The RAG-1/2 endonuclease causes genomic instability and controls CNS complications of lymphoblastic leukemia in p53/Prkdc-deficient mice

Rebecca A. Gladdy,[1,3] Michael D. Taylor,[3,5] Christine J. Williams,[1,2] Ildiko Grandal,[1,2] Jana Karaskova,[4,6] Jeremy A. Squire,[4,6] James T. Rutka,[3,5] Cynthia J. Guidos,[1,2,7] and Jayne S. Danska[1,2,4,7,*]

[1]Program in Developmental Biology, The Hospital for Sick Children
[2]Department of Immunology
[3]Department of Surgery
[4]Department of Medical Biophysics
[5]Arthur and Sonia Labatt Brain Tumor Research Laboratory, The Hospital for Sick Children and Division of Neurosurgery
[6]Ontario Cancer Institute/Princess Margaret Hospital, University Health Network and Department of Laboratory Medicine and Pathobiology Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada
[7]These authors contributed equally to this work.
*Correspondence: jayne.danska@sickkids.ca

Summary

Double-strand DNA breaks (DSB) induce chromosomal translocations and gene amplification in cell culture, but mechanisms by which DSB cause genomic instability in vivo are poorly understood. We show that RAG-1/2-induced DSB cause *IgH/c-Myc* translocations in leukemic pro-B cells from *p53/Prkdc*-deficient mice. Strikingly, these translocations were complex, clonally heterogeneous and amplified. We observed reiterated *IgH/c-Myc* fusions on dicentric chromosomes, suggesting that amplification occurred by repeated cycles of bridge, breakage and fusion. Leukemogenesis was not mitigated in *RAG-2/p53/Prkdc*-deficient mice, but leukemic pro-B cells lacked *IgH/c-Myc* translocations. Thus, global genomic instability conferred by *p53/Prkdc* disruption efficiently transforms pro-B cells lacking RAG-1/2-induced DSB. Unexpectedly, *RAG-2/p53/Prkdc*-deficient mice also developed leptomeningeal leukemia, providing a novel spontaneous model for this frequent complication of human lymphoblastic malignancies.

Introduction

Human malignancies frequently display evidence of genomic instability, as revealed by chromosomal translocations, amplifications, deletions, and aneuploidy (Mitelman et al., 1997; Lengauer et al., 1998). These genetic alterations can induce ectopic expression of protooncogenes or disrupt expression of tumor suppressor genes to disregulate cell growth and survival during initiation or progression of tumorigenesis (Marx, 2002). Gene amplification is a common means by which tumor cells acquire resistance to chemotherapeutic agents (Schimke et al., 1978; Gorre et al., 2001). An important mechanism of gene amplification is the breakage-fusion-bridge (BFB) cycle, in which critical shortening or defective capping of telomeres triggers sister chromatid or end-to-end chromosomal fusions. The resulting dicentric chromosomes are unstable because they form anaphase bridges that are broken during mitosis (Maser and DePinho, 2002). The formation of end-to-end chromosomal fusions (Chin et al., 1999) and gene amplification in vitro (Livingstone et al., 1992; Yin et al., 1992) can be suppressed by the p53-dependent DNA damage response pathway, presumably by preventing cells from replicating chromosome with dysfunctional telomeres and DNA double-strand breaks (DSB) (Paulson et al., 1998). Experimental induction of DSB (Pipiras et al., 1998; Richardson and Jasin, 2000), chromosomal fragile sites (Windle et al., 1991; Coquelle et al., 1997; Hellman et al., 2002), and defects in DSB repair by nonhomologous end-joining (NHEJ) (Karanjawala et al., 1999; Difilippantonio et al., 2000; Ferguson et al., 2000) can also trigger translocations and gene amplification in cultured cells. Mice with defects in NHEJ are highly tumor-prone (Ferguson and Alt, 2001; Sharpless et al., 2001), demonstrating that unrepaired DSB can be tumorigenic. However, mechanisms by which DSB induce genomic instability and gene amplification in vivo are poorly understood.

SIGNIFICANCE

Genomic instability is a hallmark of human malignancies, and is manifested in tumor cells by the presence of numerous chromosomal translocations, amplifications, deletions, and aneuploidy. However, mechanisms that cause genomic instability in vivo are poorly understood. We show that in mice with disrupted DNA damage surveillance and DSB repair, physiologically introduced DSB induce chromosomal translocations and gene amplification through repeated bridge-breakage-fusion cycles in vivo. In addition, we show that the pattern of genomic instability influences the clinical behavior of leukemic pro-B cells, providing a new animal model to elucidate molecular mechanisms by which leukemic lymphoblasts invade and grow within the CNS.

The developmentally regulated process of V(D)J recombination requires the introduction of site-specific DNA double-strand breaks in lymphocyte progenitors, and provides a useful model to study general mechanisms by which improper surveillance and repair of DSB might cause genomic instability and oncogenesis in vivo. V(D)J recombination is used to generate clonally unique and highly diverse variable (V) region genes for antigen recognition by immunoglobulins (Ig) and T cell receptors (TCR) (Bassing et al., 2002). It is initiated when the RAG-1/2 lymphocyte-specific endonuclease introduces DSB at recombination signal sequences adjacent to V, D, and J gene segments (Fugmann et al., 2000), generating signal end and coding end DSB intermediates that are then repaired by NHEJ. NHEJ proteins with critical functions in V(D)J recombination also function ubiquitously in DSB repair and include the DNA-dependent protein kinase (DNA-PK or Prkdc), KU70, KU80, XRCC4, DNA ligase IV (LIG4), and Artemis (Bassing et al., 2002). While these NHEJ proteins function in concert during V(D)J recombination, KU70, KU80, XRCC4, and LIG4 also regulate telomere function, replicative senescence, cell growth, and embryonic neurogenesis (Bassing et al., 2002).

Although V(D)J recombination generates valuable somatic diversification of the germline repertoire of V genes, it may also compromise genomic stability in lymphocyte precursors. Indeed, many human lymphoid tumors harbor recurrent chromosomal translocations between antigen receptor loci and protooncogenes (Look, 1997; Faderl et al., 1998; Kuppers and Dalla-Favera, 2001) that are thought to arise by illegitimate rejoining of V(D)J coding ends to DSB on other chromosomes (Vanasse et al., 1999a; Vaandrager et al., 2000). V(D)J coding ends accumulate in NHEJ-deficient lymphocyte precursors (Roth et al., 1992), and inefficient repair of these DSB intermediates impairs the generation of functional antigen receptor genes, arresting lymphocyte development at the pro-B and pro-T stages. We have previously shown that the abnormal accumulation of RAG-1/2-mediated DSB in thymocytes from DNA-PK-deficient (*Prkdc*$^{scid/scid}$) mice induces the p53-dependent DNA damage checkpoint (Guidos et al., 1996). Thus, although RAG-1/2-induced DSB are restricted to lymphocyte progenitors, they can elicit DNA damage surveillance mechanisms thought to be important in many cell types. This surveillance is particularly important when lymphoid progenitors have defective NHEJ, since mice lacking both p53 and various NHEJ components rapidly develop lethal pro-B cell leukemia and/or thymic (immature T cell) lymphoma (Guidos et al., 1996; Nacht et al., 1996; Difilippantonio et al., 2000; Frank et al., 2000; Gao et al., 2000; Lim et al., 2000).

Leukemic pro-B cells from p53/NHEJ-deficient mice display many cytogenetic aberrations, such as aneuploidy, chromosomal translocations, and amplification (Guidos et al., 1996; Vanasse et al., 1999b; Difilippantonio et al., 2000), demonstrating that the combined loss of p53 and NHEJ causes genomic instability which neoplastically transforms B cell progenitors very efficiently. Recurrent translocations involving the immunoglobulin heavy chain (*IgH)* locus on chromosome 12 and *c-Myc* on chromosome 15 occur in leukemic B cells from *p53*$^{-/-}$*KU80*$^{-/-}$, *p53*$^{-/-}$*LIG4*$^{-/-}$, and *p53*$^{-/-}$*XRCC4*$^{-/-}$ mice (Difilippantonio et al., 2000; Gao et al., 2000), suggesting that RAG-1/2-mediated DSB contribute to genomic instability and leukemogenesis in these models. However, these potentially oncogenic translocations were not observed in leukemic pro-B cells from *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice (Vanasse et al., 1999b). Moreover, lymphoid oncogenesis was absolutely dependent on RAG-1/2 function in a small cohort of *p53*$^{-/-}$*XRCC4*$^{-/-}$ mice (Zhu et al., 2002), but was RAG-1/2-independent in small cohorts of *p53*$^{-/-}$*KU80*$^{-/-}$ or *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice (Vanasse et al., 1999b; Difilippantonio et al., 2002). Thus, it remains unclear to what extent RAG-1/2-mediated DSB versus global genomic instability contribute to lymphoid oncogenesis in mice with combined p53/NHEJ deficiencies.

In contrast to a previous report (Vanasse et al., 1999b), we show here that leukemic pro-B cells from *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice harbor recurrent *IgH/c-Myc* translocations. Strikingly, these translocations were typically complex (involving other chromosomes), clonally heterogeneous, and amplified, and we present evidence that they participate in BFB cycles. To determine if RAG-1/2-mediated DSB are essential to induce genomic instability and oncogenic transformation of *p53/Prkdc*-deficient lymphoid progenitors, we bred *RAG-2/p53/Prkdc*-deficient mice. We show that while RAG-1/2-mediated DSB are essential for generating *IgH/c-Myc* translocations in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ pro-B cells, the global genomic instability conferred by loss of p53 and DNA-PK efficiently transforms pro-B cells that lack V(D)J recombinase activity. Unexpectedly, *RAG-2/p53/Prkdc*-deficient leukemias showed a unique capacity for dissemination to the central nervous system (CNS), causing leptomeningeal leukemia, an important clinical complication of human lymphoblastic malignancies. Collectively, our data demonstrate that physiologically introduced DSB can promote chromosomal translocations and gene amplification by repeated BFB cycles in vivo, and we present a novel animal model that provides a unique opportunity to elucidate the molecular mechanisms by which leukemic lymphoblasts invade and grow within the CNS.

Results

Recurrent translocations involving chromosomes 12 and 15 in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ pro-B cell leukemias

We previously reported that *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice develop a rapidly lethal pro-B cell leukemia that closely resembles acute lymphoblastic leukemia, and that these leukemic pro-B cells display significant levels of aneuploidy (Guidos et al., 1996). To gain insights into mechanisms leading to pro-B cell transformation in this model, we performed spectral karyotype analysis (SKY) to see if they displayed recurrent cytogenetic abnormalities. We observed a high level of chromosomal instability in all *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ leukemias, as expected from the combined disruption of NHEJ and p53. Clonal and nonclonal abnormalities were seen, and included whole chromosome losses or gains, segmental multiplications, dicentric chromosomes, evidence of gene amplification in homogeneously staining regions, nonreciprocal translocations, and inversions (Table 1). Among the clonal abnormalities, translocations involving either chromosomes 12 and 15 {t(12;15)} or 12 and 16 {t(12;16)} (Table 1 and Figure 1A) were recurrent in all leukemias. Cytogenetic analyses of the t(12;15) breakpoints were consistent with involvement of *IgH* at 12F1 and *c-Myc* at 15D3 (Table 1). We therefore performed dual color fluorescent in situ hybridization analyses (FISH) with BAC probes containing the *IgC*μ/*JH* and *c-Myc* loci. Importantly, we observed colocalization of the *IgH* and *c-Myc* signals in metaphases from 5/5 leukemias harboring a t(12;15). Thus, like the endemic form of Burkitt's lymphoma in humans

Table 1. Clonal chromosomal abnormalities in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ pro-B cell leukemias

Leukemia	Structural	Numerical
528	der(11;14)(A1;A1) der(12)t(12;16)(F1;B1) der(12)t(6;12)(C1;D1)	−6, +11, −16
544	der(2;15)(A1;A1) der(3)t(3;15;3)(H4;?;F2) der(12)t(12;15)(F1;D3) der(15)dupinv(15)(?A1F2)t(15;16)(?A1;B1) der(15)dupinv(15)(A1F2)t(3;15)(F2;A1)	
556	der(12)t(12;16)(F1;B1)	−Y, −6 , −16
584	dup(12)(E;F2) der(12)t(12;13)(F1;B) der(12)t(12;15)(F1;D3) qdp(15)(B1 D3) hsr(16)(C)	+3
596	der(1;3)(A1;A1) der(12)t(12;15)(F1;D3) dup(15)(**)	+3
599	der(12)t(12;15)(F1;D3) t(14;15)(E3;E)	+3, −6
601	der(3)t(3;3)(A1;D) der(12)t(12;15)(F1;D3)	

Ten metaphases were analyzed by SKY for each DM leukemia. Karyotype descriptions are based on ISCN 1995 guidelines and standard mouse chromosome nomenclature. Multiple nonclonal changes were also observed in all leukemias, reflecting ongoing genomic instability in these cells (data not shown).
?, chromosome band not identified.
**, different levels of amplification in different metaphases.

(Faderl et al., 1998), the pro-B cell leukemias that arose in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice are characterized by recurrent translocations that juxtapose *IgH* and *c-Myc*.

IgH/c-Myc translocations are complex and amplified

Surprisingly, however, we observed dramatic clonal heterogeneity in *IgH/c-Myc* fusion patterns among different metaphases within each leukemia (Figure 1B). The *IgH/c-Myc* fusions were present in 1–5 copies and were distributed between 1–3 individual chromosomes in each metaphase. In addition, the *IgH/c-Myc* fusions were frequently amplified, often to different degrees on the same chromosome. In several leukemias (528, 584, and 599), we also observed metaphases with striking amplification of the *IgH* locus on a nonrearranged chromosome 12 (Figure 1B and data not shown). These observations suggested that both the *IgH* locus and the *IgH/c-Myc* fusions were genomically unstable in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ leukemias, due to ongoing rearrangement and amplification.

The *IgH* locus on chromosome 12 is oriented with the *IgCH* region cluster located on the centromeric side of the *V(D)JH* gene cluster (D'Eustachio and Riblet, 1999). The most distal *VH* genes are found in the subtelomeric region of chromosome 12. By analogy with the human t(8;14) translocations that fuse *IgH/c-Myc* in the endemic form of Burkitt's lymphoma (Faderl et al., 1998), we expected that *c-Myc* would be relocated into the D/J region of *IgH* on murine chromosome 12, bringing *c-Myc* under transcriptional control of the intronic or 3′ *IgH* enhancers (D'Eustachio and Riblet, 1999). However, a previous study did not find *IgH/c-Myc* fusions on the derivative chromosome 12 {der(12)} in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ leukemias (Vanasse et al., 1999b). Using sequential SKY/FISH to identify the chromosomes containing the *IgH/c-Myc* fusions, we found that in 3/5 leukemias (Type I), the *IgH/c-Myc* fusions resided on a der(15) rather than on a der(12) chromosome in most metaphases (Figure 1C and Table 2). Thus, the t(12;15) were reciprocal translocations in these leukemias. We did observe the *IgH/c-Myc* fusion on der(12)t(12;15) chromosomes in 2 leukemias (type II), and these translocations appeared to be nonreciprocal (Table 2). To define the *IgH* regions relocated to the der(15)t(12;15), we compared FISH signals using BAC probes containing the *IgC*α gene and sequences centromeric to it, and another containing the telomeric *VH* genes. While these two probes colocalized on the nonrearranged chromosome 12 in pro-B cell leukemias (Figure 1C) and in normal cells (not shown), the telomeric *VH* gene cluster was lost from the both the der(15) and der(12) chromosomes (Figure 1C, right). Moreover, sequences hybridizing to the *IgC*α BAC were amplified on the der(15)t(12;15). These data suggested that the process of *IgH/c-Myc* translocation began with RAG-1/2-mediated cleavage of *VDJH* gene segments and loss of the subtelomeric *VH* gene cluster.

The clonal instability of *IgH* and the *IgH/c-Myc* fusions is further illustrated in Figure 2. For example, sequential SKY/FISH analyses of leukemia 544 showed that some der(15) chromosomes had *IgH/c-Myc* fusions at the extreme telomeric end, whereas in others, *IgH/c-Myc* fusions resided at the junctions of the der(15) with a third chromosome (eg., t[3;15] or t[15;16] in Figure 2A). Similarly complex translocations were observed in all other leukemias (Table 2 and data not shown). Interestingly, we also found the *IgH/c-Myc* fusions on "third party" chromosomes, such as 16 (Figure 2A), 2 (Figure 2B), and 3 (data not shown). Moreover, amplified *IgH* sequences without *c-Myc* were seen on chromosome 12 and on a third party chromosome (Figure 2B). Importantly, these observations suggest that RAG-1/2-mediated loss of telomeric *VH* can destabilize the *IgH* locus on chromosome 12 independently of translocation with *c-Myc*. Finally, we observed reiterated or amplified *IgH/c-Myc* fusions on dicentric chromosomes, as well as ladder-like interspersions of chromosome 15 with other chromosomes by SKY (Figure 2C). Collectively, these data strongly suggest that repeated BFB cycles generate clonally unstable and complex *IgH* translocations in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ pro-B cell leukemias.

Effect of *RAG-2* deficiency on lymphoid tumorigenesis in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice

The above cytogenetic observations provided strong circumstantial evidence that RAG-1/2-mediated DSB could initiate the process of *IgH/c-Myc* translocations in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ pro-B cell leukemias. However, given the global genomic instability in these leukemias, we could not rule out the possibility that the *IgH/c-Myc* translocations were generated by RAG-1/2-independent mechanisms but were strongly selected for during leukemogenesis. To test the requirement for RAG-1/2-mediated DSB for leukemogenesis in this model, we generated *RAG-2*$^{-/-}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ triple mutant (TM) mice and compared their morbidity due to lymphoblastic leukemia to *RAG-2*$^{+/-}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ or *RAG-2*$^{+/+}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ double mutant (DM) mice. Two breeding schemes were used to minimize modifier gene effects: *RAG-2*$^{-/-}$*Prkdc*$^{scid/scid}$ mice segregating *p53* alleles, and *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice segregating *RAG-2* alleles (Figure 3A and Experimental Procedures). All DM mice developed leukemia with a median latency of 9 weeks, as ex-

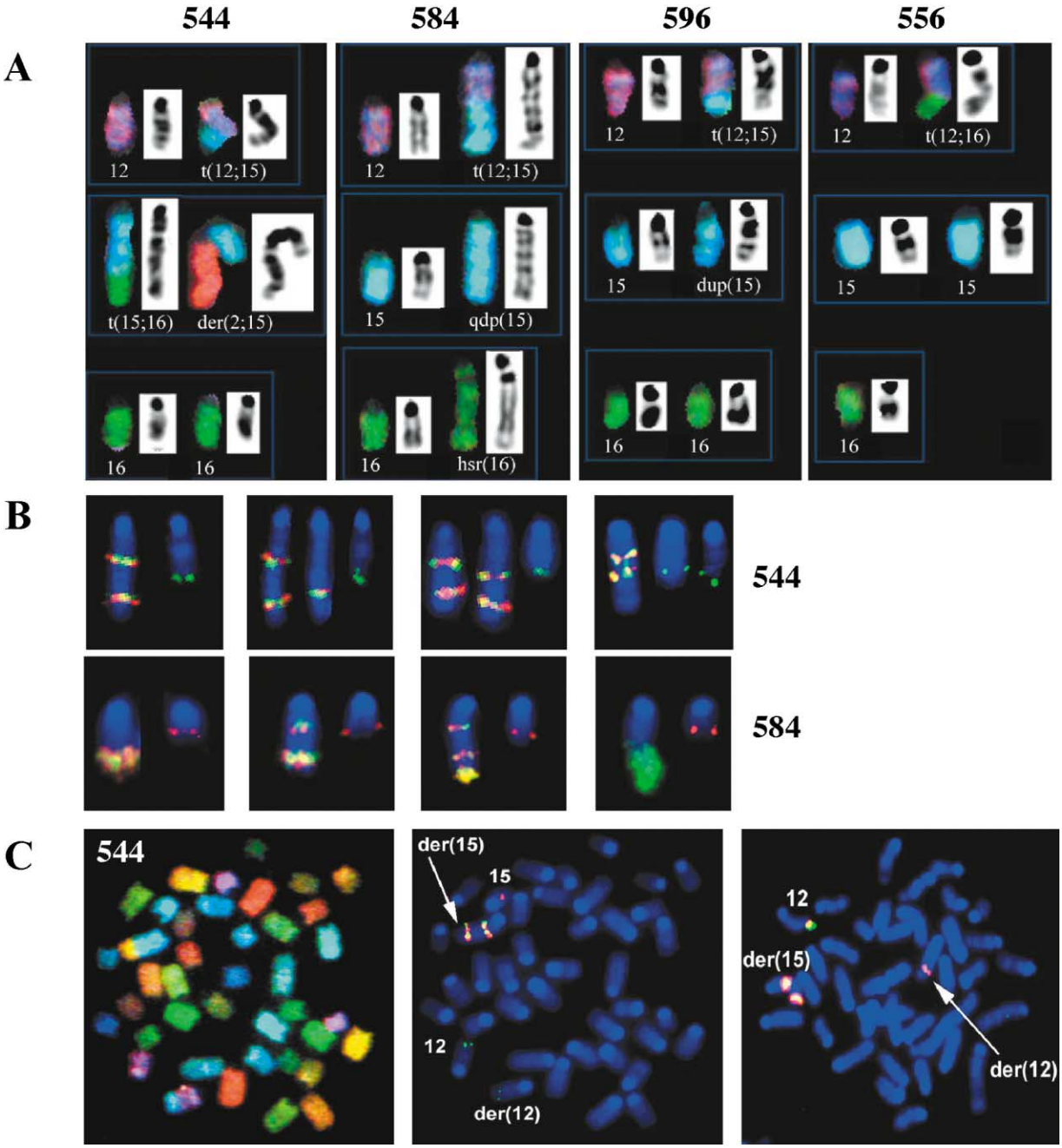

Figure 1. Chromosomal translocations involving *IgH* and *c-Myc* in DM leukemias

A: SKY and inverted DAPI images showing normal and derivative chromosomes 12, 15, and 16 from the indicated DM leukemias (see Table 1 for detailed karyotype description).

B: Amplification and mobilization of *IgH/c-Myc* fusions in DM leukemias 544 and 584. The BAC probes used for FISH contained *IgC*μ (MBAC 20B20, green) and *c-Myc* (MBAC 270G24, red). Each leukemia showed clonal heterogeneity and amplification of *IgH/c-Myc* fusions (yellow). Each panel shows a nonrearranged chromosome 12 with *IgH* only (green) or chromosome 15 with *c-Myc* (red) only on the right.

C: Chromosomal location and structure of *IgH/c-Myc* fusions in DM leukemia 544. Sequential SKY (left) and dual color FISH analysis revealed colocalization of *IgC*μ (MBAC 20B20, green) and *c-Myc* (MBAC 270G24, red) on a der(15)t(3;15) (middle) and loss of subtelomeric *VH* genes (BAC CT7-224M14, green) from the der(12) and movement of the 3′ *IgC*α region (BAC CT7-199M11, red) to the der(15)t(3;15) (right). A similar pattern was observed with DM leukemia 601 (not shown).

pected in accordance with our previous study of *RAG-2*$^{+/+}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice (Guidos et al., 1996). We have previously shown that *RAG-2*$^{-/-}$*p53*$^{+/+}$*Prkdc*$^{scid/scid}$ mice do not develop lymphoid malignancies by 6 months of age (Williams et al., 2001), and document here that heterozygosity for p53 provides significant protection, as only 3% of *RAG-2*$^{-/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ developed leukemia (median latency, 27.5 weeks). Strikingly, *RAG-2* genotype had no effect on the survival of *p53*$^{-/-}$

Table 2. Location of *IgH*, *c-Myc*, and *IgH/c-Myc* fusions in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ pro-B cell leukemias

Type	No.	Freq.	IgH	c-Myc	IgH/c-Myc	No signal
I	601	10/15	12	15, der(12)t(12;15)	der(15)t(12;15)	
		4/15	12	15	der(15)t(12;15)	der(12)t(12;15)
		rare	12, der(12)t(12;15)	15	2 on der(15)t(15;12;16) 1 on der(16)t(16;15;12)	
I	544	8/20	12, der(12)t(12;15)	15	2 on der(15)amp(15)t(3;15)	
		9/20	12, der(12)t(12;15)	der(2;15)	2 on der(15)amp(15)t(15;16)	
		3/20	12, der(12)t(12;15)	15	2 on der(15)amp(15)	
		rare	12, der(12)t(12;15)	15	der(15)t(15;12;15;16)*	
		rare	12, der(15)t(15;12;15;3)	15	der(15)t(15;12;15;3)* der(15)t(16;15;16)	der(12)t(12;15)
I	596	11/20	12, der(12)t(12;15)	15	2 on der(15)amp(15)	
		7/20	12, der(12)t(12;15)	15	3 on der(15)amp(15)	
		2/20	12, der(12)t(12;15)	15	3 on der(15)amp(15)t(15;12;...)	
		rare	12, der(12)t(12;15)	15	5 on dic(15;15)	
II	599	8/11	12	15	der(12)t(12;15)	der(14)t(14;15) der(15)t(14;15)
		3/11	12	15	der(12)t(12;15)	der(15)t(15;14;15;14)
		rare	12	15	der(12)t(12;15) dic(14;15)t(14;15;14;...)*	
		rare	12**	15	der(12)t(12;15)	
		rare	12	15	2 on der(15)t(15;12;15;12...)**	der(12)t(12;15)
II	584	8/20	12, dup(12)***	15,15	none	
		8/20	12	15	1 on der(12)t(12;15) 2 on der(12)t(12;15)**	
		rare	12	15,15	der(12)t(12;1;6)***	
		rare	12	15,15	2	
		rare	12, 12, der(16)t(12;16)*	15,15	none	

The chromosomal locations for *IgH*, *c-Myc*, and *IgH/c-Myc* fusions were determined on 11–20 metaphases from each DM leukemia by sequential SKY/FISH. Signals showing slight (*), moderate (**), or high (***) levels of amplification are indicated. In Type I leukemias, the *IgH/c-Myc* fusions are predominantly on the der(15) chromosome, whereas in Type II leukemias they are predominantly on the der(12) chromosome.

Prkdc$^{scid/scid}$ mice, as TM and DM mice became moribund with identical kinetics (Figure 3A). Flow cytometric evaluation of lymphoid tissues demonstrated that 40/42 TM mice and 39/40 DM mice examined had disseminated pro-B cell leukemia, whereas 2/42 TM and 1/40 DM mice had pre-T cell lymphoblastic leukemia/lymphoma (pre-TLL, previously called thymic lymphoma) (Morse et al., 2002). Finally, 3/42 TM and 2/40 DM mice had both pro-B and pre-TLL. Leukemia incidence and latency in TM mice generated by the two breeding strategies were indistinguishable (data not shown). Leukemic cells in the bone marrow of each strain displayed similar pro-B cell phenotypes (B220$^+$CD19$^+$CD43$^+$IgM$^-$, data not shown). Both TM and DM leukemias disseminated to lymph node, spleen, and thymus, but in many TM mice this was evident only by flow cytometric examination of these tissues. TM mice rarely displayed the gross lymphadenopathy and thymic enlargement that was typical for DM mice. We conclude that loss of RAG-1/2 function did not alter the incidence or latency of lymphoblastic leukemia in p53$^{-/-}$*Prkdc*$^{scid/scid}$ mice.

Leptomeningeal dissemination of leukemic pro-B cells impairs CNS function in TM mice

Surprisingly, however, *RAG-2* genotype exerted a profound effect on the clinical course of leukemia in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice. We observed a high incidence (73%) of neurological impairment concomitant with morbidity from systemic leukemia in TM mice (Figure 3B). Neurologic findings included a domed head (Figure 4Ai), ataxic gait, and/or hind limb paresis. Progression of CNS disease was very rapid, and TM animals commonly became paralyzed and unable to drink or feed ≤24 hr after a normal neurological exam. CNS symptoms were rarely observed in DM (4%), *RAG-2*$^{-/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ (3%), or *RAG-2*$^{+/+}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ (5%) mice, and were not seen in *RAG-2*$^{-/-}$*p53*$^{+/+}$*Prkdc*$^{scid/scid}$ mice. Normal lateral ventricular and choroid plexus architecture was seen in neurologically asymptomatic *RAG-2*$^{-/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ mice (Figure 4Aii). However, all affected TM mice showed dilation of cerebral ventricles, signifying communicating hydrocephalus (Figure 4Aiii), accounting for the domed head appearance. The cortical surface of the brain from asymptomatic *RAG-2*$^{-/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ mice was normal (Figure 4Aiv). 11 of 23 (48%) DM mice had thin patchy infiltrates (<2 cells thick) of leukemic cells in the subarachnoid (leptomeningeal) space (Figure 4Av), but they did not display neurological impairment. In contrast, symptomatic TM mice showed a prominent infiltration of mononuclear cells animals (>10 cells thick) within the leptomeninges (Figures 4Avi and 4Avii), but infiltration of the brain parenchyma was never observed. This leukemic infiltrate was absent in TM mice lacking signs of CNS impairment. Cells infiltrating the leptomeninges of TM mice expressed B220 and CD19, identifying them as leukemic B-lineage cells (Figure 4Aviii and data not shown). No significant cellular infiltrate was seen in the spinal cord of TM mice, ruling out malignant compression as the cause of hind limb paresis (data not shown). We conclude that the neurological symptoms seen in TM mice resulted from hydrocephalus caused by dissemination of leukemic pro-B cells to the leptomeningeal space. Importantly, these pathological findings closely resemble CNS (leptomeningeal) leukemia, a frequent and morbid complication of acute lymphoblastic leukemia and

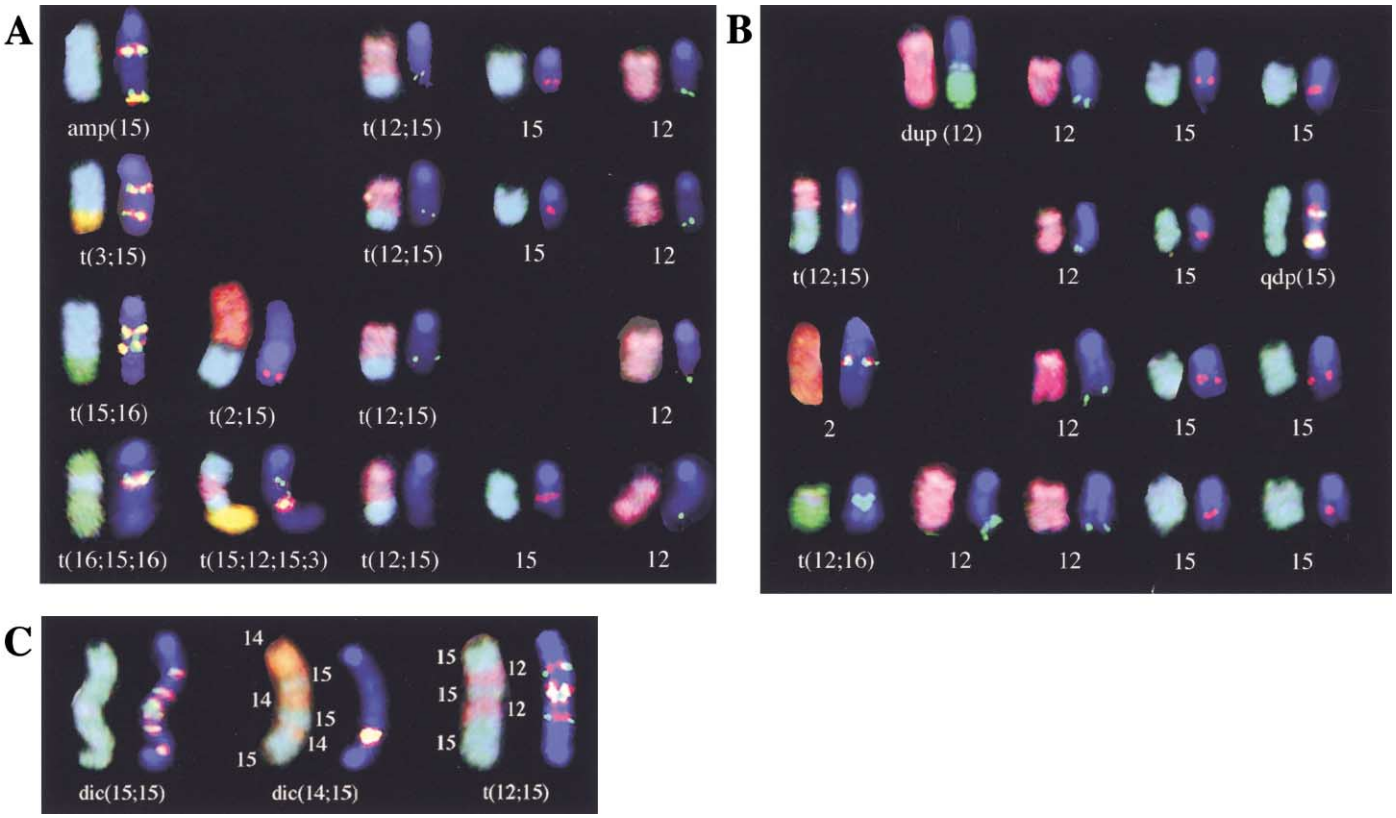

Figure 2. Clonal heterogeneity and amplification of *IgH* and *IgH/c-Myc* fusions

SKY (left) and FISH (right) images of chromosomes containing *IgCμ* (MBAC 20B20, green), *c-Myc* (MBAC 270G24, red) or fused (yellow) signals. Each row shows chromosomes from individual metaphases of DM 544 (**A**) and DM 584 (**B**). In both leukemias, the *IgH/c-Myc* fusions were found on several different chromosomes, some of which captured the telomeric end of a third chromosome.

B: In DM 584, *IgH* was amplified on a dup(12) or was inserted into a der(16)t(12;16).

C: Dicentric chromosomes and complex translocations harboring *IgH/c-Myc* fusions indicative of ongoing BFB cycles. Left: dicentric (15;15) from DM 596. Middle: dicentric (14;15;14;15;14;15) from DM 599. Right: der(15)amp(15)t(15;12;15;12;15) from DM 596.

non-Hodgkin's lymphoma (Price, 1979; Pui and Evans, 1998; Sandlund et al., 2000).

We performed adoptive transfer experiments to determine if the differential incidence of CNS leukemia in TM versus DM mice reflects genotype-dependent properties intrinsic to the leukemic pro-B cells. *RAG-2*$^{-/-}$ hosts (sublethally irradiated) were injected with leukemic pro-B cells isolated from lymphoid tissues of three TM mice with CNS impairment, or from two DM mice with no neurological involvement. Recipients of TM leukemias rapidly developed neurological symptoms (mean onset of symptoms 3 weeks), and flow cytometric analysis of their lymph nodes, spleen, thymus, and bone marrow revealed heavy infiltration of leukemic pro-B cells (data not shown). In addition, histological examination of TM recipient brains revealed CNS leukemia (Figure 4Aix). In contrast, recipients of DM leukemias rapidly succumbed to systemic leukemia without signs of CNS involvement. Thus, leptomeningeal dissemination reflected cell-intrinsic differences between TM and DM leukemic pro-B cells.

Genomic instability in TM pro-B cell leukemias

Like the DM leukemias, all TM pro-B cell leukemias analyzed displayed a wide spectrum of clonal and nonclonal chromosomal aberrations, documenting that they were also genomically unstable (Table 3). However, no recurrent aberrations were observed among all TM leukemias, and translocations involving chromosomes 12 and 15 were never seen. Interestingly, however, 3/5 had translocations in which one chromosomal segment was fused with multiple different partners (Table 3 and Figure 4B), suggesting that multiple rounds of breaking and rejoining occurred at one site. For example, TM leukemia 485 contained several derivative chromosomes 14 {der(14)} in which 14D was fused with portions of chromosome 3, 4, 12, or 19 (Figure 4B, top). Several observations suggest that repeated BFB cycles could account for these chromosomal anomalies in TM leukemias. First, we observed end-to-end fusions of the multiply translocated chromosome with other chromosomes, creating dicentrics (dic[4;17] in TM 560 and dic[2;14] in TM 485, Figure 4B). Second, we observed broken versions (del[14D] in TM 485 and del[2G] in TM 518) of the multiply translocated chromosomes. Finally, several TM leukemias had amplification of large chromosomal segments (Table 3).

Southern blot analyses confirmed that the *IgJH* region remained in germline configuration in TM leukemias (Figure 5A). In contrast, all DM leukemias harbored *IgH* rearrangements and/or loss of *IgJH4* germline sequences, demonstrating deletion of this genomic region. As expected from the cytogenetic studies,

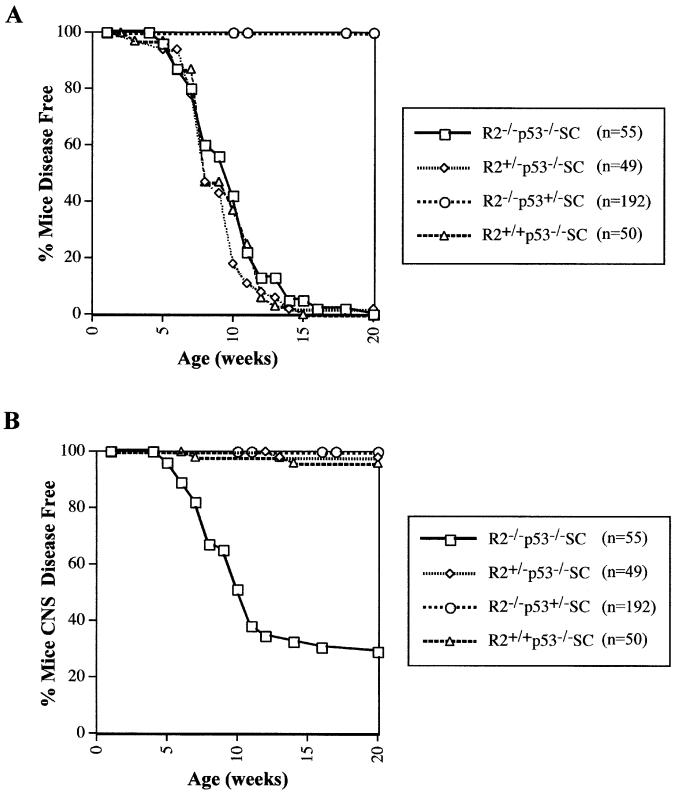

Figure 3. Effect of *p53* and *RAG-2* genotype on survival of *Prkdc*$^{scid/scid}$ mice

A: Kaplan-Meier analysis of mortality of *Prkdc*$^{scid/scid}$ (SC) mice up to 20 weeks of age according to *RAG-2* (R2) and p53 genotype. Mice were euthanized and tissues removed for histology and flow cytometry when they showed signs of morbidity (wasting, labored breathing, lymphadenopathy, domed head, or paresis). The survival of *RAG-2*$^{+/+}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice is shown for comparison.

B: Frequency of CNS pathology in the indicated mouse strain. Most *RAG-2*$^{-/-}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice (73%) exhibited symptoms of neurological disease (domed head, ataxia, and/or hind limb paresis) at the time of sacrifice. Neurologic signs were observed in the other strains as follows: *RAG-2*$^{+/-}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ (2/49), *RAG-2*$^{-/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ (5/192), and *RAG-2*$^{+/+}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ (2/50) mice. Control *RAG-2*$^{-/-}$*p53*$^{+/+}$*Prkdc*$^{scid/scid}$ mice did not develop leukemia or signs of CNS pathology.

clonal heterogeneity of *IgH* rearrangements was evident in some DM leukemias: rearrangements were present in subgenomic quantities in DM 556 and 601, and DM 584 contained several *IgJH* rearrangements that were amplified to different degrees. Amplification of *c-Myc* sequences was also evident in some DM leukemias, but not in three TM leukemias examined (Figure 5A). Based on the absence of t(12:15) translocations and *IgH* rearrangements from TM leukemias, we conclude that *RAG-1/2* is absolutely required to generate the recurrent *IgH/c-Myc* translocations seen in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ leukemias. Nonetheless, the global genomic instability conferred by loss of p53 and DNA-PK is sufficient to rapidly transform pro-B cells in the absence of RAG-1/2-induced translocations.

c-Myc mRNA was markedly overexpressed in most pro-B cell DM leukemias relative to normal pro-B cells (Figure 5B), regardless of whether the *c-Myc* gene was amplified. Some TM leukemias also overexpressed *c-Myc*, indicating that dysregulation of *c-Myc* expression can occur independently of translocation to the *IgH* locus. Interestingly, we also observed overexpression of *N-Myc* in 2/9 pro-B cell leukemias (Figure 5B). In one case, this was accompanied by *N-Myc* amplification (data not shown). Thus, ectopic expression of *c-Myc* and/or *N-Myc* is a common feature of both TM and DM leukemias.

Discussion

Induction of translocations and BFB cycles by RAG-1/2 in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ pro-B cells

Complex translocations with amplification have been previously noted in solid tumors and treatment-related leukemias and lymphomas (Felix et al., 1998; Tanaka and Kamada, 1998; Padilla-Nash et al., 2001), but mechanisms underlying the generation of these chromosomal aberrations in vivo are poorly understood. Here, we show that RAG-1/2-induced DSB can cause *IgH/c-Myc* translocations in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ pro-B cells that become rearranged and amplified by BFB in vivo, generating complex chromosomal aberrations similar to those caused by telomere dysfunction (Artandi et al., 2000; Rudolph et al., 2001). Two groups have recently reported very similar cytogenetic findings during pro-B cell leukemogenesis in *p53*$^{-/-}$*Ku80*$^{-/-}$ and *p53*$^{-/-}$*LIG4*$^{-/-}$ mice (Difilippantonio et al., 2002; Zhu et al., 2002). Thus, combined disruption of p53 and any NHEJ component promotes pro-B cell leukemogenesis by a common mechanism involving RAG-1/2-induced *IgH/c-Myc* translocations and their amplification by BFB. This process is initiated by RAG-1/2-induced DSB, which cause loss of the *VH* gene cluster from the extreme telomeric end of chromosome 12. The presence of *IgH/c-Myc* fusions on either der(12) or der(15) chromosomes suggests two different scenarios for resolving this broken chromosome 12 (Figures 6A and 6B). In the first scenario, a spontaneous DSB centromeric to *c-Myc* could allow a large telomeric segment of chromosome 15 to be aberrantly joined to the broken chromosome 12, generating an *IgH/c-Myc* fusion on a der(12) chromosome (Type II leukemias, Table 2). This nonreciprocal exchange would leave a broken chromosome 15 that gets lost from the cell, or undergoes BFB cycles (Figure 6A). Consistent with the latter notion, we observed multiple translocations involving chromosome 15 in metaphases harboring the *IgH/c-Myc* fusion on a der(12) chromosome in leukemia 599 (Table 2).

In the second scenario, reciprocal translocations occur between chromosomes 12 and 15 (Type I leukemias, Table 2), generating a dicentric (12;15) harboring the *IgH/c-Myc* fusion, and a der(12)t(12;15) with *IgH* only (Figure 6B, steps 1–3). The dicentric (12;15) would likely break during anaphase, initiating the BFB cycle and leaving the *IgH/c-Myc* fusion on a broken der(15) (step 4). In accordance with this notion, we observed several aberrant der(15) chromosomes that likely represent intermediates in this iterative process. For example, we typically saw *IgH/c-Myc* at the extreme ends of der(15) chromosomes (Figure 2A), suggesting that they were broken and lacked telomeres (Figure 6B, steps 5 and 7). In addition, we observed der(15) chromosomes with 1–3 copies of *IgH/c-Myc* interspersed with segments of chromosome 15 (Figure 2A, top) or chromosome 12 (Figure 2C, right), as would be predicted by the BFB model. Finally, we observed a dicentric (15;15) chromosome with a ladder-like distribution of 5 *IgH/c-Myc* fusions (Figure 2C, left), indicative of ongoing BFB cycles (Figure 6B, step 8a). Our data suggest that broken der(15) chromosomes can also capture segments of third party chromosomes, resulting

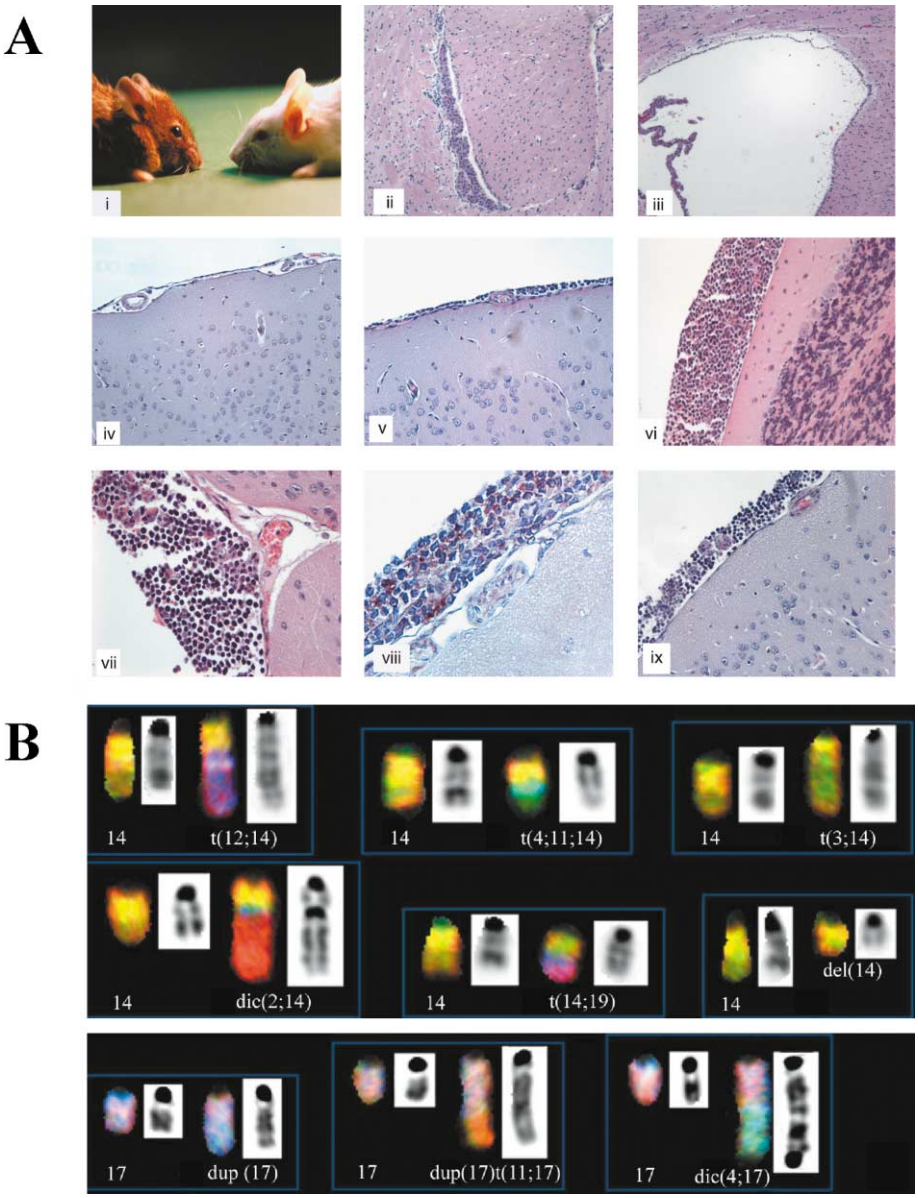

Figure 4. CNS leukemia and chromosomal instability in TM mice

A: CNS histopathology.
(i) Gross cervical lymphadenopathy and domed head in a TM mouse with ataxia and hind limb paresis (left) compared to an asymptomatic *RAG-2*$^{-/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ littermate (right).
(ii) H&E stained coronal brain section from a *RAG-2*$^{-/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ mouse that did not show CNS signs demonstrating normal lateral ventricular architecture and the choroid plexus.
(iii) H&E stained coronal brain section from a TM mouse with hind limb paresis and ataxia showing massive dilation of the lateral ventricle, characteristic of hydrocephalus.
(iv) Cortical brain surface of a *RAG-2*$^{-/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ mouse without neurological signs showing normal leptomeninges with no leukemic infiltrate.
(v) A thin layer of leukemic cells is seen in the subarachnoid space of a DM mouse that did not have any neurological signs. This mouse, representative of 48% (11/23) of this genotype, had scanty subarachnoid cellular infiltrate but no signs of neurological impairment.
(vi) Extensive leptomeningeal infiltration in the cerebellum in a *RAG-2*$^{-/-}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mouse with hind limb paresis and ataxia. The cells were not observed in the brain parenchyma and were limited to the subarachnoid space.
(vii) Higher power view of the surface of the cerebral hemisphere from the same mouse in (vi) demonstrating the extensive distribution of leukemia throughout the subarachnoid space.
(viii) Immunohistochemical staining with anti-B220 confirms that the cellular infiltrate in coronal sections from a TM brain is pro-B cell leukemia. B220 positive cells were not seen within the brain parenchyma.
(ix) H&E stained coronal section of a sublethally irradiated *RAG-2*$^{-/-}$ mouse 3 weeks after injection of 10^7 leukemic cells from a TM donor with hind limb paresis and ataxia. In all recipients of TM leukemic cells, the subarachnoid space was heavily infiltrated and the mice developed CNS signs.
B: Chromosomal instability in TM pro-B cell leukemias.
(i) Translocations or deletions of chromosome 14D seen in different metaphases from leukemia 485; clockwise from top left: t(12;14), t(4;11;14), t(3;14), dic(2;14), t(14;19), and del(14). Although the TCRαδ locus maps near band 14D, FISH analysis with probes to the extreme proximal and distal regions of this >1 Mb locus revealed that the TCRαδ locus was not disrupted by these rearrangements (data not shown).
(ii) Translocations of chromosome 17E5 seen in different metaphases from leukemia 560; from the left to right: dup(17), dup(17)t(11;17), and dic(4;17).

in complex translocations. For example, the dicentric (14;15) we observed (Figure 2C, middle) could have been generated by end-to-end fusion of a broken der(15) with chromosome 14 (Figure 6B, step 8b). If this dicentric breaks during anaphase, the *IgH/c-Myc* fusion could move to chromosome 14 (step 9). Alternatively, a broken der(15) could capture the telomeric portion of a third chromosome (step 8c). Indeed, we frequently observed *IgH/c-Myc* fusions at the junction of the der(15) and telomeric regions of other chromosomes (Figure 2A).

Interestingly, we also observed considerable instability of the *IgH* locus independently of translocation with chromosome 15. This was manifested by dramatic amplification of *IgH* sequences on a nonrearranged chromosome 12 (Figure 2B, top, and Table 2), and by movement of amplified *IgH* sequences to chromosome 16 (Figure 2B, bottom). Since the *IgH* locus remained in germline configuration in TM leukemias, it is likely that this process was also initiated by the RAG-1/2-mediated loss of telomeric *VDJH* sequences on chromosome 12, generating a broken chromosome 12 that participates in BFB cycles (Figure 6C).

Role of ectopic c-Myc expression and p53 deficiency in transforming DNA-PK-deficient pro-B cells

Most pro-B cells from *Prkdc*$^{scid/scid}$ mice undergo apoptosis because they fail to make in-frame *IgH* rearrangements that allow them to express a pre-B cell receptor (Muljo and Schlissel, 2000). It seems likely that this developmental checkpoint is circumvented in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ pro-B cells by the *IgH/c-Myc*

Table 3. Clonal chromosomal abnormalities in TM pro-B cell leukemias

Leukemia	Structural	Numerical
485	der(14)t(14;*)(D;*)	−Y, −6, −7
513	der(2)t(2;5)(F;C1)	+5, +14
518	der(2)t(2;*)(G;*) del(2)(G) der(5;6)(A1;A1)	+5
560	dic(17;*)(E5;*) dup(17)(D;E5)	−13
605	der(3;19)(A1;A1) der(7;15)(A1;A1) der(15;15)(A1;A1) dup(16)(B4C4) dup(17)(CE5)	+3, +15

Ten metaphases were analyzed by SKY for each TM leukemia, as described for Table 1.
*, different translocation partners in each metaphase.

translocations, which cause ectopic expression of *c-Myc*. Our data suggest two mechanisms for promoting *c-Myc* overexpression in p53/DNA-PK-deficient pro-B cell leukemias. First, a translocation could place *c-Myc* under control of the intronic or 3′ *IgH* enhancers. This mechanism is likely operative in leukemias 599 and 601, which harbor *IgH/c-Myc* fusions which are only amplified in rare metaphases (Table 2 and Figure 5A). Alternatively or additionally, the amplification of *c-Myc* that occurs during repeated BFB cycles could contribute to its overexpression in some DM leukemias.

p53 deficiency likely serves two important functions during pro-B cell leukemogenesis in DNA-PK deficient mice. First, p53 deficiency prevents apoptosis or cell cycle arrest in response to short telomeres (Chin et al., 1999) or unrepaired RAG-1/2-mediated DSB (Guidos et al., 1996). This would allow pro-B cells to replicate broken chromosomes which could undergo sister chromatid or end-to-end chromosomal fusions to generate dicentrics that perpetuate BFB cycles. Second, ectopic expression of *c-Myc* triggers normal cells to undergo p53-dependent apoptosis, and p53 mutations are highly selected for in B cell lymphomas induced by transgenic overexpression of *c-Myc* (Eischen et al., 1999; Schmitt et al., 1999). Similarly, a high proportion of Burkitt's lymphomas have p53 mutations (Krug et al., 2002). Thus, the p53-deficiency in our model obviates the need for somatic inactivation of p53 to allow survival of c-Myc overexpressing cells.

RAG-1/2 independent lymphoid oncogenesis in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice

We demonstrate that RAG-1/2-deficiency did not impact the incidence or latency of pro-B and pre-T cell leukemogenesis in a cohort of 55 *RAG-/2*$^{-/-}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice. In contrast, another group reported that in a small cohort of RAG-/2-deficient *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice, 8/14 developed pre-TLL (and 1 of these also had leukemic pro-B cells in the thymus) or sarcoma, and the other 6 died from undocumented causes (Vanasse et al., 1999b). Thus, RAG-1/2-deficiency altered the lineage specificity of tumorigenesis and prolonged the lifespan of *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ mice in that study. The genetic background of TM mice generated by Vanasse et al. was similar to ours, and included 129/Sv, C57BL/6, and ICR, but our genetic background also included C.B-17 (see Experimental Procedures).

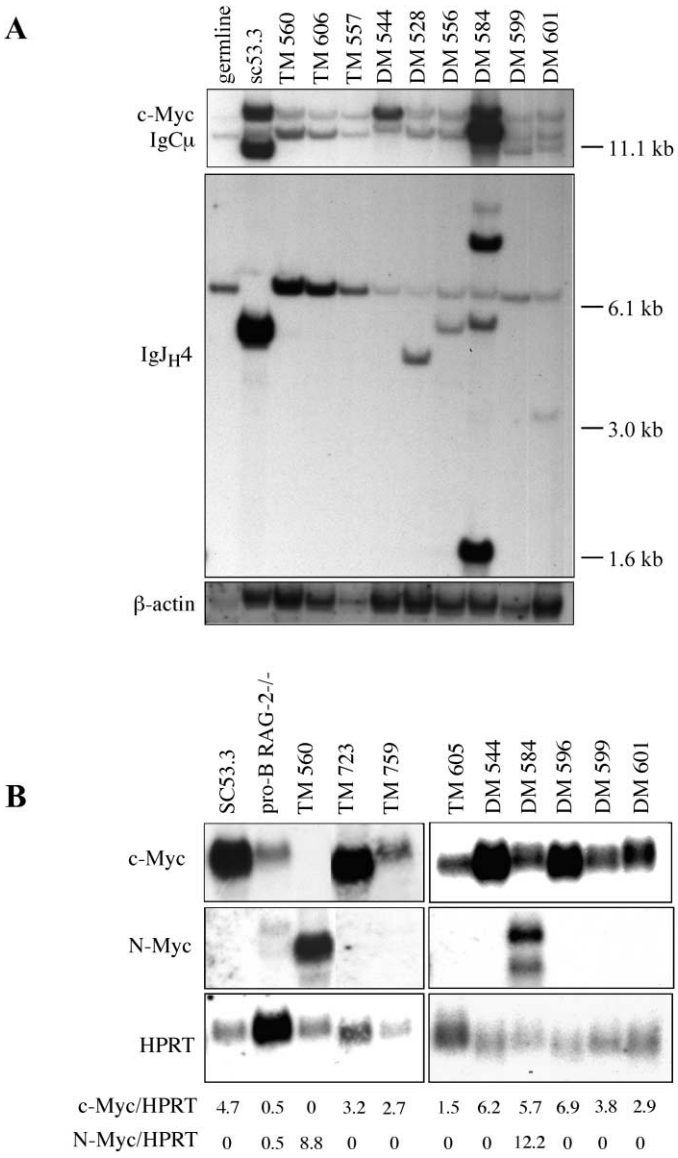

Figure 5. A: Southern analysis of *IgH* and *c-Myc* loci in TM and DM leukemias. A Southern blot containing the indicated samples of EcoR1-digested genomic DNA was sequentially hybridized to *c-Myc* followed by *Ig-Cμ* (top), *IgJH4* (middle), and β-actin (bottom) cDNA probes. SC53.3 is a *RAG-2*$^{+/+}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ leukemic pro-B cell line (Guidos et al., 1996). Amplification of *c-Myc* and *IgCμ* was seen in DM leukemia 584 and amplification of *c-Myc* alone was seen in DM leukemia 544 when standardized to a β-actin loading control.
B: Northern analysis of *c-Myc* and *N-Myc* expression in DM and TM leukemias. Densitometry ratios of *c-Myc* to HPRT or *N-Myc* to HPRT are shown for each sample. Note that normal *RAG-2*$^{-/-}$ pro-B cells and DM 584 have 2.0 and 2.5 kb *N-Myc* transcripts, but TM560 lacks the 2.5 kb transcript.

However, we have found that pro-B cell leukemia was similarly prevalent in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ (129/Sv and ICR) and *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ (129/Sv and C.B-17) mice (C.J.W., C.J.G., and J.S.D., unpublished data). Moreover, the much higher prevalence of pro-B cell leukemia relative to pre-TLL was identical in our DM and TM mice. Thus, we do not believe that the presence of C.B-17 background genes in our TM mice explains why we observed a high incidence of pro-B cell leukemia and

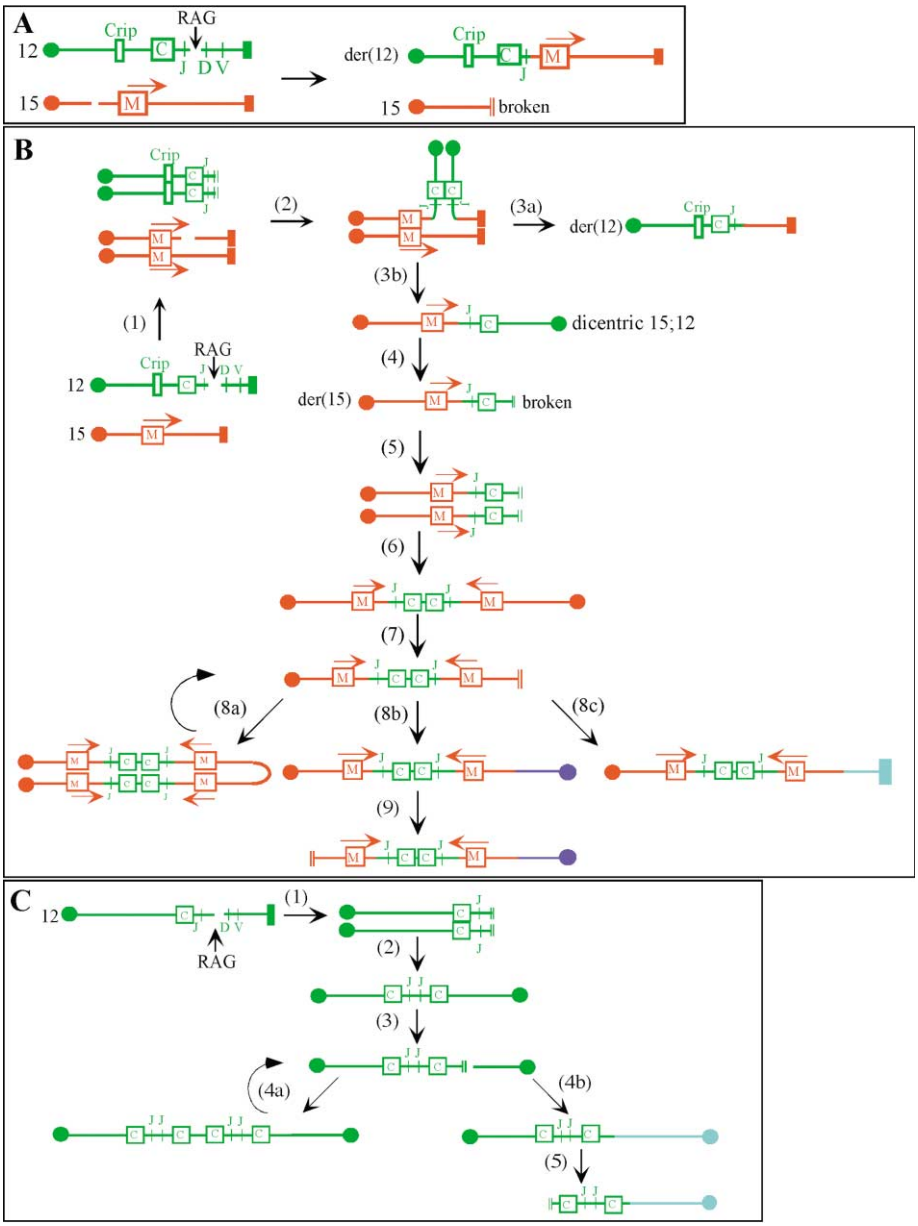

Figure 6. Model for generation of *IgH/c-Myc* translocations and amplification by BFB

A: Nonreciprocal translocation of chromosomes 12 and 15 (Type II leukemias, Table 2). A spontaneous DSB on the centromeric side of *c-Myc* on 15 and a second RAG-1/2-induced DSB on 12 facilitate fusion of the *c-Myc*-containing telomeric portion of 15 to the *IgC* gene cluster on 12. This process would generate a der(12)t(12;15), leaving a "broken" 15 lacking a telomere.

B: Reciprocal translocation of chromosomes 12 and 15 (Type I leukemias, Table 2). Failure to repair RAG-1/2-induced DSB at *IgH VDJ* gene segments causes loss of the telomeric segment of chromsome 12 (1). After replication of this broken chromosome (facilitated by p53 deficiency), one broken 12 chromatid joins to a DSB telomeric of *c-Myc* on 15 (2), generating a dicentric (12;15) intermediate (3b). The other broken 12 chromatid joins to the telomeric segment of 15, generating a der(12)t(12;15) with *IgH* but not *c-Myc* (3a). The unstable dicentric (12;15) breaks centromeric to *IgC*α during anaphase, generating a der(15) chromosome harboring the *IgH/c-Myc* fusion but lacking a telomere (4). The broken der(15) replicates (5) and the chromatids fuse forming a dicentric (15;15) with 2 copies of the *IgH/c-Myc* fusion (6). The dicentric breaks again during anaphase (7). Data shown in Figure 2B and Table 2 suggest 3 possible fates for this broken der(15) chromosome. After it replicates, sister chromatid fusion generates another dicentric (15;15) that reenters the BFB cycle (8a). The broken der(15) could also undergo end-to-end fusion with a different chromosome (8b). Breakage of the resulting dicentric during anaphase could result in the *IgH/c-Myc* fusions moving to this third party chromosome (9). Finally, the broken der(15) could capture the telomeric portion of a third chromosome (8c).

C: Model for *IgH* amplification. RAG-1/2-mediated DSB induce loss of the telomeric *VDJH* sequences. After replication of this broken chromosome 12 (1), sister chromatid fusion could generate a (12;12) dicentric (2) that participates in BFB cycles leading to *IgH* amplification on chromosome 12 (3 and 4a). Alternatively, the broken 12 could form a dicentric with a second chromosome (4b). If this dicentric breaks appropriately, *IgH* could move to this other chromosome (5), as we observed in Figure 2B.

low incidence of pre-TLL, whereas Vanasse et al. reported the opposite in their TM mice (Vanasse et al., 1999b). Nonetheless, genetic background has a well-documented effect on the incidence and latency of lymphoid tumorigenesis in *Prkdc*$^{scid/scid}$ and *RAG-/2*$^{-/-}$ mice. For example, pre-TLL occurs with much higher incidence and shorter latency in NOD.*Prkdc*$^{scid/scid}$ relative to C.B-17.*Prkdc*$^{scid/scid}$ mice (Custer et al., 1985; Prochazka et al., 1992). Moreover, 44% of NOD.*RAG-/2*$^{-/-}$ mice develop spontaneous pre-TLL and pro-B cell leukemia by 10 months of age (Chiu et al., 2002), whereas no lymphoid tumors have been reported in RAG-2-deficient mice on other genetic backgrounds. Thus, it is possible that the latency of lymphoid tumorigenesis was skewed by modifier genes in the small TM cohort analyzed by Vanasse et al. Finally, Vanasse et al. did not document brain histopathology in their TM mice, so undetected CNS leukemia may have accounted for the unexplained death of nearly 50% of their cohort. This unexpected complication was rapidly progressive and fatal in our TM mice, and often developed in the absence of bulky extramedullary tumors.

The RAG-1/2-dependence of pro-B cell leukemogenesis has also recently been assessed in small cohorts of other p53/NHEJ-deficient mice, with discordant results. Alt's group reported no lymphoid malignancies in 6 *RAG-2/p53/XRCC4*-deficient mice (Zhu et al., 2002), whereas Nussenzweig and colleagues reported that 4 *RAG-/2/p53/KU80*-deficient mice developed pre-TLL but not pro-B cell leukemia (Difilippantonio et al., 2002). If verified with larger cohorts, these data, in conjunction with ours, would suggest that pro-B cell leukemogenesis is completely RAG-1/2-dependent in *p53/XRCC4*-deficient and *p53/KU80*-deficient mice, but completely RAG-1/2-independent in *p53/Prkdc*-deficient mice. This dichotomy may reflect the differential effect of these NHEJ mutations on DSB repair: V(D)J joining is more severely affected by loss of XRCC4/LIG4 or KU70/KU80 than by Prkdc deficiency, suggesting that an alternative repair pathway can function in the absence of Prkdc (Bogue et al., 1998; Ferguson and Alt, 2001). This putative alternative repair pathway could affect RAG-1/2-independent aspects of leukemogenesis in least two ways. First, it could actually enhance genomic instability in p53/Prkdc-deficient pro-B cells relative to those from p53/KU80- or p53/XRCC4-deficient mice by promoting translocations through misrepair of RAG-1/2-independent DSB. Supporting this possibility is our observation that 6/7 DM leukemias had at least 1 other clonal translocation besides the t(12;15) (Table 1), whereas such additional translocations were rare in p53/KU80-deficient pro-B cell leukemias (Difilippantonio et al., 2000). Moreover, the extensive clonal instability of *IgH/c-Myc* translocations and *IgH* we observed in DM leukemias was not noted in leukemias from *p53*$^{-/-}$*/KU80*$^{-/-}$ or *p53*$^{-/-}$*/XRCC4*$^{-/-}$ mice. Thus, it appears that loss of p53 and Prkdc imparts a greater degree of genomic instability than loss of p53 and KU80 or XRCC4. Second, loss of XRCC4/LIG4 or KU70/KU80, but not Prkdc, severely compromises cell growth (Difilippantonio et al., 2000; Frank et al., 2000; Gao et al., 2000). Therefore, *p53/XRCC4*-deficient pro-B cells may stringently depend on RAG-1/2-dependent *IgH* translocations to induce overexpression of *c-Myc*, allowing them to survive and proliferate when repair and cell growth are both severely compromised. In contrast, alternative repair pathways in *p53/Prkdc*-deficient pro-B cells may facilitate their survival and neoplastic transformation by *IgH/c-Myc*-independent mechanisms. Consistent with this notion, we found that TM leukemias overexpress *c-Myc* or *N-Myc* by mechanisms not involving translocation to the *IgH* locus (Figure 5). These two genes are functionally redundant in developing lymphocytes (Malynn et al., 2000).

Interestingly, we showed that RAG-1/2-deficient pro-B cell leukemias displayed many chromosomal aberrations indicative of genomic instability, but no recurrent *IgH/c-Myc* translocations. Thus, RAG-1/2 function is required to generate these translocations in *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ pro-B cell leukemias. However, DSB that occur spontaneously due to replication blocks, oxidative damage, or at chromosomal fragile sites likely induce chromosomal instability that is sufficient to transform TM pro-B cells. Indeed, nonlymphoid cells from NHEJ-deficient and p53/NHEJ-deficient mice display striking genomic instability and elevated frequencies of nonreciprocal translocations (Ferguson et al., 2000; Gao et al., 2000), demonstrating that spontaneously generated DSB are often misjoined when NHEJ is compromised. The telomere capping defect conferred by the DNA-PK mutation (Bailey et al., 1999; Gilley et al., 2001; Goytisolo et al., 2001) may also promote the formation of dicentric chromosomes that undergo repeated BFB cycles in TM pro-B cells. Our observation that several TM leukemias harbor translocations in which a particular chromosomal segment is translocated to multiple different partners is consistent with these notions.

A novel mouse model of spontaneous CNS leukemia

Although RAG-1/2 function did not affect the incidence or latency of lymphoid oncogenesis in *p53/Prkdc*-deficient mice, most TM mice displayed signs of acute neurological impairment and had prominent infiltration of leukemic pro-B cells within the leptomeninges. In contrast, DM mice rarely showed neurological impairment or leptomeningeal dissemination of leukemic pro-B cells. Thus, TM mice provide a novel spontaneous model of CNS leukemia, a frequent and morbid complication of human lymphoblastic malignancies. Survival rates for children afflicted with acute lymphoblastic leukemia (ALL) have improved dramatically, but remain poor for adult ALL (Cortes and Kantarjian, 1995; Ferrando and Look, 2000; Pui, 2000). Treatment failure for ALL and non-Hodgkin's lymphoma is frequently due to dissemination of leukemic cells to the CNS (Berg et al., 2000; Sandlund et al., 2000). Intrathecal chemotherapy and cranial irradiation prevent CNS relapse in most patients, but they are associated with significant morbidity, such as cognitive delay, endocrine failure, and a 2% 15-year risk of treatment-related brain tumors (Pui and Evans, 1998; Walter et al., 1998; Pui, 2000). Currently, cytogenetic markers are used to stratify pro/pre-B ALL patients, so that the most aggressive treatments are reserved for those with the highest risk of relapse (Ferrando and Look, 2000). Unfortunately, up to 40% of pediatric ALL patients require CNS prophylaxis (Silverman et al., 2000). The differential and highly reproducible incidence of CNS leukemia in DM versus TM mice will provide a valuable model system for elucidating the molecular pathways that control leptomeningeal dissemination of leukemic lymphoblasts. This information may allow redefinition of risk factors for CNS relapse based on the molecular characteristics of leukemic cells, ultimately providing novel therapeutic targets, as well as a more extensive panel of markers to more accurately identify patients with the highest risk of CNS relapse.

Experimental procedures

Mice

All mice were bred and housed in specific pathogen-free conditions at the Hospital for Sick Children animal facility. *p53*$^{-/-}$*Prkdc*$^{scid/scid}$ (DM) mice and their *p53*$^{+/-}$*Prkdc*$^{scid/scid}$ littermates were bred from *p53*$^{-/-}$ 129/Sv and *Prkdc*$^{scid/scid}$ ICR parental strains as previously described (Guidos et al., 1996). The generation of *RAG-2*$^{-/-}$*Prkdc*$^{scid/scid}$ mice from *RAG-2*$^{-/-}$ (129/Sv and C57BL/6) and *Prkdc*$^{scid/scid}$ (C.B-17) mice has also been described (Guidos et al., 1996). *RAG-2*$^{-/-}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ (TM) mice were created by interbreeding *p53*$^{+/-}$*Prkdc*$^{scid/scid}$ mice and *RAG-2*$^{-/-}$*Prkdc*$^{scid/scid}$ mice to generate *RAG-2*$^{+/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ progeny. The latter mice were then intercrossed to produce *Prkdc*$^{scid/scid}$ mice segregating wild-type and mutant alleles of *p53* and *RAG-2*. Two strategies were then used to produce a higher frequency of TM and *RAG-2*$^{+/-}$ segregants: (1) *RAG-2*$^{-/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ parents were intercrossed, or (2) *RAG-2*$^{-/-}$*p53*$^{-/-}$*Prkdc*$^{scid/scid}$ male and *RAG-2*$^{+/-}$*p53*$^{+/-}$*Prkdc*$^{scid/scid}$ females were mated. PCR amplification of tail DNA was used to genotype alleles at the *p53* and *RAG-2* loci (Guidos et al., 1996; Williams et al., 2001). Adoptive transfer of TM and DM leukemic pro-B cells from lymph node, spleen, or thymus was performed by intravenous injection of 10^7 cells in 200 μl of PBS into sublethally irradiated (500 cGy) C57BL/6 *RAG-2*$^{-/-}$ hosts, 6–8 weeks of age. Recipients were monitored daily for CNS signs and/or for peripheral lymphadenopathy. At the first sign of morbidity, animals were sacrificed and their brains processed for histological assessment of CNS leukemia. In addition, lymphoid tissues were analyzed by flow cytometry for the presence of leukemic pro-B cell lymphoblasts.

Flow cytometry

Flow cytometric analyses of thymocytes, splenocytes, lymph node, and bone marrow cells using antibodies specific for CD4, CD8, and B220 were performed as previously described (Danska et al., 1994; Guidos et al., 1996). In addition, biotinylated-CD19 (6D5) was purchased from Pharmingen (San Diego, CA). Streptavidin-phycoerytherin was purchased from Caltag (South San Francisco, CA) and used as a second stage reagent with biotinylated primary antibodies.

Histology

Mice were sacrificed using carbon dioxide inhalation. Brains and spines were dissected and fixed in 37% formalin for 2 weeks. Brains were cut in the coronal plane and subsequently embedded in paraffin. Spines were decalcified in 50% formic acid/20% sodium citrate solution for several weeks and then cut in the axial plane and embedded in paraffin. 10 μm paraffin-embedded tissue sections were mounted on glass slides and stained with hematoxylin and eosin using standard techniques. For immunohistochemistry, brain sections were deparaffinized in xylene, then rehydrated in a decreasing ethanol series. Endogenous peroxidase activity was blocked by incubation of slides in 3% hydrogen peroxide solution diluted in methanol. Slides were then incubated sequentially with blocking serum, anti-B220, biotinylated anti-rat IgG, and avidin-horseradish peroxidase. Blocking serum and secondary reagents were prepared from a Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA). Diaminobenzidine-nickel peroxidase substrate solution was prepared according to manufacturer's instructions (Vector Laboratories Inc.) and dripped onto the surface of slides. Slides were counterstained with hematoxylin and mounted in AquaPerm (Immunon Shandon, Pittsburgh, PA). Images were obtained using a Leica microscope and Adobe Photoshop 3.0.4 software.

Spectral karyotyping

Primary leukemic cells were cultured from lymphoid tissues of moribund DM or TM mice as described (Guidos et al., 1996) for 1–6 days prior to the induction of metaphase arrest by treatment with colcemid for 2–4 hr (0.05 μg/ml) (Dracopoli, 2000). Cells were exposed to 0.075 M KCl for 10 min at room temperature, followed by three changes of methanol/acetic acid (3:1) prior to making metaphase spreads. The SKY™KIT probe cocktail (Applied Spectral Imaging; ASI, Carlsbad, CA) was hybridized to cytogenetic preparations following the manufacturer's instructions, and slides were then counterstained with DAPI (Vector, Burlingame, CA). Images were captured using an SD200 spectral bioimaging system (ASI Ltd., Migdal Haemek, Israel) attached to a Zeiss Axioplan 2 microscope and analyzed with ASI Spectral Imaging software.

Fluorescence in situ hybridization

Metaphase preparations from DM leukemias 544, 584, 596, 599, and 601 were analyzed by standard two-color FISH methods (Dracopoli, 2000). BAC clones containing the *TCR*α constant region (MBAC 77) or the most 5′ Vα gene cluster (MBAC01, Genbank accession AF259071) were kindly provided by Dr. I. Lee, University of Washington (Seattle, WA). These two probes were used to show that the breakpoints on the der(14) chromosome in TM leukemia 485 did not disrupt the *TCR*αδ locus. BAC clones containing sequences from *IgCγ2b* to the *JH* cluster (MBAC 20B20) and *c-Myc* (MBAC 270G24) were obtained by screening a mouse BAC library (RPCI-22; http://www.chori.org/bacpac) with *IgC*μ and *c-Myc* cDNA probes, respectively. BAC clones containing sequences 3′ of the *IgC* gene cluster (from Crip to *IgC*α, CT7-199M11) or the most distal *VH* gene cluster (CT7-224M14) were provided by Dr. Roy Riblet (San Diego, CA). For use as FISH probes, BAC DNA was labeled by nick translation, either with biotin-14-dATP (GibcoBRL, Gaithersburg, MD) or digoxigenin-11-dUTP (Boehringer Mannheim). The probes were then denatured for 10 min at 75°C, preannealed at 37°C for 1 hr, and hybridized to denatured slides overnight at 37°C. Probes were detected by using rhodamine-conjugated anti-digoxigenin antibody (Boehringer Mannheim) and FITC-avidin (Ventana, Tucson, AZ) antibodies. Chromosomes were counterstained with DAPI. Sequential SKY and FISH was performed as described to identify chromosomes containing *IgH*/*c-Myc* fusions (Bayani et al., 2000).

Northern and Southern blotting

DNA was prepared from infiltrated thymus or lymph nodes of TM and DM mice with disseminated pro-B cell leukemia, tail clips DNA of wild-type mice, and a DM leukemic cell line (SC53.3) (Guidos et al., 1996). DNA samples were digested with EcoRI and electrophoresed on 0.8% agarose gels. Southern (Guidos et al., 1996) and Northern (Groves et al., 1995) blot filters were prepared as described and probed with the following ^{32}P-dCTP labeled probes: *c-Myc* and *N-Myc* (Feder et al., 1990), *IgC*μ (Fox and Danska, 1997), *IgJH4* (Atkinson et al., 1991), β-actin (Groves et al., 1995), or HPRT (Groves et al., 1995).

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