of using chimeric mice made from multiply targeted ES cells to rapidly model human cancers that are dependent on specific chromosomal translocations.

Discussion

The fast-throughput translocator model

The importance of modeling human cancers in mice is to produce manipulable models that can both provide data on the molecular and cell biology of disease origin and preclinical models where *de novo* cancer is otherwise unavailable. One practical problem with mouse models of cancer is to emulate the single-cell origin and at the same time incorporate the multifaceted mutational landscape that characterize full blown neoplasias. This requires *de novo* mutation at the single-cell level and conditional secondary gene activation following that first genetic change. To fulfill these requirements, we have developed a rapid preclinical modeling system for *de novo* formation of chromosomal translocations during mouse ontogeny (fast-throughput chromosomal translocations). This is applicable to recapitulating any human chromosomal translocation such as those found in leukemia/lymphoma, sarcomas, or carcinomas (1). The characteristic feature is the use of sequentially, multiply targeted ES cells to generate chimeric mice for tumor cohorts, building on the concept of using chimeras in tumor biology, which we suggested from the initial fusion protein that we showed with the initial knock-in study (10).

In the fast-throughput translocator technology, the "tumor cohort" comprises chimeric pups and does not require interbreeding that can be very time consuming if multiple, non-linked genes are involved, thus comparing with at least more than 18 months of interbreeding with three mouse strains mice. Moreover, the frequency of triple carriers is low until homozygous carriers can be used for interbreeding, adding another 4 to 6 months. Overall, the use of chimeras is a practical advantage in terms of speed to tumor phenotype (10, 17), an economic advantage in terms of limiting the mouse numbers required to obtain critical preclinical analysis data and, crucially, a significant reduction in animal usage. A critical additional feature of the translocator approach is the natural consequence of creating haploinsufficiency in cells with the reciprocal translocations. There are increasing results to suggest this is a complementary outcome of balanced translocations and may partly explain the recurrence of specific translocations in particular cell types.

Activation of secondary genes following chromosomal translocations

When one of the recurrent chromosomal translocations occurs, it is the initial genetic alteration in cancer-initiating

cells. Models of translocation-dependent or other genetic rearrangements in tumors would therefore benefit from a tagging technology that can mark the initiating cells and their progeny. The approach described in Fig. 1 allows for this by bringing together a transcriptional promoter with a fluorescent protein gene following Cre-*loxP*-mediated recombination. Cells with the aberrant chromosome express YFP and continue to do so after cell division.

Furthermore, the products of these aberrant chromosomes are not sufficient to elicit full blown neoplasia (e.g., the MLL-ENL fusion) but require mutations in other genes to supplement the oncogenic effect. Thus, the addition of complementary mutations in models of human cancer is required. It will be possible to add cooperating oncogenes to generate secondary oncogenic events directly and specifically in cells that acquire aberrant chromosomes by the promoter-gene linkage technology. This could be augmented to encompass multiple genes using viral 2A peptides (18) to facilitate the expression of multiple oncogenes from one translocated allele. Finally, future versions of this technology could use inducible forms of Cre so that temporal control of secondary events could be achieved. This will provide the capability to build a fully integrated, sequential gene activation oncogenic system into mice while retaining the single-cell origin of tumors. Thus, our method offers the potential to rapidly generate realistic mouse models of human cancer, based on chromosomal translocations in cancer stem cells that represent optimal settings for drug testing and biologic assessments using chimeric mice.

Myeloproliferation dependent on MLL-ENL fusion

The neoplastic cells that arise in this new version of the *Mll-Enl* translocator mice appear very rapidly as did those of the first-generation translocator model (9). This suggests a leucocytosis stage of malignancy only requires the creation of the *Mll-Enl* fusion by chromosomal translocation. Furthermore, the fact that cells derived from the spleens of diseased translocator mice do not transplant and are dependent on GM-CSF growth factor in culture suggests a state that is not fully transformed, although dependent on the MLL-ENL protein. Full transformation may require one or more additional genetic changes subsequent to the translocation. One clear candidate is mutant *FLT3* that is frequently altered in MLL-fusion-associated myeloid malignancies (19, 20). The fast-throughput translocator method will allow further elaboration of the interrelationships of genes in the MLL fusion protein pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Development of methodology: T. Tanaka, T. Brend, H. Ali, T. Rabbitts

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Chambers, T. Brend, N. Geisler, J.C. Cigudosa, K. MacLennan, T. Rabbitts

Writing, review, and/or revision of the manuscript: J. Chambers, T. Tanaka, T. Brend, J.C. Cigudosa, T. Rabbitts

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Chambers, N. Geisler, L. Khazin, N. Dear

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