Retroviral Insertional Mutagenesis Identifies Genes that Collaborate with NUP98-HOXD13 during Leukemic Transformation

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Abstract

The t(2;11)(q31;p15) chromosomal translocation results in a fusion between the NUP98 and HOXD13 genes and has been observed in patients with myelodysplastic syndrome (MDS) or acute myelogenous leukemia. We previously showed that expression of the NUP98-HOXD13 (NHD13) fusion gene in transgenic mice results in an invariably fatal MDS; approximately one third of mice die due to complications of severe pancytopenia, and about two thirds progress to a fatal acute leukemia. In the present study, we used retroviral insertional mutagenesis to identify genes that might collaborate with the NHD13 gene in the MDS transformed to an acute leukemia. Newborn NHD13 transgenic mice and littermate controls were infected with the MOL4070LTR retrovirus. The onset of leukemia was accelerated, suggesting a synergistic effect between the NHD13 transgene and the genes neighboring retroviral insertion events. We identified numerous common insertion sites located near protein-coding genes and confirmed dysregulation of a subset of these by expression analyses. Among these genes were Mels1, a known collaborator of HOX and NUP98-HOX fusion genes, and Mn1, a transcriptional coactivator involved in human leukemia through fusion with the TEL gene. Other putative collaborators included Gata2, Erg, and Epor. Of note, we identified a common insertion site that was >100 kb from the nearest coding gene, but within 20 kb of the mir29a/mir29b1 microRNA locus. Both of these miRNA were up-regulated, demonstrating that retroviral insertional mutagenesis can target miRNA loci as well as protein-coding loci. Our data provide new insights into NHD13-mediated leukemogenesis as well as retroviral insertional mutagenesis mechanisms.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Introduction

The NUP98-HOXD13 (NHD13) fusion gene arises from the t(2;11)(q31;p15) translocation, which occurs in the malignant cells of patients with myeloid malignancies (1). We previously characterized a transgenic mouse model resulting from the expression of this fusion gene under the control of a pan-hematopoietic promoter which caused a myelodysplastic syndrome (MDS) progressing to an acute leukemia (2). This phenotype is highly penetrant; however, the latency period preceding the onset of acute leukemia is consistently longer than 6 months. This latency period suggests the possibility that further genetic or epigenetic events are required for progression to acute leukemia. This is consistent with the hypothesis that mutations affecting multiple cellular pathways are required for oncogenesis. Of particular importance in leukemia are two types of mutations: those that lead to impaired differentiation and those that result in inappropriate proliferation and/or apoptosis (3). NUP98-HOX fusion genes have been shown to inhibit hematopoietic differentiation (2, 4, 5); therefore, one might anticipate that a complementary event affecting one or more proliferative or apoptotic pathways would be required before the NHD13 transgenic cell could progress to leukemia.

Retroviral insertional mutagenesis is a powerful screening technique used for identifying genes that can lead to malignant transformation. Upon infection into newborn mice, the retrovirus inserts into the genome of the host cells (6–8) and, in doing so, can affect the expression of nearby genes (9). If the altered expression of these genes is oncogenic, clonal expansion of the cell in which that particular insertion occurred will ensure that that clone will be predominant in the resultant tumor tissue. One important advantage of insertional mutagenesis over chemical mutagenesis is that the introduction of foreign sequence into the genome tags the affected genes, simplifying the subsequent identification of the genes. The approach has been widely shown as useful for identifying proto-oncogenes (10–12) and, specifically, for identifying collaborating events in sensitized models such as transgenic or knock-out mice (13).

In our initial studies (2, 14), we noted that the transgenic NHD13 model results in a spectrum of leukemic phenotypes, most commonly acute myelogenous leukemia (AML) and pre-T LBL. For the retroviral insertional mutagenesis experiment, we attempted to bias the system toward myeloid rather than lymphoid malignancies because the NHD13 fusion gene has been observed only in patients with myeloid malignancies. For this reason, we chose to use the MOL4070LTR retrovirus, created by replacing most of the U3 region of the Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) with that of 4070A. Unlike the parental MMLV, which induces exclusively lymphoblastic T cell lymphomas in unsensitized FVB mice, MOL4070LTR produces predominantly myeloid neoplasms (15).

Materials and Methods

Retroviral infection. The MOL4070LTR retrovirus was produced by seeding 10⁵ NIH3T3 cells chronically infected with virus with an equal number of uninfected NIH3T3 cells in a 100-mm dish. The cells were propagated for 4 days, the medium was replaced with fresh medium, and on day 5, the virus-containing medium was harvested, and titer was
determined by the Xc assay (16). Newborn mice were inoculated i.p. with 4 x 10⁶ infectious particles in 0.05 ml of culture medium.

**Phenotype analysis.** Mice were under daily observation for early signs of leukemia. These signs included lethargy, labored breathing, enlarged lymph nodes, or abdominal masses. Mice were euthanized upon observation of symptoms, and blood, bone marrow, and tissues were harvested for analysis. H&E, CD3 (DAKO), B220 (CDM5R, Pharmingen), anti-myeloperoxidase (MPO; DAKO), and F4/80 (Caltag) stained sections from tissues including the thymus, lymph nodes, spleen, liver, kidney, lung, and thymia were produced using conventional staining techniques. Bone marrow cells were harvested from femurs by flushing with Iscove’s modified minimal essential media and assessed microscopically by May-Grunwald-Giemsa-stained cytosin preparations. Two-color flow cytometry was used to determine the immunophenotype of a single-cell suspension prepared from thymus, spleen, and/or bone marrow. The cells were stained with FITC-conjugated anti-mouse CD4, CD20, Gr-1, and c-kit and phycoerythrin-conjugated anti-mouse CD4, immunoglobulin M, Mac-1, and Sca1 (Pharmingen). Diseases were classified according to the Bethesda proposals (17, 18).

**Inverse PCR.** One microgram of spleen DNA from leukemic mice was digested for 16 h with BamHI in a 20-μl final volume. DNA fragments were ligated in a 500-μl final volume using T4 DNA Ligase (Invitrogen) at 16°C for 16 h and were resuspended in 20 μl of water after precipitation. Two PCRs were done from each template; one using the 5’ LTR as template, and the other using the 3’ LTR. The PCR mix included 2 μl of template DNA, 400 ng of primer, 10 μl of 10× PCR buffer, 0.4 μl of each primer, and 2.5 units of Taq enzyme (Expand Long Template PCR, Roche), in a 50-μl final volume. Primer sequences are available upon request. Primary PCRs were done with a denaturation step (5 min at 94°C), followed by 30 cycles of amplification (each cycle included 30 s of denaturing at 94°C, 1 min annealing at 60°C, and 10 min extension at 68°C), and a final extension step (5 min at 68°C). Secondary PCRs were done using nested primers and 1 μl of the primary PCR as a template. Products from the secondary PCR were analyzed by electrophoretic separation in a 1% agarose gel and sequenced directly using the secondary PCR primers.

**Ligation-mediated PCR.** Genomic DNA was digested with NcoI or MseI and ligated to linkers constructed by annealing the oligonucleotides 5’-GTAATACGACTCACTATAGGGCTCCGCTTAAGGGAC-3’ and 5’-Phos-GTCCCTTAAAGGAGC-3’-3’-spac3’ for NcoI-digested DNA, and 5’-GTTAGATCGACTCATATAGGCGCTCGTAAGAGGCAC-3’ and 5’-Phos-TAGCCCTTAAGGAGGCGG-3’-spac3’ for MseI-digested DNA (Integrated DNA Technologies). Primary PCR was done using primers complementary to the linkers and the LTR of the MOL4070LTR retroviral sequence. Secondary PCR was done using nested primers after 1:50 dilution of the primary PCR product. All primer sequences are available upon request. Products were ligated into pGEM-T Easy (Promega) and transformed into DH5α cells (Invitrogen). DNA isolated from ampicillin-resistant colonies was sequenced using an SP6 primer with BigDye Terminator reagents and analyzed on a 3730 DNA Analyzer (Applied Biosystems). Sequences were matched to the genomic build of March 2005 (National Center for Biotechnology Information Build 34) using the UCSC genome browser and will be submitted to the Retrovirus Tagged Cancer Gene Database (RTCGD).3

**Southern blot analysis.** Genomic DNA was isolated from mouse spleen tissue and digested with either BgIII, EcoRI, EcoRV, or HindIII. Digested DNA was size-fractionated on a 0.8% agarose gel, denatured, neutralized, and transferred to a nitrocellulose membrane. The nitrocellulose membrane was hybridized to a ³²P-labeled virus-specific or locus-specific probe generated by PCR. Primer sequences used to generate probes are available upon request.

**Northern blot mRNA analysis.** RNA was isolated from spleen using the TRIzol reagent and protocols (Invitrogen). RNA samples (10 μg each) were run on 15% acrylamide denaturing (urea) precast gels (Invitrogen) and then transferred onto Nytran Supercharge membrane (Schleicher & Schuell). Probes were labeled with [α-³²P]ATP using the Starfire oligo labeling kit (Integrated DNA Technologies). Probe hybridization was done at 37°C in 7% SDS/0.2 mol/L NaPO₄ overnight. Membranes were washed twice with 2× saline-sodium phosphate-EDTA/0.1% SDS at 37°C. Blots were reprobed with a 3S RNA probe as a loading control.

**Reverse transcription-PCR.** RNA was isolated from spleen using the TRIzol reagent (Invitrogen), and 1 μg RNA was reverse transcribed using Superscript II reverse transcriptase (RT) with random hexamer primer (Invitrogen). A forward Mst1 primer (5’TACCTCAACCCCTGACAGCTAT-GG-3’) and a reverse Moloney pol primer (5’TCTCCCGATCTCCATTTGTT-GATC-3’) were used for amplification of a fusion product. After 3 min at 94°C, 35 or 45 cycles (as noted) of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s were used, followed by a terminal 5-min extension at 72°C. PCR products were analyzed by agarose gel electrophoresis.

**Real-time PCR.** First-strand cDNA templates were generated as described above. Real-time reverse transcription-PCR (RT-PCR) was carried out on a 7500 Fast Real-Time TaqMan PCR System (Applied Biosystems). Primer and probe sets were purchased from Applied Biosystems and used under recommended conditions. Primer details are available upon request. The expression of Gapdh was used as an endogenous control. As controls, cDNA from the spleen and bone marrow of three wild-type mice were used, and the average value taken. All reactions were done in triplicate, and the ΔΔCt mean and SE were calculated for each sample (19). Values were normalized to the value generated for normal spleen.

**Results**

**MOL4070LTR infection accelerates leukemogenesis in NHD13 mice.** The recombinant MOL4070LTR virus retains the majority of the genomic sequence of MMLV, but the LTR regions have been replaced with those from the 4070A virus. Infection of wild-type FVB mice with this recombinant virus results in induction of leukemia with a mean latency of 31.7 weeks (15). NHD13 and wild-type littermate mice were infected with MOL4070LTR on the day after birth, and a cohort of 34 transgenic and 41 wild-type mice were then monitored for disease symptoms. Complete blood counts were obtained from a subset of five NHD13 and five wild-type littermates at the age of 2 months to confirm that these mice showed an MDS phenotype consistent with that observed in noninfected NHD13 (Supplementary Table S1). Leukemia developed more rapidly in NHD13-infected mice than either wild-type infected mice (P = 5 x 10⁻¹¹) or NHD13 noninfected mice (P = 2 x 10⁻⁵; Fig. 1A; Supplementary Table S1). The median latency of disease in NHD13-infected mice was 19.4 weeks (mean, 19.9). Mice were euthanized when leukemic symptoms were identified, and tissues were harvested for molecular and histologic analysis to confirm and classify the leukemia.

The number of retroviral insertions in each leukemic sample was determined by Southern blot analysis using a probe specific for the viral genome (Fig. 1B). This allowed assessment of the number of insertions per tumor and an estimation of the clonality of each tumor. The relative intensity of bands obtained by Southern blot analysis suggests that many of the tumors were oligoclonal. This leukemic phenotype was determined by fluorescence-activated cell sorting (FACS) or immunohistochemistry for 26 of the 31 mice. In total, there were 5 mice with acute lymphocytic leukemia (ALL) and 22 mice with acute non-lymphocytic leukemia (ANLL; one mouse had an immunohistologically apparent biclonal
identified in these mice (Table 1). Additionally, some insertion events that were not recurrent are of interest based on the known function of the gene or the presence of similar insertion events in other models (Table 2).

**Meis1.** Meis1 was the most commonly detected insertion site in the present study, occurring in 12 mice as determined by PCR, consistent with previous findings by other investigators (21, 22). All 12 of the Meis1 insertion events detected by PCR occurred within 155 kb of one another (Fig. 24). Nine were in the reverse orientation and within 8 kb 5’ of the gene. The remaining three were in the forward orientation, in intron 7 (no. 89), the 3’ untranslated region (no. 99), or 8 kb 3’ of Meis1 (no. 118). A screen for additional Meis1 insertions by Southern blot revealed insertions in two additional mice (nos. 7 and 26) that were not detected by PCR (Fig. 2B).

We examined the expression of Meis1 in six mice that had Meis1 insertions (nos. 3, 12, 30, 89, 101, and 118). In five of the six, Meis1 expression was increased more than 4-fold compared with normal spleen and bone marrow (Fig. 2C), the exception being mouse no. 12. However, because no abnormal Meis1 band was evident for this sample by Southern blot, the leukemic clone containing the Meis1 insertion in this mouse may well be a minor clone.

**Mn1.** We identified four insertion sites in the same intron of Mn1, all in the forward orientation with respect to Mn1 (Fig. 3A). Southern blots showed that the clone with the Mn1 insertion was a prominent clone in all four cases (Fig. 3B, left and top right). Because an MN1-TEL fusion has been identified in human leukemia patients and the insertions we detected were in the same intron as the MN1-TEL breakpoints, we suspected that a Mn1-viral fusion may be produced in these mice. We used RT-PCR to amplify a Mn1-viral fusion transcript in all four mice that had an intronic insertion (Fig. 3B, bottom right). Sequencing of this product revealed Mn1 exon 1 spliced to a cryptic splice acceptor in the viral pol gene, resulting in an in-frame Mn1-pol fusion (Fig. 3C). This in-frame

### Table 1. CISs in NHD13 mice

<table>
<thead>
<tr>
<th>CIS</th>
<th>Incidence</th>
<th>Mouse identification</th>
<th>Occurrence in RTCGD</th>
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<tbody>
<tr>
<td>Meis1</td>
<td>14</td>
<td>3, 12, 30, 89, 99, 101, 106, 112, 116, 118, 119, 121</td>
<td>27</td>
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<tr>
<td>Mn1</td>
<td>8</td>
<td>19, 26, 28, 84, 110, 112, 113, 118</td>
<td>3</td>
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<td>Gata2</td>
<td>5</td>
<td>7, 12, 86, 99, 119</td>
<td>0</td>
</tr>
<tr>
<td>Erg</td>
<td>4</td>
<td>24, 83, 88, 92</td>
<td>2</td>
</tr>
<tr>
<td>Runx2</td>
<td>3</td>
<td>8, 11, 106</td>
<td>7</td>
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<td>Ras2</td>
<td>2</td>
<td>106, 113</td>
<td>32</td>
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<tr>
<td>Epor</td>
<td>2</td>
<td>29, 89</td>
<td>1</td>
</tr>
<tr>
<td>mir29a and mir29b</td>
<td>2</td>
<td>3, 11</td>
<td>11</td>
</tr>
<tr>
<td>Pom1</td>
<td>2</td>
<td>12, 76</td>
<td>34</td>
</tr>
<tr>
<td>Gfi1B</td>
<td>2</td>
<td>11, 25</td>
<td>1</td>
</tr>
<tr>
<td>Basgrp1</td>
<td>2</td>
<td>28, 76</td>
<td>31</td>
</tr>
<tr>
<td>Ephl</td>
<td>2</td>
<td>106, 113</td>
<td>3</td>
</tr>
<tr>
<td>Evi1</td>
<td>2</td>
<td>11, 84</td>
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<td>6qA3.3</td>
<td>2</td>
<td>106, 118</td>
<td>0</td>
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</tbody>
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NOTE: RTCGD data derived from transposon insertion studies are not included.
We examined the expression of the gene. We found that the level of expression was substantially increased over the levels of expression in both normal bone marrow and normal spleen, suggesting that the impact of these insertions is to up-regulate the expression level of the Epor in these mice (Supplementary Fig. S2C). Interestingly, one of these mice (no. 29) had ANLL with marked basophilia, and FACS analysis showed that the major leukemic clone was positive for CD41 and CD117 and negative for Mac1 and ter119 (data not shown), indicating that Epor overexpression was associated with characteristics of trilineage (erythroid, megakaryocyte, mast) precursor cells (23–25).

**miRNA insertions.** Two mice (nos. 3 and 11) had insertion sites on chromosome 6qA3.3 separated by only 250 bp. This locus is not near any characterized protein-coding genes, but is 20 kb from a miRNA cluster containing miR29a and miR29b1. We examined the expression of these miRNAs by Northern blot. Both miR29a and miR29b1 are expressed at high levels in the spleens of both

**Table 2. Selected single insertion sites in NHD13 leukemia**

<table>
<thead>
<tr>
<th>Insertion sites present in RTCGD</th>
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<tbody>
<tr>
<td><strong>Signal</strong></td>
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<tr>
<td><strong>transduction</strong></td>
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<tr>
<td><strong>Cell cycle</strong></td>
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<tr>
<td><strong>Differrentiation</strong></td>
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<tr>
<td><strong>Other</strong></td>
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<table>
<thead>
<tr>
<th>Insertion sites not present in RTCGD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Signal</strong></td>
</tr>
<tr>
<td><strong>transduction</strong></td>
</tr>
<tr>
<td><strong>Cell cycle</strong></td>
</tr>
<tr>
<td><strong>Differrentiation</strong></td>
</tr>
<tr>
<td><strong>MicroRNA</strong></td>
</tr>
<tr>
<td><strong>Other</strong></td>
</tr>
</tbody>
</table>

NOTE: RTCGD data derived from transposon insertion sites are not included.

fusion transcript encodes a protein that fuses the NH2-terminal portion of MN1 to the final 115 residues of the integrase peptide encoded by the pol gene (accession number AAC98548).

In addition, we found a second CIS 80 to 166 kb 3′ of Mn1 that was present in four additional mice (nos. 19, 110, 113, and 118; Fig. 3A). These insertions were not all in the same orientation, excluding the possibility that a Mn1-pol fusion gene (or at least a Mn1-pol fusion gene) could be formed. We designed a real-time RT-PCR assay to assess the expression levels of wild-type Mn1 transcripts in all eight mice. All eight mice overexpressed wild-type Mn1 transcripts, in one case by 100-fold (Fig. 3D). Therefore, in the first set of four mice, the insertion seems to have two effects: the creation of a fusion transcript and an overexpression of the wild-type Mn1. In the second set of four mice, overexpression of wild-type Mn1 is the only detected outcome.

**Gata2, Erg, and Epor.** We assayed expression levels of Gata2, Erg, and Epor in mice from which these CISs were cloned. Five mice (nos. 7, 12, 86, 99, and 119) had insertions that were thought to potentially affect the expression of Gata2. The location of these insertions was widely varied; however, from 130 kb 5′ to 65× (Supplementary Fig. S2A). Four mice (nos. 24, 85, 88, and 92) had insertions in the Erg gene, three in intron 2, and one in intron 4. Erg expression in the leukemic spleens was up-regulated compared with normal spleen in all four samples, between 2.5-fold and 14-fold (Supplementary Fig. S2B). Two mice (nos. 29 and 89) had insertions near the Epor gene; one in intron 2 and the other immediately 3′ of the gene. We examined the expression of the Epor transcript in these mice and found that the level of expression was substantially increased over the levels of expression in both normal bone marrow and normal spleen, suggesting that the impact of these

**Figure 2. Meis1 insertions are frequent and result in overexpression of the Meis1 transcript.** A, exon/intron structure of the Meis1 locus and orientation of viral insertion sites. Black boxes, Meis1 exons. Arrow, direction of transcription. Arrowheads, viral insertion site position and orientation. White box, position of the cDNA Southern probe. E, EcoRV restriction site. Numbers, mouse identification. B, Southern analysis of Meis1 locus. *, expected germ line band. Arrows, insertion-derived bands of predicted size based on LM-PCR data. Arrowheads, bands resulting from viral insertion events not detected by LM-PCR. C, real-time PCR analysis of Meis1 expression in spleens of mice with Meis1 insertion events. Expression was calculated using the ΔΔCt method (19) and is shown in log 2 scale. Expression level was normalized to that of wild-type spleen. Columns are labeled with mouse identification number. S, wild-type spleen; BM, wild-type bone marrow.
nos. 3 and 11. We compared the expression levels of the microRNAs in some of the other retroviroly induced tumors of similar phenotype, in which retroviral insertions at this locus were not detected. The expression level of both miR29a and miR29b1 in five other tumors was very low by comparison, demonstrating up-regulation associated with retroviral insertion near the locus (Fig. 4A and B).

We examined the predominance of the clone containing this insertion site in the spleens of each of these mice by Southern blot. A non-germline band was evident in both mice, although it was fairly faint in mouse no. 3, suggesting that the leukemic clone with the miR29 insertion was a minor clone in this mouse (Fig. 4C), consistent with the more dramatic up-regulation of miR29a and miR29b1 in mouse no. 11 (Fig. 4A and B).

Because the up-regulation of a microRNA locus by a retroviral insertional event has not been previously described, we sought other potential examples of microRNA up-regulation in our data set. We identified five instances of retroviral insertions within 100 kb of known microRNA loci (Table 2), and did expression analyses for microRNAs at each of these insertion loci. The only locus that showed any suggestion of microRNA overexpression was miR21 in mouse no. 88 (Fig. 4D, top). We did a Southern blot to determine the relative contribution of the miR21-containing clone to the spleen tissue used for expression analysis (Fig. 4D, bottom); this experiment confirmed that the miR21 insertion derived from an expanded clone but also that the clone comprised only a minor portion of the spleen tissue. Therefore, the relatively modest up-regulation observed by Northern blot is consistent with the Southern blot, indicating that the clone with the miR21 insertion is a relatively minor clone.
**Single insertion sites.** Several loci that were only identified once in our cohort are nonetheless of interest because of the known or expected function of the gene. A selection of these loci are listed in Table 2 and includes a large number of genes implicated in leukemia, such as Flt3, Stat5b, Cend1, Cend2, Cend3, Flt1, Gfi1, Myb, Trp53, Pdgfrb, Egfr1, Flt1, Akt2, and Mllt3. Comparison of this list with the retroviral insertion mutagenesis data contained within the RTCGD (26) indicated that a large number of integration sites had not previously been identified.

**Discussion**

Previously, we showed that the NHD13 fusion protein, generated by the (t(2;11)(q31:p15) chromosomal translocation associated with MDS and AML, leads to impaired hematopoietic differentiation and MDS in transgenic mice (2). However, it is likely that this fusion protein is insufficient to induce leukemia because the NHD13 mice develop overt leukemia only following a long latent period. An evolving paradigm suggests that AML results from one mutation that impairs differentiation and at least one complementary mutation that increases cell proliferation and/or decreases apoptotic cell death (27). We sought to identify events that could complement the NHD13 fusion during leukemic transformation by using retroviral insertional mutagenesis.

Leukemia was induced with a latency of 3 to 6 months by injecting the MOL4070LTR retrovirus into newborn NDH13 pups. The efficiency of induction was much greater than either injecting the retrovirus into wild-type pups (3–10 months) or NDH13 pups that did not receive virus (4–14 months). This provided evidence that the NHD13 transgene and the retroviral insertions were indeed collaborating leukemogenic events. We identified a total of 14 CIS, defined as the occurrence of two insertions within 100 kb (20). Among the CIS, several had been observed in other retroviral insertion screens for proto-oncogenes or tumor suppressor genes, but others have not been previously reported as CIS in retroviral insertional mutagenesis for proto-oncogenes or tumor suppressor genes, defined as the occurrence of two insertions within 100 kb (20). We identified at least one clone that did not receive virus (4–14 months). This provided evidence of nearby genes, we chose a subset of CIS genes and examined the regulatory mutation that increases cell proliferation and/or decreases apoptotic cell death (27). We sought to identify events that could complement the NHD13 fusion during leukemic transformation by using retroviral insertional mutagenesis.

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To verify that insertional mutagenesis altered the transcription of nearby genes, we chose a subset of CIS genes and examined the level of expression in the leukemic spleen tissues from which the insertions were cloned. We investigated three genes (Meis1, Mn1, Gata2, Erg, and Epor), and in most cases examined, the gene was overexpressed with respect to normal spleen. This suggests that up-regulation of the gene is a consequence of the insertion and, therefore, is likely to be contributing to the oncogenicity of the leukemic clone. For those mice in which the gene at the CIS was not markedly overexpressed, it is possible that the clone in question is a minor clone.

Meis1 was first identified as a CIS in a spontaneously recombinant retrovirus (BXH2) leukemogenic model (28) and has since been well established as a collaborator of overexpressed HOX genes in leukemogenesis (29–31). Meis1 is also known to collaborate with NUP98-HOX fusion genes (22, 32), including NHD13 (21). It was therefore not surprising that Meis1 would be a CIS identified by this screen. We identified Meis1 insertions in a total of 14 mice, more than for any other gene. Almost all of these insertions were near the 5’ end of the gene; therefore, the mechanism by which this insertion contributes to leukemic transformation is most likely overexpression of the normal Meis1 transcript, consistent with previous findings. By quantitative real-time PCR analysis of mice known to contain Meis1 insertions, the majority of tumors showed clear up-regulation of Meis1 expression. For the minority that did not, it is possible that the Meis1 clone was a minor clone in an oligoclonal tumor, such as mouse no. 12.

The second most frequent CIS identified in our screen was Mn1. Mn1 is a transcriptional coactivator known to synergize with retinoic acid receptor–mediated transcription (33) and has been shown to be involved in leukemia by at least two mechanisms. Mn1 is involved in formation of a fusion transcript with the TEL oncogene in AML (34), and high levels of Mn1 expression have recently been shown to be a poor prognostic marker for AML patients with normal cytogenetics (35). Mn1 has not been identified as a CIS in any other retroviral mutagenesis experiment contained in the RTCGD, supporting the possibility that Mn1 is a specific collaborator with NHD13. The insertion sites in Mn1 identified in our study fell into two distinct groups. Four mice had an insertion in the forward orientation in the homologous intron of Mn1 in which the MN1-TEL breakpoint occurs. These mice expressed a fusion transcript between Mn1 and the integrase viral gene, perhaps recapitulating the MN1-TEL fusion. Of note, a recent study showed that MN1-TEL collaborated with HOXA9 to induce myeloid leukemia in mice (36), so collaboration of an Mn1-integrase fusion gene with NHD13 may occur via a similar mechanism. If these mechanisms are indeed similar, then the TEL and integrase portions of the respective fusions may not contribute any required function to the fusion protein, and the amino terminal portion of MN1 may be sufficient to promote leukemic transformation. Alternatively, it may be that some non–sequence-specific scaffolding function is required of the MN1 fusion partner; for instance, the retained portions of TEL and integrase each encode a DNA-binding domain (37). Four other mice had insertions 3’ of Mn1, and all of these mice, as well as the mice with intronic insertions, overexpressed wild-type Mn1 transcripts by real-time PCR.

Gata2, identified as the third most frequent CIS in our screen, was not present in the RTCGD. This was somewhat surprising, as GATA2 is an important regulator of hematopoietic differentiation and has been implicated in leukemia by overexpression, especially in patients with 3q21 aberrations (38, 39). Gata2 expression was strongly up-regulated in those mice that had nearby insertions, suggesting that it is indeed contributing to the leukemic phenotype.

The insertions at the miR29a and miR29b1 microRNA cluster result in the overexpression of the mature form of both of these microRNAs. The likely mechanism is transcriptional up-regulation of the pri-microRNA transcript, followed by processing to the mature form. Whether a high level of expression of both microRNAs contributes to leukemic transformation, or whether up-regulation of either miR29a or miR29b1 alone would be sufficient, is unknown. Microarray studies have shown that miR29b2, the mature sequence of which is identical to miR29b1, is frequently up-regulated in breast, colon, pancreatic, and prostate cancer (40) and modestly up-regulated in papillary thyroid carcinoma (41). Little is known about the normal function of miR29a or miR29b1, although both have been shown to be expressed in a subset of hematopoietic cell lines (42).

To our knowledge, the insertions near the miR29a and miR29b1 microRNA cluster are the first demonstration of microRNA transcript up-regulation by retroviral insertion. Of interest, a previous study (43) observed a common retroviral integration site near this locus in brain tumors, reinforcing insertion at this locus (and subsequent up-regulation of these microRNAs) as oncogenic events. This finding also suggests that miR29a/b1 up-regulation may not be a specific collaborator of NHD13, nor even specific to
leukemia. Because up-regulation of a microRNA by retroviral insertion has not been previously shown, we sought to uncover a second instance of this phenomenon in our cohort. We identified five candidate single insertion sites and analyzed expression of a corresponding mature microRNA in the appropriate mouse. Only one additional microRNA (mir21) was observed to be overexpressed with respect to other leukemia samples of similar phenotype, and the overexpression was fairly subtle. mir21 has been previously implicated in oncogenesis (40, 44–46), supporting the evidence for its overexpression having a role in the outgrowth of this clone.

Many of the leukemias generated in this study contained more than one CIS, suggesting the possibility that a single retroviral integration is insufficient to complement NHD13, and that multiple integrations are required. However, because many of the leukemias seemed to be oligoclonal by Southern blot, it is also possible that each individual clone in the oligoclonal sample was generated by a single leukemogenic insertion (discounting bystander events). This question will be addressed by functional complementation studies.

Several loci that were identified only once in our cohort are nonetheless of interest because of the known or expected function of the gene. The list was searched against the RTCGD database to determine whether these events were common in retroviral insertion screening. A surprisingly high number of these events were not listed in the RTCGD, suggesting that they are unique to our screen, possibly because of either the sensitizing background (NHD13) or the virus used (MOL4070LTR). It is also possible that these insertion sites were not selected for but were merely bystander events.

The findings of the present study are likely to be relevant for human AML. Although translocations involving homeobox genes are relatively rare in patients with AML, translocations involving the MLL gene are thought to exert their oncogenic effect through the activation of homeobox genes (47), and HOXA cluster genes are among the genes most commonly up-regulated in patients with AML (48). In this context, it is important to note that recent reports have implicated the overexpression of MN1 (35) or ERG (49), two of the four genes most frequently identified in our screen, to be important prognostic factors for patients with AML.

Our data show that NHD13-induced leukemia can be accelerated using the MOL4070LTR retrovirus. Some of the genes that we identified as potential collaborators (Mtn1, Gata2, Erg) have not previously been reported as CIS in retroviral mutagenesis screens and are potentially specific NHD13 collaborators in the same mode as Meis1, rather than more general collaborators associated with malignant transformation, such as Stat5b, Ccn1, or Trp53. Indeed, some may be even more specific than Meis1, given their absence from the NHA9 BXH2 screen done previously. Of particular interest was the up-regulation of two microRNAs in response to retroviral insertion. Because insertion at this locus has been previously observed in retrovirally induced brain tumors, up-regulation of either mir29a or mir29b1 may well be a more general oncogenic event. Our findings present novel implications for mechanisms of NHD13 leukemogenesis and again show the utility of retroviral insertional mutagenesis as a means of identifying such information.

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