Insertional Mutagenesis Reveals Progression Genes and Checkpoints in MYC/Runx2 Lymphomas

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Abstract
In this study, we have exploited the power of insertional mutagenesis to elucidate tumor progression pathways in mice carrying two oncogenes (MYC/Runx2) that collaborate to drive early lymphoma development. Neonatal infection of these mice with Moloney murine leukemia virus resulted in accelerated tumor onset with associated increases in clonal complexity and lymphoid dissemination. Large-scale analysis of retroviral integration sites in these tumors revealed a profound bias towards a narrow range of target genes, including Jdp2 (Junmd), D cyclin, and Pim family genes. Remarkably, direct PCR analysis of integration hotspots revealed that every progressing tumor consisted of multiple clones harboring hits at these loci, giving access to large numbers of independent insertion events and uncovering the contrasting mutagenic mechanisms operating at each target gene. Direct PCR analysis showed that high-frequency targeting occurs only in the tumor environment in vivo and is specific for the progression gene set. These results indicate that early lymphomas in MYC/Runx2 mice remain dependent on exogenous growth signals, and that progression can be achieved by constitutive activation of pathways converging on a cell cycle checkpoint that acts as the major rate-limiting step for lymphoma outgrowth. [Cancer Res 2007;67(11):5126–33]

Introduction
Overexpression of the c-MYC gene is widespread in human cancer and was the first oncogenic lesion to be modeled in transgenic mice (1, 2). These mouse strains have been used extensively to uncover oncogenes and tumor suppressor genes that can collaborate with Myc in tumorigenesis, which include the Runx gene family of transcription factors (3). The RUNX genes also play important roles in human cancer, with evidence of both gain and loss of function in the context of different lineages and tumor types (reviewed in ref. 4). Indeed, RUNXI is frequently involved in human leukemias, where it is subject to a variety of chromosomal translocations causing gene fusions as well as gene amplification, deletion, and inactivating point mutations (4, 5). Of note for the present study, RUNX1 and MYC are among the most highly overexpressed genes in childhood acute lymphocytic leukemias (6).

We identified all three murine Runx genes as targets for insertional mutagenesis and overexpression in a MYC transgenic model in which this oncogene is directed to the T-cell compartment under the control of a CD2 expression cassette (3, 7, 8), suggesting that the Runx genes share a redundant oncogenic function in the context of deregulated Myc. To explore this aspect of Runx function, we have studied CD2-Runx2 transgenic mice that are prone to lymphoma development and display impaired thymocyte maturation with an accumulation of immature CD8 cells. Crossing with CD2-MYC mice leads to early tumor onset (9), and our recent studies of the underlying mechanism have indicated that ectopic Myc over-rides the Runx2-imposed proliferation block, whereas Runx2 expression confers a low apoptotic rate, apparently neutralizing the propensity of Myc to induce apoptosis in tumor cells (10, 11).

Despite the rapid onset of tumors in MYC/Runx2 transgenic mice, it seems that further events are required to complete oncogenic transformation. Rearranging gene analyses indicate that the tumors arise as outgrowths from an initially polyclonal population in the postnatal thymus (9, 12). The identification and characterization of progression genes in MYC/Runx2 tumors is therefore of considerable interest for the further elucidation of this collaboration mechanism.

Retroviral insertional mutagenesis is a classic method of identifying genes relevant to cancer and has been particularly effective in the study of hematopoietic malignancies (13). Based on the assumption that retroviral insertion is effectively random, the occurrence of a common insertion site in independent tumors is indicative of a selective process driving tumorigenesis and the proximity of a gene whose expression or function is affected by retroviral integration. The development of high-throughput PCR methods and completion of human and murine genome sequences has led to a resurgence of interest in the use of retroviruses as genetic screening tools in cancer. Analysis of mice infected with strains of murine leukemia virus (MLV) or retrotransposons has revealed many genes with the potential to be targeted. More refined approaches of this type include collaboration tagging, where the technique is used to detect cooperating genes in mice carrying a dominant oncogene or with a defect in a tumor suppressor gene (3, 14–17), and complementation tagging, where mutagenesis is used to tag functional homologues of genes in mice deleted in one or more known targets (18). More recently, infection of mice with a defect in DNA repair has been employed to shift the target gene spectrum towards tumor suppressor loci (19). In this study, we show that...
retroviral insertional mutagenesis can be used to elucidate the rate-limiting steps in tumor progression and identify the genes families and pathways that drive this process.

Materials and Methods

Transgenic mice and lymphomas. CD2-MYC/CD2-Runx2 transgenic mice (hereafter described as MYC/Runx2) on a C57Bl/6xCB/Ca strain were generated as previously described (9). Newborn bitransgenic mice were infected with 10^3 infectious units of Moloney MLV (MMLV; ref. 15) within 24 h of birth. Genotypes of mice were identified by Southern blot hybridization analysis carried out on DNA extracted after tail biopsy. MYC/Runx2 animals revealed exclusively multicentric lymphomas from which high molecular weight DNA was isolated. All animal work was carried out in line with the UK Animals (Scientific Procedures) Act of 1986.

Cloning of proviral insertion sites. Proviral insertion sites were amplified using the splinkerette-based approach as previously described (18), with slight modifications. Briefly, 3 μg of tumor DNA was digested with BstXI (New England Biolabs). Following inactivation of enzyme, 300 ng of digested DNA was ligated to 0.12 pmol of the splinkerette adaptor with 4U T4 DNA ligase (Roche) overnight at 16°C. To avoid subsequent amplification of the internal 3′ MMLV fragment, the ligated mixture was digested with an excess of EcoRI followed by DNA purification using Qiajan columns. Proviral/genomic DNA junction fragments were isolated after two rounds of PCR amplification; 100 ng of ligated DNA was used in the primary PCR containing 4U Pfu Turbo hotstart (Stratagene) and 200 nmol/L of each primer (Splnk1 and LTR5, sequences available on request). The hotstart PCR conditions were 3 min at 94°C (1 cycle); 15 s at 94°C, 30 s at 68°C, 5 min at 72°C (2 cycles); 15 s at 94°C, 30 s at 66°C, 5 min at 72°C (27 cycles); 5 min at 72°C (1 cycle). A nested PCR was carried out using 2 μL of the primary PCR with 200 nmol/L of primer (Splnk2 and LTR5, primer sequences available on request) and 12.5 μL Qiajan Multiplex PCR kit mix (Qiagen). The PCR conditions were 15 min at 94°C (1 cycle); 15 s at 94°C, 90 s at 60°C, 3 min at 72°C (25 cycles); 5 min at 72°C (1 cycle). PCR products were visualized on 4% polyacrylamide gels and a ladder of fragments obtained ranging from −100 to −2,000 bp; 1 μL of the nested PCR reaction was shotgun cloned in the Topo TA vector (Invitrogen) and DNA from 24 transformants isolated and analyzed by both EcoRI digestion followed by gel electrophoresis and DNA sequencing (BigDye terminator mix V2.0, Applied Biosystems). Gel electrophoresis allowed comparison of the cloned products with the nested PCR to ensure that all of the prominent bands in the PCR were represented in the shotgun cloning.

Sequence analysis. Homology searches of all of the sequences isolated were carried out using the publicly available BLASTn in Genbank databases and mouse genome database (February 2006 draft assembly) and identified annotated candidate genes located near each retroviral insertion site. We compared these sites with previously identified insertion sites in the Mouse Retroviral Tagged Cancer Gene Database (RTCGD).1

Direct DNA PCR. Amplification was carried out on 1-μg aliquots of genomic DNA with 50 pmol of primer pairs in 2× Reddy Mix (Abgene). MLV-LTR–specific primers were LTR sense (5′-CCACCCTAGTTGGGCGGACGC) and LTR antisense (5′-CCGGCCTGAGTCTCTTC or 5′-CCTGTTCA-ACGTGTCTCCAGACC). Gene-specific primers were as follows: Ifp2 exon 3 (JDP-R, 5′-CATCTGGCTGGAGACTTTG), Pim1 exon 6 (PIM-F, 5′-GGA-CAGCCAATGACAATCCTAC or 5′-GAAATCCGGGACCATCaTG), C/EBPα exon 1(C/EBPα, 5′-CCATGGGCTGGGCGGGGAGTCC), Gfi-1 exon 1 (Gfi-1-R, 5′-ACATTGCTTTGGACTACCCAG), Barc exon 2 (BARC-F, 5′-GCTGCTGCA-CGAGGAAGACCT), Myc exon 1 (MYC-F, 5′-CATCTGAGTCTCTACCTCAAGACC) and Myc exon 6 (MYC-F, 5′-CATCTGGCTGGAGACTTTG). Amplification conditions were 5 min at 94°C (1 cycle); 1 min at 94°C, 1 min at 60°C, 1 min at 72°C (30 cycles) and 5 min at 72°C (1 cycle) for all MLV gene–specific amplifications with the exception of Pim/F-LTR antisense PCR. Amplification conditions were 5 min at 94°C (1 cycle); 1 min at 94°C, 1 min at 55°C, 1 min at 72°C (30 cycles); and 5 min at 72°C (1 cycle). PCR products were separated on 1% to 2% Tris-borate EDTA (TBE) agarose gels and visualized by staining in ethidium bromide.

Reverse transcription-PCR. One microgram of total RNA was reverse transcribed using ImProm-II Reverse Transcription System (Promega). Amplification was done on aliquots of one twentieth of the sample with 40 pmol each of primer pair MLV-RU5 (5′-GCGCTGCTGGAGACTTTG).

2 http://genome.ucsc.edu/
and JDP-R. Amplification was done in 2× Reddy Mix (Abgene) at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 30 cycles. Aliquots (12.5 μL) were separated on 2% TBE agarose gels and visualized by staining in ethidium bromide.

**DNA hybridization analysis.** High molecular weight DNA from mouse lymphomas and radiolabeled probes were prepared as previously described (9). MLV proviral sequences were detected using a probe derived from the U3 domain of the LTR (20). Pim1 probe is (Pim1A) as previously described (19).

**Results**

Neonatal infection with Moloney MLV accelerates tumor onset and increases clonal complexity in MYC/Runx2 mice. To test the ability of MLV to drive tumor progression, MYC/Runx2 mice were infected at birth and monitored for development of disease. Uninfected MYC/Runx2 mice develop lymphomas in the thymus and peripheral lymphoid tissues at an average age of 36 days (9, 12). MLV caused a significant acceleration ($P \leq 0.0001$) of tumor development, with infected animals developing multicentric lymphomas by 28 days (Fig. 1A). Although tumor pathology was grossly similar, it was notable that the virus-accelerated tumors displayed greater involvement of extra-thymic lymphoid tissues (Fig. 1B). Analysis of these lymphomas for clonal complexity by T-cell receptor gene rearrangement revealed a pattern of increased complexity in the infected tumors, suggesting that viral infection increases the number of expanding tumor cell clones and not merely the growth rate of transformed cells (data not shown). As an independent marker of tumor cell clonality and a clue to the role of insertional mutagenesis in rapid tumor progression, the pattern of proviral integration junction fragments was assessed by hybridization with a U3 probe. This analysis showed that the lymphomas from MLV-infected mice contained multiple viral copies with some evidence of emerging dominant clones as identified by unique junction fragments (Fig. 1C). Screening for rearrangements at candidate target genes was carried out using common insertion site probes, including Pim-1, Pim-2, c-Myb, Ahi-1, Bmi-1, Evi-5, and Gfi-1, most of which have been found as targets in virus-accelerated tumors of Runx2 mice (12). The results were negative, apart from Pim-1, where we noted a clonal and subclonal rearrangement (Fig. 1C), and Pim-2, where a single tumor with a subclonal rearrangement was observed (data not shown).

**Direct PCR reveals multiple insertions at Pim-1 in all virus-accelerated MYC/Runx2 lymphomas.** Detection of Pim-1 as a potential progression gene was consistent with the fact that an Eμ-Pim-1 transgene accelerates tumor onset in MYC/Runx2 mice (12). As MLV insertions that activate Pim-1 frequently cluster within

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**Figure 2.** Pim1 direct DNA PCR assay reveals multiple sense MLV integrations in 3’UTR. A, top, genomic organization of Pim1 gene with location and number of mapped MLV integrations indicated (RTCGD hits). 3’UTR integration hotspot (bold). Top arrow, transcription start site; bottom arrows, direction of PCR primers, LTR sense (LTR-S), LTR antisense (LTR-AS), and Pim1 exon 6 (PIM-F). Bottom, PCR products amplified from a selection of MYC/Runx2+MLV lymphomas, MYC/Runx2 cell lines, and control cells (N, NIH3T3) infected in vitro. B, lack of antisense MLV integrations in MYC/Runx2+MLV lymphomas. *, single PCR product amplified in one lymphoma; M, low molecular weight DNA markers. Primer pairs used for individual assays are indicated.
the 3′ untranslated region (UTR; ref. 15), we considered the possibility that the Southern blot analysis underestimated the prevalence of hits at *Pim-1* due to the clonal complexity of the tumors and devised a direct PCR assay to detect insertions at this site. As shown in Fig. 2A, this analysis revealed a remarkably high number of hits with every tumor containing multiple independent MLV insertions, lying in the same orientation as *Pim-1*, distributed throughout the 3′UTR. The authenticity of these insertions was confirmed by probe analysis and sequencing. In contrast, use of a LTR primer to detect proviruses in the antisense orientation at this site revealed only a single insertion from the tumor panel, confirming the bias in orientation of activating insertions at *Pim-1* observed in previous studies (Fig. 2B). The detection of multiple insertions in the MYC/Runx2 + Pim-1 tumors contrasted with longer-latency tumors of Runx2 mice infected with MLV and harboring insertions at *Pim-1*, which displayed a much simpler pattern, indicative of only one or a small number of cell clones with these insertions (Fig. 2C). The band intensities in these cases were significantly higher and from dilution experiments we estimate that most of the oligoclonal insertions in the MYC/Runx2 tumors represent from 0.1% to 1% of the tumor cell mass, indicating that the cells carrying these insertions have undergone significant clonal expansion (data not shown).

**High-frequency gene targeting is a specific feature of the in vivo tumor environment.** To investigate the possibility that the sensitive direct PCR assay was detecting an intrinsic bias of MLV integration rather than the result of growth selection of clones carrying insertions at *Pim-1*, we infected cells with MMLV *in vitro* and grew these for 28 days to recapitulate the *in vivo* tumor latent period after neonatal infection. We infected NH3T3 fibroblasts and a series of T-cell tumor cell lines established from the MYC/Runx2 background to model as closely as possible the genetic and transcriptional environment of the *in vivo* target cell. Direct PCR on DNA from these cells revealed no detectable hits at the *Pim-1* 3′UTR hotspot in fibroblasts (Fig. 2A). Southern blot analysis and hybridization with a U3-specific probe confirmed successful infection with MLV (data not shown). Faint bands seen on the stained gels were mostly nonspecific and did not hybridize to a *Pim-1* probe, although low-level insertions could be detected in one of the *in vitro* infected lymphoma lines (data not shown).

**Large-scale analysis of retroviral insertion sites reveals multiple hits at a narrow range of target genes including *Jdp2*, *D cyclin*, and *Pim*.** The discovery of *Pim-1* as a frequent progression target encouraged us to conduct a wider analysis for other genes that might play an analogous role. For an unbiased screen of proviral insertion sites, we used a splinkerette-based PCR approach (18). Shot-gun libraries of clones were generated from individual tumors, and insert sizes were determined to ensure that all unique integrants were sequenced (see Materials and Methods). In total, 480 putative viral junction fragments were isolated, cloned, and sequenced. Following elimination of non-informative clones and duplicate clones from the same tumor, 272 independent retroviral integration sites were identified. Homology (BLASTn) searches of the sequences isolated were carried out, and candidate genes located near each tag were identified. Genes targeted more than once in the data set are shown in Table 1 (see Supplementary Table S1 for precise chromosomal coordinates). It is evident that the target loci detected in this screen are far from a random selection from the murine genome. Comparison with the RTCGD3 shows that 12 of the 18 genes targeted more than once correspond to known common insertion sites. Of the remaining six genes, five have annotated functions of potential relevance to cancer. Notably, genes that regulate survival and cell cycle progression are highly represented in the target set.

The most frequently targeted gene, *Jdp2* ([Jun-dimerizing protein 2 or *Jundm2*]), has been observed previously as a common insertion site in a number of MLV-induced tumors from p27-deficient or wild-type mouse backgrounds (17, 21) and encodes a b-ZIP protein.

### Table 1. Summary of genes represented more than once in a library of 272 insertion sites cloned from 20 lymphomas arising in MLV-infected MYC/Runx2 transgenic mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. hits (tumors)*</th>
<th>Known CIS</th>
<th>Product</th>
<th>Reported function relevant to cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jdp2</td>
<td>16 (8)</td>
<td>Y</td>
<td>b-ZIP protein</td>
<td>Transformation, survival</td>
</tr>
<tr>
<td>Ccn1</td>
<td>12 (9)</td>
<td>Y</td>
<td>Cyclin</td>
<td>Cell cycle, survival</td>
</tr>
<tr>
<td>Pim2</td>
<td>8 (6)</td>
<td>Y</td>
<td>Pim family protein kinase</td>
<td>Survival, proliferation</td>
</tr>
<tr>
<td>Basgp1</td>
<td>5 (4)</td>
<td>Y</td>
<td>Ras guanyl releasing protein</td>
<td>Proliferation, transformation</td>
</tr>
<tr>
<td>Pim1</td>
<td>4 (4)</td>
<td>Y</td>
<td>Pim family protein kinase</td>
<td>Survival, proliferation</td>
</tr>
<tr>
<td>Ccn3</td>
<td>4 (4)</td>
<td>Y</td>
<td>Cyclin</td>
<td>Cell cycle, survival</td>
</tr>
<tr>
<td>Cst4</td>
<td>4 (4)</td>
<td>Y</td>
<td>Mitochondrial protein</td>
<td>Mitochondrial biogenesis</td>
</tr>
<tr>
<td>Otx2</td>
<td>3 (3)</td>
<td>N</td>
<td>Homeodomain protein</td>
<td>Survival, tissue specification</td>
</tr>
<tr>
<td>Pik3r5</td>
<td>3 (2)</td>
<td>Y</td>
<td>PI3K regulatory subunit</td>
<td>Survival, cell cycle</td>
</tr>
<tr>
<td>Tnfsf13b</td>
<td>3 (2)</td>
<td>N</td>
<td>TNF family cytokine (Baff, BlyS)</td>
<td>Lymphocyte survival, cell cycle</td>
</tr>
<tr>
<td>Akt1</td>
<td>2 (2)</td>
<td>Y</td>
<td>Akt protein kinase</td>
<td>Survival, cell cycle</td>
</tr>
<tr>
<td>Bfip1</td>
<td>2 (2)</td>
<td>N</td>
<td>Cytoskeletal protein</td>
<td>—</td>
</tr>
<tr>
<td>Ccrk</td>
<td>2 (2)</td>
<td>N</td>
<td>Cell cycle–related kinase (p42, PNQARLE)</td>
<td>Survival, cell cycle</td>
</tr>
<tr>
<td>Eif3y</td>
<td>2 (2)</td>
<td>N</td>
<td>Translation initiation factor</td>
<td>Cell growth</td>
</tr>
<tr>
<td>Mad1l1</td>
<td>2 (2)</td>
<td>N</td>
<td>Mitotic spindle assembly checkpoint protein</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>Ral17</td>
<td>2 (2)</td>
<td>Y</td>
<td>MIZ zinc finger domain</td>
<td>Localization to replication foci</td>
</tr>
<tr>
<td>Slec9a9</td>
<td>2 (2)</td>
<td>Y</td>
<td>Solute carrier</td>
<td>—</td>
</tr>
<tr>
<td>Sbp3</td>
<td>2 (1)</td>
<td>Y</td>
<td>Single-stranded DNA binding</td>
<td>DNA replication</td>
</tr>
</tbody>
</table>

Abbreviations: CIS, common insertion site; Y, yes; N, no; PI3, phosphatidylinositol 3-kinase; TNF, tumor necrosis factor.

*Numbers in parenthesis represent number of tumors carrying gene-specific insertions.
that binds to Jun family proteins and represses activator protein-1 (AP-1) transcription (22). The D cyclin genes Ccnd1 and Ccnd3 were also highly represented in the progression gene set, with a total of 16 hits. The insertions corresponded to favored sites for gene activation, either upstream and in the reverse orientation or in the sense orientation within the 3’UTR. Two members of the Pim kinase family, Pim-1 and Pim-2, are also represented in the progression gene set with 4 and 8 hits, respectively. As expected from the direct PCR analysis in Fig. 2A, insertions cluster in the 3’UTR hotspot for Pim-1 and upstream of Pim-2 in the opposite orientation, the preferred mode for MLV activation of each gene (13). Although all of the frequently targeted gene set have been observed previously in wild-type mouse models, the percentage hit rates recorded here are unprecedented.

Genes targeted twice or thrice in this study might conceivably have been observed by chance (23), but this seems unlikely in cases where the pattern and clustering of insertions are indicative of gene activation (Pik3r5, Akt1, BlyS, and Otx2) or inactivation (Mad1l1; Supplementary Table S1). Moreover, these rarer targets include known common insertion sites (Pik3r5 and Akt1) and novel targets (Ccrk, BlyS, and Mad1l1) with functional relevance to cancer (24–26).

**Direct PCR confirms positive and specific selection of the progression gene set.** As a further test for the specificity of the progression set, we devised direct PCR assays based on hotspots in Ccnd1, which was frequently targeted in the MYC/Runx2+MLV tumors, and Gfi-1, which was not detected. The lack of hits at Gfi-1 was of interest as this is a potent collaborator with Myc that has also been observed as a target in virus-infected Runx2 mice (12). These assays reinforced the findings of the large-scale random cloning analysis, with frequent insertions detected at Ccnd1 (Fig. 3A), but none at the Gfi-1 cluster (Fig. 3B). We also generated a PCR assay for a locus that was targeted only once in the random cloning panel (Rorc). This assay detected a product only from the index tumor and showed no amplification products in the rest of the panel (Fig. 3C). Direct DNA PCR was carried out on MLV-induced tumors from strain matched nontransgenic control mice, revealing clonal insertions in only a subset of the tumors (Ccnd1, 2 of 13; Pim-1, 3 of 13; Jdp2, 4 of 13; data not shown), emphasizing the bias towards these loci in the MYC/Runx2 tumors where 28 of 28 scored positive. Again, a contrast was evident with Gfi-1, where direct PCR assay revealed 2 of 13 insertions in the infected wild-type and 0 of 28 in the MYC/Runx2 tumors (data not shown).

A major integration hotspot in Jdp2 reveals selection for fusion transcripts and protein truncation. The most highly targeted gene in the large-scale analysis was Jdp2, where 80% (13 of 16) of the tags mapped to the 3’end of intron 2. A direct Jdp2 DNA PCR assay was devised to detect similar insertions (Fig. 4A). This analysis revealed multiple insertions in all of the MYC/Runx2 lymphomas, and the authenticity of these products was again confirmed by blot hybridization analysis with a Jdp2-specific probe and by sequencing of selected clones. Integrations were detected in both the sense and antisense orientation, with a slightly greater representation of antisense amplicons. Despite the very high hit rate in the tumors, we again found no evidence of insertions at this site in cells infected in vitro (Fig. 4A).

The presence of insertions in the sense orientation suggested that these might drive the expression of hybrid transcripts, and this prediction was confirmed by reverse transcription-PCR (RT-PCR) analysis (Fig. 4A). We mapped a total of 83 independent insertion sites in intron 2 by either direct PCR or RT-PCR, and the locations

![Figure 3. Direct PCR confirms specificity of progression gene set.](image-url)
of these are shown in Fig. 4B. Direct sequence analysis of the RT-PCR products showed that the fusion transcripts derive from the viral 3LTR, suggesting that they arose by read-through or de-repression of the 3LTR promoter. An interesting difference between the sense and antisense insertions is that the latter do not occur close to the exon boundary (Fig. 4B). Scanning of the intron sequence for polII promoter sequences reveals a marginal prediction at the point shown by an arrow in Fig. 4B, suggesting that the insertions in the antisense orientation may activate a cryptic promoter element.

The fusion transcripts contain in-frame ATGs derived from Jdp2 intron 2 close to the exon boundary or from further upstream due to splicing into exon 3 (Fig. 4B). Insertions in the opposite orientation would be predicted to drive the expression of similar products if the cryptic promoter element in intron 2 is activated. As shown in Fig. 4C, a notable feature of the fusion proteins predicted from these insertions is that they lack an NH2-terminal domain that has been shown to be important for histone binding and inhibition of p300-mediated histone acetylation at target promoters (27). The products should therefore be defective in the AP1 repressive activity of the wild-type Jdp2 protein but might be expected to have novel biological properties as they retain the b-ZIP domain through which Jdp2 heterodimerizes with c-Jun and related proteins (22).

Discussion

This study has shown that retroviral insertional mutagenesis can be used to drive tumor progression in vivo and to identify relevant target genes in tumors arising in highly tumor-prone mice. Large-scale analysis of integrated proviruses was able to detect a strong skew towards a subset of MLV targets that can be linked
mechanistically to the initiating oncogenic programme. The ability of one of the frequent targets, Pim-1, to collaborate independently with MYC and Runx2 (12, 28) and with the combination of both genes (12) confirms the relevance of the progression gene set detected in this study. Moreover, direct PCR of integration hotspots validated the findings of random sampling and confirmed that the bias towards specific loci is a function of selection operating on cells in the in vivo tumor environment rather than an intrinsic integration bias of Moloney MLV. It seems likely that each clone arises from a single mutagenic hit, as the probability of hitting two loci simultaneously in a single round of infection is very low, and tumor onset is probably too fast to allow the generation and spread of MLV recombinants that could circumvent envelope-mediated interference (29).

The skew towards integrations at specific target genes observed here is even more profound than that seen previously in panels of retrovirus-induced tumors from wild-type mice or those carrying a single genetic lesion (17, 18, 30, 31), presumably reflecting the limited repertoire of genes capable of efficient synergy with the MYC/Runx2 oncogenic program. The predominance of hits at Ccn2 is somewhat surprising in light of its low endogenous expression levels in lymphoid cells (32), and this observation lends further support to the argument that gene targeting by retroviruses in cancer is not simply a function of preferred integration into highly expressed genes (23).

An unanticipated advantage of the progression tagging approach we have described here is that the polyclonal nature of the tumors allows many insertions to be sampled from a relatively small series of tumors, providing superior insights into underlying mechanisms of gene deregulation. Thus, insertions within the Pim1 3′UTR were entirely consistent with the established enhancer insertion mechanism (33), displaying a strong bias with regard to orientation but no evident selection for precise location within this domain. In contrast, insertions in Jdp2 intron 2 often appeared to select for disruption of the coding sequence and the expression of truncated proteins.

Figure 5 presents a model based on our current understanding of the oncogenic collaboration of MYC and Runx2 (4, 11), incorporating the progression set as accessory factors interacting with the basic feedback loop. Our previous studies have indicated that Runx2 and MYC collaborate by neutralizing each other’s fail-safe responses. The induction of growth arrest by ectopic Runx2 (10) is not fully understood but may conceivably involve cyclin-dependent kinase inhibitors, such as p21Waf1, that can be induced or repressed by Runx (34) and can be counteracted by ectopic Myc (35). Runx2 seems to inhibit Myc-induced apoptosis in vivo, although the underlying pathways remain to be identified (11). The Pim kinases have recently been shown to phosphorylate Runx proteins directly (36) but may also feed into G1 checkpoint controls through phosphorylation and inactivation of p21Waf1 (37). However, it should be noted that this model may represent only a subset of relevant Pim functions as these kinases have additional targets in cell cycle and apoptosis control (38).

The frequent targeting of D cyclins implies that G1 checkpoint controls are rate limiting for the growth of MYC/Runx2–expressing cells. This observation is reminiscent of classic studies in fibroblasts where Myc was characterized as a competence factor capable of mediating G0–G1 transition but unable to complete progression to S phase unless complemented by progression factors (39, 40). It is conceivable that progression signals are provided in the MYC/Runx2 thymus by stromally presented growth factors or TCR ligation, allowing limited expansion of preneoplastic cells. In this model, tumor progression results from genetic or epigenetic changes mediating release from the requirement for external mitogenic signals. The progression gene set we have observed fits this model well, as the expression of both D cyclins and Pim kinases is strongly dependent on mitogenic signals (38, 41), and this process should be short circuited by proviral gene activation. The model is also consistent with the biology of virus-accelerated tumors which display wider lymphoid dissemination (Fig. 1B). In further support of this model, some of the less frequently targeted common insertion sites we identified, such as Akt1, P3kr5, and Blys, would also be expected to feed into this checkpoint through induction of D cyclin expression (25, 42).

The role of Jdp2 in cancer has been the subject of conflicting findings, but we favor a model in which the truncated proteins feed into the same D cyclin–responsive checkpoint through mimicry of growth factor–induced c-Fos activation. Jdp2 protein was discovered as a repressor of AP-1 signaling that is expressed in quiescent cells where it forms heterodimers with Jun family proteins and is displaced by c-Fos upon serum induction (22). Recent studies support its putative tumor suppressor role by showing that the full-length protein can suppress both Ras transformation of 3T3 fibroblasts and the tumorigenicity of prostate cancer xenografts (43). Notably, the truncated proteins that are predicted to be expressed as a result of retroviral insertions in Jdp2 intron 2 lack a domain that has been identified recently to be critical for histone
binding and transcriptional repression (27). These observations suggest a scenario where the truncated Jdp2 proteins block repression of AP-1–responsive promoters by homodimerizing with wild-type Jdp2 or by forming nonfunctional heterodimers with other Jun family members. Such an explanation leaves us with a puzzle with regard to those cases where MLV has inserted upstream of the gene and seems to be associated with enhanced transcription (17, 21), although it should be noted that the integrity of the Jdp2 sequence has not been analyzed in such cases. Functional analysis of full-length and truncated Jdp2 variants will be required to answer these questions.

Our findings show that insertional mutagenesis is a powerful genetic tool in the study of tumor progression. However, there is a darker side to these findings as the ability of viral insertions to drive tumor progression with unexpectedly high efficiency raises further concerns about the safety of retroviral vectors in human cancer treatment, where they have been used for the purposes of cell marking (44) and delivery of prodrug-activating enzymes (45). In light of these results, it would seem prudent to avoid the use of integrating vectors for such applications, or at least ensure that their powerful mutagenic potential has been neutralized.

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