

Artemis and p53 cooperate to suppress oncogenic *N-myc* amplification in progenitor B cells

Sean Rooney*, JoAnn Sekiguchi*, Scott Whitlow*, Mark Eckersdorff*, John P. Manis*, Charles Lee†, David O. Ferguson*, and Frederick W. Alt*‡

*Howard Hughes Medical Institute, Children's Hospital, Department of Genetics, Harvard Medical School, and Center for Blood Research, Boston, MA 02115; and †Division of Cyto genetics, Department of Pathology, Brigham and Woman's Hospital, Harvard Medical School, Boston, MA 02115

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The nonhomologous DNA end-joining (NHEJ) pathway contains six known components, including Artemis, a nuclease mutated in a subset of human severe combined immunodeficient patients. Mice doubly deficient for the five previously analyzed NHEJ factors and p53 inevitably develop progenitor B lymphomas harboring der(12)t(12;15) translocations and immunoglobulin heavy chain (*IgH*)/*c-myc* coamplification mediated by a breakage-fusion-bridge mechanism. In this report, we show that Artemis/p53-deficient mice also succumb reproducibly to progenitor B cell tumors, demonstrating that Artemis is a tumor suppressor in mice. However, the majority of Artemis/p53-deficient tumors lacked der(12)t(12;15) translocations and *c-myc* amplification and instead coamplified *IgH* and *N-myc* through an intra- or interchromosome 12 breakage-fusion-bridge mechanism. We discuss this finding in the context of potential implications for mechanisms that may target *IgH* locus translocations to particular oncogenes.

The nonhomologous end-joining (NHEJ) pathway ligates broken DNA ends irrespective of homology and is required for both general double-strand break (DSB) repair and repair of developmentally programmed DSBs introduced by the recombination-activating gene 1/2 (RAG) endonuclease (1). There are six known mammalian NHEJ factors: Ku70, Ku80, XRCC4, and Ligase 4 are evolutionarily conserved and function in all known NHEJ reactions, whereas the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Artemis evolved more recently and together provide a nuclease activity for NHEJ reactions that require end-processing (2, 3). In this regard, RAG cleaves between V-, D-, and J-coding segments and flanking recombination signal sequence (RS) to form hairpin coding ends and blunt RS ends. Although the four conserved NHEJ factors are needed to join both coding and RS ends, DNA-PKcs and Artemis are relatively dispensable for RS joining but absolutely required for coding end-joining because of their role in hairpin opening (3, 4).

Inactivation of NHEJ results in increased ionizing radiation sensitivity, genomic instability, and severe combined immunodeficiency (SCID) (1, 3) resulting from the inability to join RAG-cleaved gene segments in progenitor (pro)-B and T lymphocytes. Despite their inability to repair DSBs, NHEJ-deficient mice show, at most, a modest predisposition to lymphomas, because cells with unrepaired breaks are eliminated by the checkpoint protein p53 (5–8). Inactivation of p53 restores pro-B lymphocyte numbers, although it does not rescue NHEJ or lymphocyte development (5, 6, 9). Combined deficiencies for p53 and all NHEJ factors except Artemis have been analyzed and lead to consistent development of early-onset pro-B lymphomas (5–12).

The molecular mechanisms underlying transformation in NHEJ/p53 pro-B lymphocytes have been elucidated (10, 13, 14). Almost all lymphomas exhibit amplification of *c-myc* coupled with consistent cytogenetic abnormalities, including a nonreciprocal der(12)t(12; 15) translocation, referred to as C12;15, and a complex translocation, referred to as a complicon, containing amplified immunoglobulin heavy chain (*IgH*) and *c-myc*. NHEJ/

p53-deficient lymphomas have been proposed to result from unrepaired DSBs persisting into S phase, where they are replicated and fused downstream of *c-myc*, generating the signature C12;15 and a dicentric 12;15 (15), which then leads to *c-myc* amplification through a breakage-fusion-bridge (BFB) mechanism (10, 13, 14). A major unresolved question is why *c-myc* is nearly invariably amplified. Nonmutually exclusive possibilities include strong selection for *c-myc* amplification or preferential targeting of the translocation to chromosome 15.

Mutations in *Artemis* form the basis for radiosensitive SCID in humans (16). Despite its more restricted role in NHEJ, Artemis-deficiency also leads to increased genomic instability in cultured murine cells, suggesting that it might function as a tumor suppressor (3, 17). In this study, we have asked whether Artemis has tumor suppressor functions in mice that are unmasked in the context of a p53-deficient background.

Methods

Generation of *Art^{N/N}p53^{N/N}* Mice. 129Sv *Art^{N/N}* mice (3) were crossed to 129Sv/C57BL/6 *p53^{N/N}* mice (18) to generate *Art^{N/+}p53^{N/+}* mice, which were then bred to generate all cohort mice. Mice were genotyped for *Artemis* by PCR with the following primers: ART5–3 (CAAGAGGCATTTCGTGTAT-ATGGGTGGC) and ART3–2 (CCCGTAACAGAGCTATGACAGAACCGGG), yielding a 250-bp wild-type band, or ART5–3 and neoRev (ACCGCTATCAGGACATAGCGT), yielding a 1-kb targeted band. Mice were genotyped for *p53* as described (19).

Characterization of Mice/Tumors. All mice were regularly monitored and killed at first sign of illness. Mice were fixed in bouins and subjected to complete histological analysis. Lymphoid tissue samples were characterized by flow cytometry with anti-CD4, CD8, CD3, B-220, CD43 (PharMingen) and anti-IgM (Southern Biotechnology Associates). pro-B cell lymphomas were grown in culture as described (13), and T cell lymphomas were cultured in RPMI medium 1640 supplemented with 15% FCS/25 units per ml IL-2 (BD Biosciences)/25 ng/ml IL-7 (PeproTech, Rocky Hill, NJ). Spectral karyotyping (SKY) was performed on metaphases prepared from each tumor (20). The *c-myc* (13) and 3'*IgHRR* (19) fluorescence *in situ* hybridization (FISH) probes have been described. The *N-myc* FISH probe is a bacterial artificial chromosome (RP23-246B9) overlapping the murine *N-myc* locus (Research Genetics, Huntsville, AL). Southern and Northern analyses were performed as described (13). Probes were as follows: J_H1.1, a 1.1-kb NaeI-EcoRI fragment downstream of *J_H4*; *mycA*, a 1.6-kb Xba fragment upstream of *c-myc*; *N-myc*, a 1.6-kb *Clal*-EcoRI fragment containing exon 3 of the

Abbreviations: NHEJ, nonhomologous end-joining; *IgH*, Ig heavy chain; DSB, double-strand break; BFB, breakage-fusion-bridge; pro, progenitor; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; SKY, spectral karyotyping; FISH, fluorescence *in situ* hybridization; RAG, recombination-activating gene 1/2; SCID, severe combined immunodeficiency.

†To whom correspondence should be addressed. E-mail: alt@enders.tch.harvard.edu.

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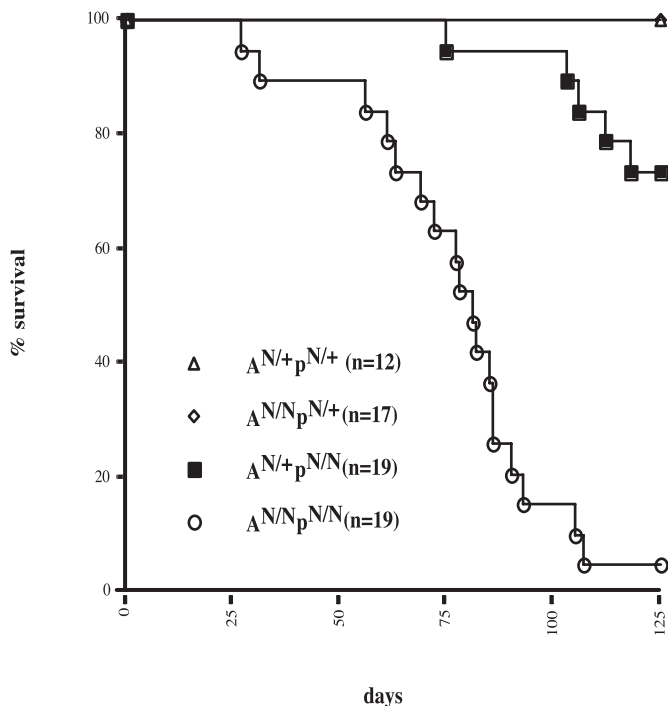


Fig. 1. Increased mortality in $A^{N/N}p^{N/N}$ double-deficient mice. Shown is a Kaplan-Meier curve representing the percent survival of $A^{N/+}p^{N/+}$ ($n = 12$), $A^{N/N}p^{N/+}$ ($n = 17$), $A^{N/+}p^{N/N}$ ($n = 19$), and $A^{N/N}p^{N/N}$ ($n = 19$) cohort mice versus age in days.

murine *N-myc* gene (21); *c-myc* northern probe, a 1.5-kb *Pst*I fragment from the *c-myc* cDNA that hybridizes to exon 2 and 3; *LR8* control probe, a 600-bp *Eco*R1 fragment upstream of the *LR8* locus (19); and the 3' *IgH* enhancer probe, a 700-bp *Hinc*II-receptor I fragment downstream of HS4 (22).

Cloning and Sequencing Translocation Junctions. All translocation junctions were cloned by circular PCR and sequenced as described (13). The non-*IgH* sequence was identified by using the Celera database.

Results

Increased Mortality in $Art^{N/N}p53^{N/N}$ Mice. To determine whether Artemis and p53 cooperate in tumor suppression, we crossed a line of $Art^{+/N}$ mice to p53-deficient ($p53^{+/N}$) mice and then bred progeny. These breedings yielded cohorts of $Art^{N/N}p53^{N/N}$ ($A^{N/N}p^{N/N}$) mice ($n = 19$) and $A^{N/+}p^{N/+}$ ($n = 12$), $A^{N/N}p^{N/+}$ ($n = 17$), and $A^{N/+}p^{N/N}$ ($n = 19$) mice. All genotypes were born at Mendelian ratios and appeared normal at birth. However, we observed a dramatic increase in the mortality rate of $A^{N/N}p^{N/N}$ mice, compared with littermate controls (Fig. 1). The majority of $A^{N/N}p^{N/N}$ mice died (or were killed because of severe morbidity) by 12 weeks of age (84 days), with 18 of 19 (95%) dead by 18 weeks (126 days). In contrast, only 5 of 19 (26%) $A^{N/+}p^{N/N}$ mice died by 18 weeks of age, similar to $p53^{N/N}$ mice alone (18, 23, 24). Finally, all $A^{N/N}p^{N/+}$ and $A^{N/+}p^{N/+}$ mice lived past 18 weeks.

The majority of $A^{N/N}p^{N/N}$ mice analyzed by histology (10/16) developed aggressive pro-B cell ($B220^{+}IgM^{-}CD43^{+}$) lymphomas (Table 1) similarly to those reported for other NHEJ/p53 double-deficient mice (5–12). However, this was not the exclusive cause of morbidity, because other tumors were observed (Table 1). By comparison, T cell lymphomas were the most common lesion observed in $A^{N/+}p^{N/N}$ mice (6/19), consistent

Table 1. Tumor spectrum in $Art/p53$ mice

	$A^{+/N}p^{+/N}$ ($n = 12$)	$A^{N/N}p^{+/N}$ ($n = 17$)	$A^{+/N}p^{N/N}$ ($n = 19$)	$A^{N/N}p^{N/N}$ ($n = 19$)
B cell lymphoma	0	0	1	10
T cell lymphoma	0	3	6	3
Teratoma	0	0	1	2
Colonic polyps	0	0	0	1
Medulloblastoma	0	0	2	1
Hemangiosarcoma	0	0	1	0
Cause of death unknown	1	2	4	2

Numbers shown here represent all tumors observed including those appearing beyond the 18 weeks represented in Fig. 1.

with what has been reported for $p53^{N/N}$ mice (18, 23). We did observe a pro-B cell tumor in one $A^{+/N}p^{N/N}$ mouse (Table 1).

Novel Chromosome 12 Aberrations in Most $A^{N/N}p^{N/N}$ pro-B Cell Tumors.

To analyze cytogenetic abnormalities in $A^{N/N}p^{N/N}$ pro-B cell tumors, we examined metaphase spreads from cultured tumor cells by SKY. Surprisingly, of eight tumors examined, only three (AP138, AP143, and AP424) $A^{N/N}p^{N/N}$ (Table 2, which is published as supporting information on the PNAS web site, and data not shown) exhibited predominant translocations involving both chromosomes 12 and 15, as observed in most other NHEJ/p53-deficient tumors. All three of these tumors had translocations involving chromosome 15, and two also had the signature C12;15. The remaining five $A^{N/N}p^{N/N}$ pro-B cell tumors had predominant chromosomal aberrations that involved translocations and/or intrachromosomal gains of chromosome 12 material but did not involve chromosome 15 (Fig. 2 and Table 2). Each of these tumors (AP10, AP87, AP145, AP269, and AP270) had a chromosome 12 that by SKY appeared larger than the normal chromosome 12 (Figs. 2 and 3 and Table 2). Each large chromosome 12 did not appear to be dicentric intermediates, given that only one centromere was usually observed (Fig. 2 and data not shown). Additionally, a telomere-specific probe failed to identify any internal telomeric sequence on the enlarged chromosome 12 of tumor AP270 (Fig. 6, which is published as supporting information on the PNAS web site), suggesting that this aberration is not the result of telomeric fusions, which have been observed in $Art^{N/N}$ cultured cells (17). A number of the tumors, each of which had an altered chromosome 12, also had a heterogeneous spectrum of nonclonal translocations, including C12;16 (AP10), C12;19 (AP145, AP269), C12;3 (AP269), and C12;15 (AP143) (Table 2).

Activation of *c-myc* in a Minority of $A^{N/N}p^{N/N}$ pro-B Tumors.

We examined the amplification status of *c-myc* and *IgH* from $A^{N/N}p^{N/N}$ pro-B cell tumors. Southern blot analyses with *IgH*-specific (J_H) probes revealed that most $A^{N/N}p^{N/N}$ tumors harbored either clonal rearrangements of J_H or loss of the J_H germline allele because of deletions extending downstream of the J_H probe (Fig. 4A). A number of the tumors showed amplification of J_H where it was retained (Fig. 4A), and all tumors exhibited amplification of *IgH* as detected by a 3'*IgH* regulatory region probe (Table 2 and data not shown). However, analysis with a *c-myc* probe revealed that only the three tumors with predominant translocations involving chromosomes 12 and 15 also contained significant amplification of *c-myc* (Fig. 4B and Table 2). Moreover, Northern blot analysis revealed that increased *c-myc* expression also was restricted to those tumors with C12;15 translocation and *c-myc* amplification (AP138, AP143, and AP424) (Fig. 4C), ruling out ectopic activation of *c-myc* as a mechanism contributing to the remaining six $A^{N/N}p^{N/N}$ tumors. Metaphase spreads of one *c-myc*-amplified tumor (AP138) were

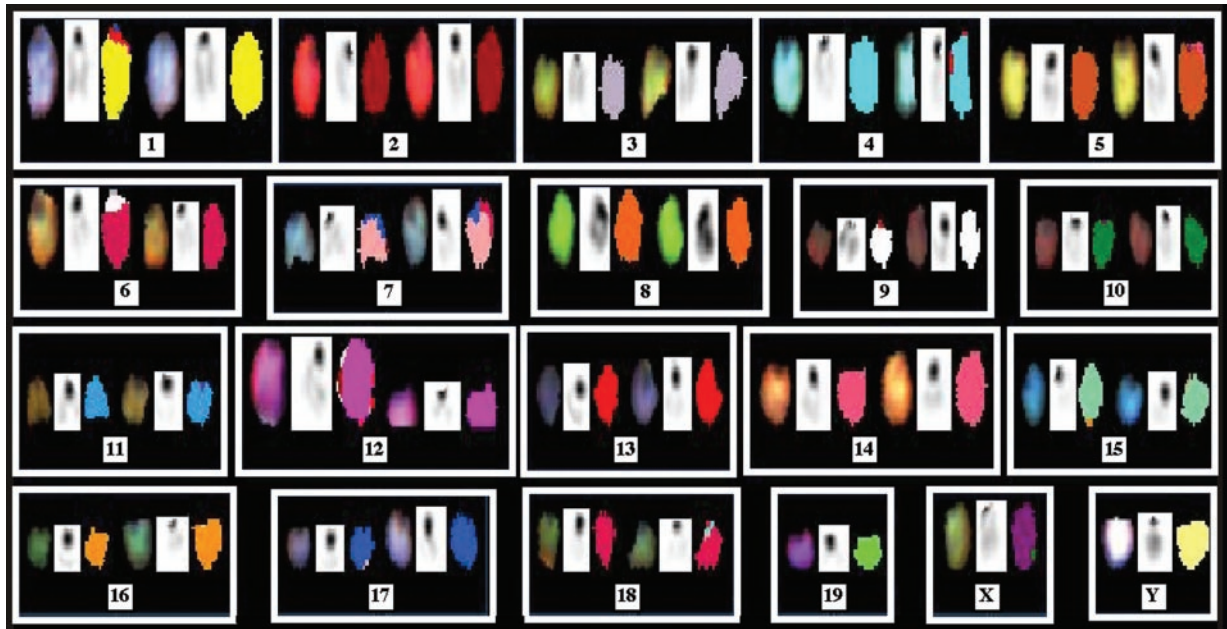


Fig. 2. A majority of $A^{N/N}p^{N/N}$ pro-B cell tumors are cytogenetically distinct from other NHEJ/p53 tumors. Shown are SKY images of metaphase spreads from AP87, representative of the majority of $A^{N/N}p^{N/N}$ pro-B cell tumors harboring a novel aberration, presenting as an enlarged copy of chromosome 12. SKY images are on the left, 4',6-diamidino-2-phenylindole-stained images are in the middle, and computer-classified colors are on the right.

found by FISH to be karyotypically similar to those of previously analyzed NHEJ/p53 tumors (10, 13, 14), with one normal chromosome 15, one C12;15 (lacking *c-myc*), one normal chro-

somosome 12, and coamplified *IgH* and *c-myc* within a compicon (Fig. 3B). Nucleotide sequence analysis of the J_H region junction from AP138 confirmed that it was fused to chromosome 15,

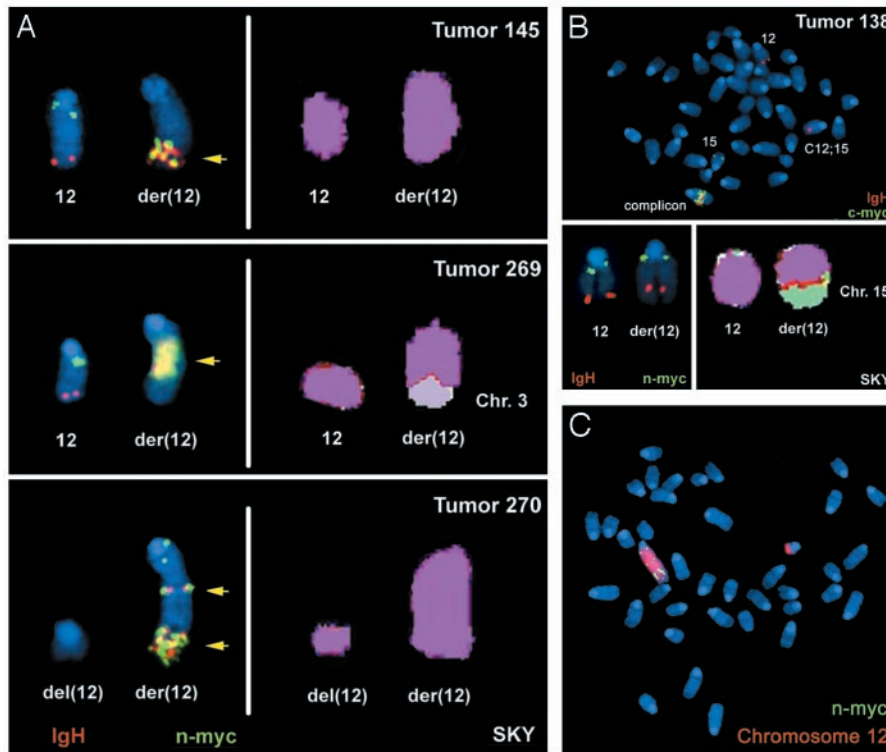


Fig. 3. *IgH* and *N-myc* are coamplified on chromosome 12. (A) Representative FISH (Left) and SKY (Right) analyses of three tumors (AP 145, AP269, and AP270) exhibiting *N-myc* amplification by Southern blotting. The *IgH* FISH probe is red, the *N-myc* probe is green, and regions of coamplification are highlighted by yellow arrows. (B) Representative FISH (Upper, Lower Left) and SKY (Lower Right) analyses of tumor AP138 that exhibits *c-myc* amplification by Southern blotting. The *IgH* FISH probe is red, and the *c-myc* (Upper) or *N-myc* (Lower Left) is green. (C) Representative metaphase from AP270 analysis with a chromosome 12 paint (red) and the *N-myc* FISH probe (green).

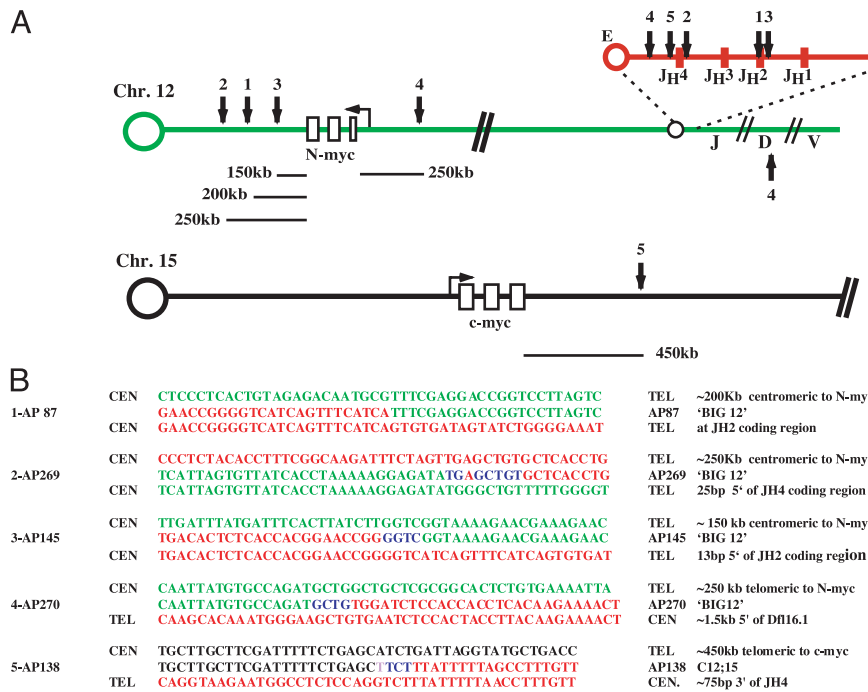


Fig. 5. *N-myc* amplification is initiated by the translocation of *IgH* sequence around *N-myc*. (A) Schematic representation of chromosome 12 and 15 showing breakpoints of indicated tumors within the *J_H* locus and *N-myc* (chr 12) or *c-myc* (chr 15). Breakpoint no. 4 (AP270) represents a *D_HJ_H* rearrangement translocated telomeric to *N-myc*. (B) Sequences of the cloned translocation breakpoints from *A^{N/N}p^{N/N}* pro-B cell tumors. AP87, AP269, AP145, and AP270 have *N-myc* amplification, and AP138 is amplified for *c-myc*. *J_H* sequences are in red, *N-myc* sequences are in green, and *c-myc* sequences are in black. Regions of homology are in blue, and nontemplated nucleotides are in purple. The locations and orientation of the translocation breakpoints are indicated to the right. TEL, telomeric; CEN, centromeric.

for NHEJ in tumor suppression in humans have remained unclear (15). However, inactivating mutations of Ku70, Ku80, DNA-PKcs, XRCC4, and Ligase 4 have also not been observed in the context of human immunodeficiencies, possibly because of a more severe impact of NHEJ mutations on human cells (26). In contrast, mutations in *Artemis* have been identified in several cohorts of human SCID patients (16, 27, 28). Therefore, our finding that *Artemis* functions as a tumor suppressor in mice raises the possibility of a similar function in humans. In this regard, hypomorphic alleles of *Artemis* have been identified in humans and have been associated with a predisposition to lymphomas (29).

The majority of *A^{N/+}p^{N/N}* control mice died from T cell lymphomas that lacked clonal translocations (data not shown) and presumably represent the T cell lymphomas that normally arise on a p53-deficient background (12, 19, 30). In contrast, of two *A^{N/N}p^{N/N}* thymic lymphomas analyzed by SKY, both had translocations involving chromosome 14, which contains the T cell antigen receptor (TCR)- α locus. In addition, two of the three *A^{N/N}p^{N/N}* T lineage tumors analyzed exhibited amplification of the TCR- α locus (data not shown). These latter findings suggest that *A^{N/N}p^{N/N}* thymic lymphomas are distinct from those that arise in p53-deficient mice. Conceivably, the *A^{N/N}p^{N/N}* thymic lymphomas may arise from a pool of pro-T cells with unresolved RAG-induced breaks that escaped elimination because of the inactivation of p53 and/or the leakiness of *Artemis* deficiency with respect to T cell development. In this regard, we observed T cell lymphomas in older (>8 months) *Art^{N/N}* mice (Table 1) that may be analogous to those observed in Ku70-deficient or SCID (DNA-PKcs-dead) mice (9, 11, 31–33), which are also leaky with respect to T cell development.

Activation of Endogenous *N-myc* or *c-myc* in *A^{N/N}p^{N/N}* pro-B Lymphomas. Nearly all NHEJ-p53 doubly deficient mice succumb to pro-B cell lymphomas (5–12) with amplified *c-myc* expression

through a BFB mechanism (10, 13, 14). Therefore, it is striking that *c-myc* was amplified in only three of nine analyzed *A^{N/N}p^{N/N}* pro-B lymphomas and that *N-myc* was amplified and overexpressed in the others. *N-myc* was identified based on its amplification in advanced stage human neuroblastomas (34, 35). However, *N-Myc* can lead to pro-B lymphomas when deregulated in transgenic mice through linkage to an *IgH* intronic enhancer (36–38). Our current studies clearly show that translocation and amplification of endogenous *N-myc* can also participate in pro-B cell lymphomagenesis.

N-Myc is a putative basic region/helix-loop-helix transcription factor and shares substantial similarity to *c-Myc* with regards to genomic organization and protein sequence (21, 34). Although both *c-Myc* and *N-Myc* knockouts are embryonic lethal (39, 40), replacement of endogenous *c-myc* with *N-myc* allows normal development, suggesting the two proteins have largely equivalent activities and that their specificity lies in differential expression (41). We detected no *c-myc* expression in *A^{N/N}p^{N/N}* pro-B cell tumors with amplified *N-myc*, despite the fact that normal murine pro-B cells express both *N-myc* and *c-myc* (42). Conversely, the three *A^{N/N}p^{N/N}* tumors with amplified *c-myc* did not express detectable *N-myc*. Similar crossregulation of *Myc* family members has been reported in the context of *N-myc*-related transformation (36, 38, 43, 44), which further supports the role of *N-myc* amplification in the transformation of *A^{N/N}p^{N/N}* pro-B cells.

***N-myc* Complicon Formation Initiated from Intrachromosome 12 Translocations.** We have proposed a molecular model for the events leading to the translocations and amplifications that underlie complicon formation in XRCC4/p53 or Ligase 4/p53-deficient mice (13). In the absence of p53, pro-B cells with unrepaired DSBs at the *J_H* locus enter S phase, where the broken chromosome 12 is replicated. Subsequent recombination be-

tween the replicated J_H DSB and a region downstream of $c-myc$ produces a clonal C12;15 translocation and a dicentric chromosome that initiates a BFB cycle, resulting in coamplification of IgH and $c-myc$. A subset (3 of 9) of pro-B tumors from $A^{N/N}p^{N/N}$ mice share many of the same features ($c-myc$ amplification, C12;15, and compicon formation); thus, $c-myc$ amplification can occur in $A^{N/N}p^{N/N}$ pro-B cells by the same mechanism that predominates in other NHEJ/p53 pro-B tumors. Yet the majority of $A^{N/N}p^{N/N}$ pro-B cell tumors are distinct, both karyotypically and in oncogene amplification status, from nearly all previously studied NHEJ/p53-deficient tumors, having amplified $N-myc$ through translocations that involve IgH .

Translocation junctions characterized from $A^{N/N}p^{N/N}$ pro-B tumors involved direct invasion of the $N-myc$ flanking region by J_H (or upstream D_H); thus, these junctions must result from intra- or interchromosomal 12 rearrangements. Based on the locations of the junctions and cytogenetic analyses, one could envision several potential scenarios leading to $N-myc$ amplification by BFB. We have outlined two possible mechanisms (Figs. 7 and 8, which are published as supporting information on the PNAS web site) that would lead to the different cytogenetic structures and breakpoints observed (Figs. 3 and 5). Although one cannot definitively distinguish between these and other related mechanisms, it is clear that the overall mechanism by which $N-myc$ is amplified in $A^{N/N}p^{N/N}$ pro-B tumors is highly analogous to the mechanism by which $c-myc$ is amplified in other NHEJ/p53-deficient tumors, except that the initial V(D)J event targets the $N-myc$ region rather than the $c-myc$ region.

Targeting of Translocation Events. Endogenous $N-myc$ and $c-myc$ both can drive pro-B cell lymphomagenesis by means of a compicon-related mechanism and appear to have similar lymphomagenic potential once activated. Although still a formal possibility, it seems unlikely that $N-myc$ versus $c-myc$ amplifica-

tion provides a specific selective growth advantage to $A^{N/N}p^{N/N}$ pro-B cells as opposed to pro-B cells deficient for other NHEJ factors and p53. In the same context, the appearance of $A^{N/N}p^{N/N}$ tumors that have amplified $c-myc$ indicates that the oncogenic potential of $c-myc$ is not limited to pro-B cells lacking other NHEJ factors.

Thus, we favor the possibility that some factor(s) must predispose $c-myc$ versus $N-myc$ for translocation in the different genetic settings. One such factor could be the presence of unresolved J_H -coding end hairpins, which when replicated could lead to a dicentric chromosome 12 that could initiate $N-myc$ amplification. However, all characterized $A^{N/N}p^{N/N}$ junctions involve fusion of IgH to regions around $N-myc$; therefore, BFB appears to have been initiated by translocation rather than a reduplication of chromosome 12 (Figs. 7 and 8). Another possibility would be a unique impact of Artemis-deficiency, compared to other NHEJ deficiencies, on the availability of particular translocation acceptor sites around $c-myc$ and/or $N-myc$. In this context, it also is possible that some other genetic difference between $A^{N/N}p^{N/N}$ and other NHEJ/p53-deficient mouse lines, rather than the specific NHEJ deficiency *per se*, might affect availability of such sites. In either case, although the nature of the factor(s) that leads to preferential targeting of IgH translocations to $c-myc$ or $N-myc$ in certain lines will require further analysis, our current studies suggest that such factors do exist.

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