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Role of genomic instability and p53 in AID-induced *c-myc-Igh* translocations

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Abstract

Chromosomal translocations involving the immunoglobulin switch region are a hallmark feature of B-cell malignancies[1]. However, little is known about the molecular mechanism by which primary B cells acquire or guard against these lesions. Here we find that translocations between *c-myc* and the IgH locus (*Igh*) are induced in primary B cells within hours of expression of the catalytically active form of activation-induced cytidine deaminase (AID), an enzyme that deaminates cytosine to produce uracil in DNA[2,3]. Translocation also requires uracil DNA glycosylase (UNG), which removes uracil from DNA to create abasic sites that are then processed to double-strand breaks[4,5]. The pathway that mediates aberrant joining of *c-myc* and *Igh* differs from intrachromosomal repair during immunoglobulin class switch recombination in that it does not require histone H2AX[6], p53 binding protein 1 (53BP1)[7,8] or the non-homologous end-joining protein Ku80[9]. In addition, translocations are inhibited by the tumour suppressors ATM, Nbs1, p19 (Arf) and p53, which is consistent with activation of DNA damage- and oncogenic stress-induced checkpoints during physiological class switching. Finally, we demonstrate that accumulation of AID-dependent, IgH-associated chromosomal lesions is not sufficient to enhance

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c-myc–Igh translocations. Our findings reveal a pathway for surveillance and protection against AID-dependent DNA damage, leading to chromosomal translocations.

AID is essential for both class-switch recombination and somatic hypermutation of antibody genes[2,10]. In addition, it is required for the accumulation of plasmacytoma-associated *c-myc–Igh* translocations in mice[11–13], but the role of AID and immunoglobulin class switching in the aetiology of these chromosome fusions has not been determined. To examine the role of AID in *c-myc–Igh* translocations we used retroviral transduction to express AID in *Aid*$^{-/-}$ B cells stimulated with lipopolysaccharide (LPS) and interleukin (IL)-4 *in vitro* (Supplementary Fig. 1a). Under these conditions AID protein was expressed at a tenfold higher level than in wild-type B cells (Supplementary Fig. 1b), and switch recombination and switch junctions were normal (Supplementary Fig. 2). Reciprocal chromosome 12 and 15 *c-myc–Igh* translocations were detected using a polymerase chain reaction (PCR) assay sensitive to 1–2 translocations (Fig. 1 and Supplementary Fig. 1c), and were confirmed by Southern blotting with *c-myc* and *Igh* probes and by sequence analysis (Fig. 1b and Supplementary Fig. 3). Translocations were readily detected in AID-overexpressing cells, and the junctions were similar to those found in B cells expressing physiological levels of AID *in vitro* (see below) and in IL-6 transgenic mice[12] (Supplementary Fig. 3). In contrast, translocations were absent from uninfected *Aid*$^{-/-}$ B cells (2.6×10^7 cells tested; see below) and *Aid*$^{-/-}$ B cells expressing a catalytically inactive point mutant of AID (AID^{E58Q}; $>1 \times 10^7$ cells tested; Fig. 1b). We conclude that overexpression of catalytically active AID promotes *c-myc–Igh* translocations in B cells stimulated with LPS and IL-4.

To examine the kinetics of the translocation reaction, we infected *Aid*$^{-/-}$ B cells with retroviruses encoding AID-oestrogen receptor (ER) or AID^{E58Q}-ER (Supplementary Fig. 1a); *c-myc–Igh* fusions were assayed after addition of tamoxifen. Switch recombination and *c-myc–Igh* translocations were both detectable as early as 12 h after tamoxifen addition (Supplementary Fig. 4a and data not shown), and by 24 h translocations were found on average once in 2×10^4 cells (Fig. 1c). In contrast, *Aid*$^{-/-}$ B cells infected with AID^{E58Q}-ER failed to show translocations or class switching at any time (Supplementary Fig. 4a and data not shown). We conclude that *c-myc–Igh* translocations are rapidly induced by AID expression *in vitro*.

It has been suggested that AID might promote accumulation of pre-existing *c-myc–Igh* translocations by enhancing the survival of cells bearing these chromosome fusions[13]. To explore this possibility we infected *Aid*$^{-/-}$ B cells with a retrovirus encoding AID-ER or AID^{E58Q}-ER and human CD4 as a marker for infection (Supplementary Fig. 1a), and measured cell division by labelling with carboxy-fluorescein diacetate succinimidyl ester (Supplementary Fig. 4b). *Aid*$^{-/-}$ B cells infected with AID-ER or AID^{E58Q}-ER divided at equal rates (averaging one division after 12 h and four divisions after 48 h) with no difference in the rate of cell death (Supplementary Fig. 4 and data not shown). The frequency of translocations found in *Aid*$^{-/-}$ B cells was less than 4×10^{-8} (see below), and increased by orders of magnitude in 24 h in B cells overexpressing retrovirally encoded AID, by which time the cells had undergone only 2–3 divisions (Supplementary Fig. 4).

Therefore, AID could not promote sufficient outgrowth of pre-existing translocation-positive cells and instead produces *c-myc–Igh* translocations *de novo* by a mechanism that requires cytidine deaminase activity.

AID is thought to initiate the switch reaction by deaminating cytidine to produce UzG mismatches in immunoglobulin switch DNA that are processed to double-strand break (DSB) intermedi-ates[2,3,14]. To determine whether *c-myc–Igh* translocations require base excision repair we assayed $Ung^{-/-}$ B cells[15]. Occasional faint bands were detectable in $Ung^{-/-}$ B cells infected with retroviruses encoding either AID or catalytically inactive AID^{E58Q}, but these failed to hybridize with both *c-myc* and *Igh* probes in Southern blots and thus were not authentic *c-myc–Igh* translocations (Fig. 1b). *c-myc-Igh* translocations were also absent from $Ung^{-/-}$ B cells stimulated to express physiological levels of AID *in vitro* upon treatment with LPS and IL-4 (Fig. 2, $P = 0.0097$ versus wild type (see below)). Thus, like normal immunoglobulin class switching and somatic mutation, efficient translocation requires processing of AID-induced U·G mismatches by the base excision repair protein UNG.

DSB intermediates in the switch reaction are repaired by a nonhomologous end-joining (NHEJ) pathway requiring Ku80/Ku70 (refs 9, 16) and partially dependent on DNA-PKcs[17,18]. To determine whether Ku80 is also required for *c-myc–Igh* translocations we overexpressed AID by retroviral transduction in immunoglobulin transgenic $Ku80^{-/-}$ B cells stimulated with LPS and IL-4 ($Ku80^{-/-}$ HL)[9]. These cells did not undergo switching but showed authentic *c-myc–Igh* translocations (Fig. 1b), which were also independent of DNA-PKcs catalytic activity (data not shown). Thus, Ku80 is dispensable for fusing *c-myc* and *Igh*, suggesting that an alternative non-classical NHEJ pathway may be involved in aberrant interchromosomal joining.

To determine whether physiological levels of AID expression can produce translocations *in vitro* we assayed wild-type and $Aid^{-/-}$ B cells before and after stimulation with LPS and IL-4. Although translocations were infrequent in stimulated wild-type B cells they were absent in stimulated $Aid^{-/-}$ B cells (Fig. 2, $P = 0.0097$ for wild type versus $Aid^{-/-}$) and in unstimulated wild-type or $Aid^{-/-}$ B cells (Supplementary Fig. 5). Thus, physiological levels of AID can produce rare *c-myc–Igh* translocations in stimulated wild-type B cells, a finding consistent with the detection of these events in B cells isolated from immunized mice[19].

In addition to NHEJ, efficient class switching requires the DSB response proteins ATM[20,21], Nbs1 (refs 22, 23), H2AX (refs 6, 24) and 53BP1 (refs 7, 8). Absence of 53BP1 produces the most profound reduction in switching in mice[7,8] (Fig. 2), whereas milder defects are found in $Atm^{-/-}$ (refs 20, 21), $H2ax^{-/-}$ (refs 6, 24) and $Nbs1^{\Delta/-}$ (refs 22, 23) mice (Fig. 2). To determine whether DSBs produced during class switching are channelled into translocations in the absence of efficient repair, we assayed $53BP1^{-/-}$, $Atm^{-/-}$, $H2ax^{-/-}$ and $Nbs1^{\Delta/-}$ (null) B cells. None of the mutant B cells showed evidence of translocation before stimulation with LPS and IL-4 (Supplementary Fig. 5 and data not shown); after stimulation, the frequency of translocation in $53BP1^{-/-}$ and $H2ax^{-/-}$ B cells was similar to wild type (Fig. 2, $P = 0.427$ and 0.487 versus wild type, respectively). In contrast, $Atm^{-/-}$ B cells exhibited significant enhancement in translocation (Fig. 2, $P < 0.0001$ versus wild type) as

did *Nbs1*$^{\Delta/-}$ B cells, which are defective in ATM activation[25] (Fig. 2, $P = 0.0006$ versus wild type). In contrast, Nbs1 hypomorphic mutant B cells (*Nbs1*$^{657\Delta5}$), which are only mildly defective in ATM activation and normal for class switching[25], resembled wild-type B cells with respect to translocation (Fig. 2, $P = 0.611$ versus wild type). The finding that a marked reduction in class switching in *53BP1*$^{-/-}$ mice did not alter the frequency of translocation, whereas a modest defect in switching in *Atm*$^{-/-}$ mice produced a strong enhancement, indicates that DSB response proteins have distinct roles in the class-switch recombination and translocation reactions.

ATM activates p53 in response to DSBs. To determine whether DNA damage response pathways downstream of ATM are involved in protecting cells from *c-myc–Igh* translocations we examined p53-deficient mice. Whereas freshly isolated *p53*$^{-/-}$ B cells did not carry *c-myc–Igh* translocations (Supplementary Fig. 5), stimulation with LPS and IL-4 induced a marked increase in the frequency of translocations relative to wild type; this was not due to an increase in AID protein expression in *p53*$^{-/-}$ B cells compared to wild type (Fig. 2, $P < 0.0001$ versus wild type; see also Supplementary Fig. 1b). Notably, loss of a single *p53* allele was sufficient to enhance trans-locations (Fig. 2, $P = 0.004$ versus wild type). The *c-myc–Igh* translocations in *p53*$^{-/-}$ B cells were similar to translocations found in wild-type B cells (Supplementary Fig. 3) and these events were AID-dependent because B cells deficient in AID and p53 (*Aid*$^{-/-}$ *p53*$^{-/-}$) produced no *c-myc–Igh* translocations (Fig. 2, $P < 0.0001$ *Aid*$^{-/-}$ *p53*$^{-/-}$ versus *p53*$^{-/-}$). Thus, like ATM, p53 is essential for protecting B cells from *c-myc–Igh* translocations, but in contrast to ATM, p53 has no detectable effect on the switch reaction (Fig. 2).

p53 is also activated in response to abnormal mitogenic signals, such as those occurring when *c-myc* is deregulated by genomic rearrangements[26]. Because this form of oncogenic stress induces expression of the p19 (Arf) tumour suppressor, we explored the possibility that p19-mediated activation of p53 might prevent accumulation of *c-myc–Igh* translocations. Although there was no immunoglobulin switch recombination defect in *p19*$^{-/-}$ B cells, the frequency of *c-myc–Igh* translocations was significantly elevated relative to wild type after stimulation with LPS and IL-4 (Fig. 2, $P < 0.0001$ versus wild type). These data suggest that induction of p19 expression is also involved in protection against AID-induced translocations.

To determine whether susceptibility to *c-myc–Igh* translocations correlates with the presence of chromosomal lesions at the IgH locus, we examined metaphase spreads from *53BP1*$^{-/-}$, *p53*$^{-/-}$, *p19*$^{-/-}$, *Atm*$^{-/-}$ and *Nbs1*$^{\Delta/-}$ B cells that were stimulated with LPS and IL-4 (Fig. 3a). Metaphases were hybridized with a combination of painting probes for chromosome 12 (which carries the mouse IgH locus in a telomere proximal position), IgH Cα (downstream of IgSγ1) and telomere repeats[27]. Consistent with the proposed defect in synapsis during class switching in *53BP1*$^{-/-}$ B cells[7,8], and the observation that 53BP1 accumulates on the IgH locus in cells undergoing switching[20], most of the aberrations in *53BP1*$^{-/-}$ metaphases were associated with chromosome 12 (Fig. 3b and Supplementary Table 1). Furthermore, the chromosome 12 lesions in *53BP1*$^{-/-}$ metaphases were AID-dependent because they were absent in *Aid*$^{-/-}$ *53BP1*$^{-/-}$ B cells (Fig. 3b). B cells from *Atm*$^{-/-}$ mice resembled *53BP1*$^{-/-}$ B cells but accumulated a greater number of chromosome lesions (Fig. 3b and

Supplementary Table 1). Overall, 39% of the *Atm*$^{-/-}$ metaphases exhibited abnormalities and 20% of aberrations carried either a deletion (24 out of 160) or translocation (6 out of 160) involving chromosome 12. Nine of the metaphases with chromosome 12 deletions lacked a telomere while maintaining a signal for IgH Cα, as would be expected of an unresolved class-switch associated break, whereas 15 cells had also lost IgH Cα (Fig. 4b and Supplementary Table 1). Similarly, a significant fraction of *Nbs1*$^{\Delta/-}$ B cells exhibited IgH-associated lesions, but these cells also showed a much higher level of general genomic instability (Fig. 3b and Supplementary Table 1). In contrast, loss of p53, which produced no defect in class switching but a strong enhancement of *c-myc–Igh* translocations (Fig. 2), resulted in chromosomal aberrations in 5% of the metaphases analysed but none involving the IgH locus (Fig. 3b and Supplementary Table 1). We conclude that genomic instability is not sufficient to enhance *c-myc–Igh* translocations: similar numbers of translocations were found in p53 and ATM mutant B cells (Fig. 2) that harbour widely differing degrees of genomic instability, whereas *53BP1*$^{-/-}$ cells exhibit AID-dependent IgH instability without a concomitant increase in translocations.

Our experiments reveal that divergent pathways lead to class switching and *c-myc–Igh* translocation. Both reactions require cytidine deamination and base excision repair and proceed through DSB intermediates, but the factors required for resolution of DNA breaks differ in the two reactions. We would like to propose a working model to account for these findings (Fig. 4). In this scheme, switching requires formation of AID-dependent DSBs, synapsis of upstream switch Sμ with downstream switch breaks mediated by H2AX/53BP1/ATM/Nbs1 (refs 6–8, 20–24) and NHEJ (Ku70/Ku80 and DNA-PKcs)[9,16–18]. DNA lesions that remain unresolved initiate a p53-dependent checkpoint through the activation of ATM. Cells that escape this initial checkpoint make Ku80-independent translocations that activate p19 in response to deregulation of *c-myc*, thereby triggering p53 by an alternative mechanism (Fig. 4). In this way, the p19–p53 or ATM–p53 signalling axes may have complementary roles in the elimination of incipient cancer cells during different stages of the translocation reaction, while not being needed for class switching *per se*. The model predicts that combined loss of p53 and factors that facilitate immunoglobulin switch synapsis or NHEJ would lead to the accelerated appearance of mature B-cell lymphomas. Consistent with this idea, combined deficiency in H2AX or 53BP1 and p53 leads to rapid lymphoma development (of T- and B-cell lineages), and the mature B-cell lymphomas harbour recurrent *c-myc–Igh* translocations[28,29].

Mature B-cell lymphomas are the most common lymphoid malignancies in humans and many of these are associated with cytogenetic abnormalities involving immunoglobulin loci[1]. Our data suggest that mutation or decreased expression of p53 contributes early in the pathogenesis of lymphoma by facilitating AID-induced translocations.

Methods

Retroviral constructs

The AIDER-CD4-IRES construct (Supplementary Fig. 1a, lower panel) was created by replacing an *Eco*RI–*Eco*RV fragment containing the IRES-PuroR cassette of pQCXIP vector (Clontech) with a *Bam*HI CD4-IRES fragment obtained by PCR from pMACS4-

IRESII plasmid (Miltenyi Biotech) with primers 5′-CGGATCCCGCCCCTCTCCCTCCCCCCCCCCTA-3′ and 5′-CGGATCCTCAGTGCCGGCACCTGACACAGAAGA-3′. AIDER or EQER were cloned in *Not*I–*Pac*I sites after amplification with primers 5′-GCGGCCGCCGCCACCATGGACAGCCTTCTGATGAAGCA-3′ and 5′-GTTAATTAATCAGACTGTGGCAGGGAAACCCTCTGC-3′.

PCR and statistical analysis

PCR reactions were performed exactly as described[12]. Briefly, genomic DNA corresponding to 10^5 cells (unless indicated otherwise) was subjected to two rounds of 25 cycles each of nested PCR using the Expand Long Template PCR system (Roche) with the following primers[30]: for derivative chromosome 12 translocations, first round 5′-TGAGGACCAGAGAGGGATAAAAGAGAA-3′ and 5′-GGGGAGGGGGTGTCAAATAATAAGA-3′, second round 5′-CACCCTGCTATTTCCTTGTTGCTAC-3′ and 5′-GACACCTCCCTTCTACACTCTAAACCG-3′; for derivative chromosome 15 translocations, first round 5′-ACTATGCTATGGACTACTGGGGTCAAG-3′ and 5′-GTGAAAACCGACTGTGGCCCTGGAA-3′, second round 5′-CCTCAGTCACCGTCTCCTCAGGTA-3′ and 5′-GTGGAGGTGTATGGGGTGTAGAC-3′. For loading control PCR we used the *myc* primers 5′-GGGGAGGGGGTGTCAAATAATAAGA-3′ and 5′-GTGAAAACCGACTGTGGCCCTGGAA-3′. In Fig. 2b, 6–26 million cells were assayed each from three independent mice: wild type, $Ung^{-/-}$, $Aid^{-/-}$ and $p53^{-/-}$ $Aid^{-/-}$, 2.6×10^7; $Atm^{-/-}$, 2.2×10^7; $p53^{-/-}$, 1.6×10^7; $53BP1^{-/-}$, $H2ax^{-/-}$ and $p19^{-/-}$, 1.4×10^7; $Nbs1^{\Delta/-}$, 0.9×10^7; $p53^{+/-}$ and $Nbs1^{657\Delta5}$, 0.6×10^7. PCR products were cloned in a TOPO-TA vector (Invitrogen) and sequenced. For Southern blotting DNA was transferred to membranes (GeneScreen, Perkin-Elmer) by alkaline transfer and hybridized to ^{32}P-end-labelled oligo probes using QuikHyb (Stratagene) according to the manufacturer's instructions. Oligonucleotide probes were internal to the primers used in the PCR assay: derChr15 *c-myc* 5′-GGACTGCGCAGGGAGACCTACAGGGG-3′; derChr15 *Igh* 5′-GAGGGAGCCGGCTGAGAGAAGTTGGG-3′; derChr12 *c-myc* 5′-GCAGCGATTCAGCACTGGGTGCAGG-3′; derChr12 *Igh* 5′-CCTGGTATACAGGACGAAACTGCAGCAG-3′. *P*-values for pairwise comparison of translocation frequencies were calculated using one-sided exact Fisher's test (GraphPad software) from contingency tables built with numbers of positive and negative PCR reactions ($n > 150$ for every test).

Mice, B-cell cultures and retroviral transductions, flow cytometry and cell sorting, and FISH detection of IgH-associated lesions are described in Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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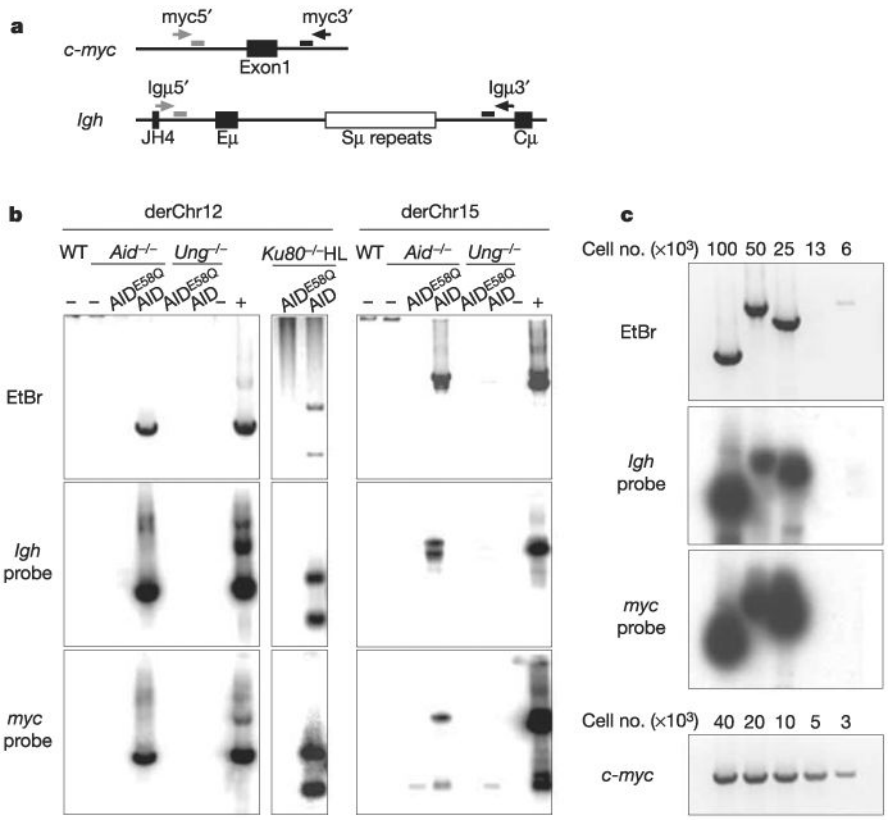

Figure 1. AID overexpression is sufficient to promote *c-myc-Igh* translocations in B cells

a, Schematic representation of the PCR assay used for *c-myc–Igh* translocations. Primers used to detect derivative chromosome 12 (derChr12) and derivative chromosome 15 (derChr15) translocations are represented as horizontal black and grey arrows, respectively. Internal oligonucleotide probes used in Southern blot experiments are shown as horizontal black and grey bars. **b,** AID overexpression promotes *c-myc–Igh* translocations in B cells. Naive B cells from wild type (WT), $Aid^{-/-}$, $Ung^{-/-}$ or $Ku80^{-/-}$ HL[9] mice were stimulated with LPS and IL-4 and retrovirally transduced ($Aid^{-/-}$, $Ung^{-/-}$, $Ku80^{-/-}$ HL) with AID or E58Q mutant AID (AID^{E58Q}) (Supplementary Fig. 1a) or left uninfected (WT and $Aid^{-/-}$), as indicated. Representative agarose gels (upper gels) and Southern blots with *Igh* or *myc* oligonucleotide probes (middle and lower gels, respectively) are shown. **c,** *c-myc–Igh* translocations can be detected after 24h of AID expression. $Aid^{-/-}$ B cells were transduced with retroviral vectors encoding AID or catalyically inactive AID^{E58Q} fused to oestrogen receptor (AID-ER or AID^{E58Q}-ER) (Supplementary Fig. 1a). GFP^{+} cells were sorted 24 h after addition of tamoxifen and translocations were analysed by PCR. Agarose gels and Southern blots are shown. Numbers above lanes indicate input cell number ($\times 10^3$). The lower gel shows *c-myc* PCR as DNA loading control.

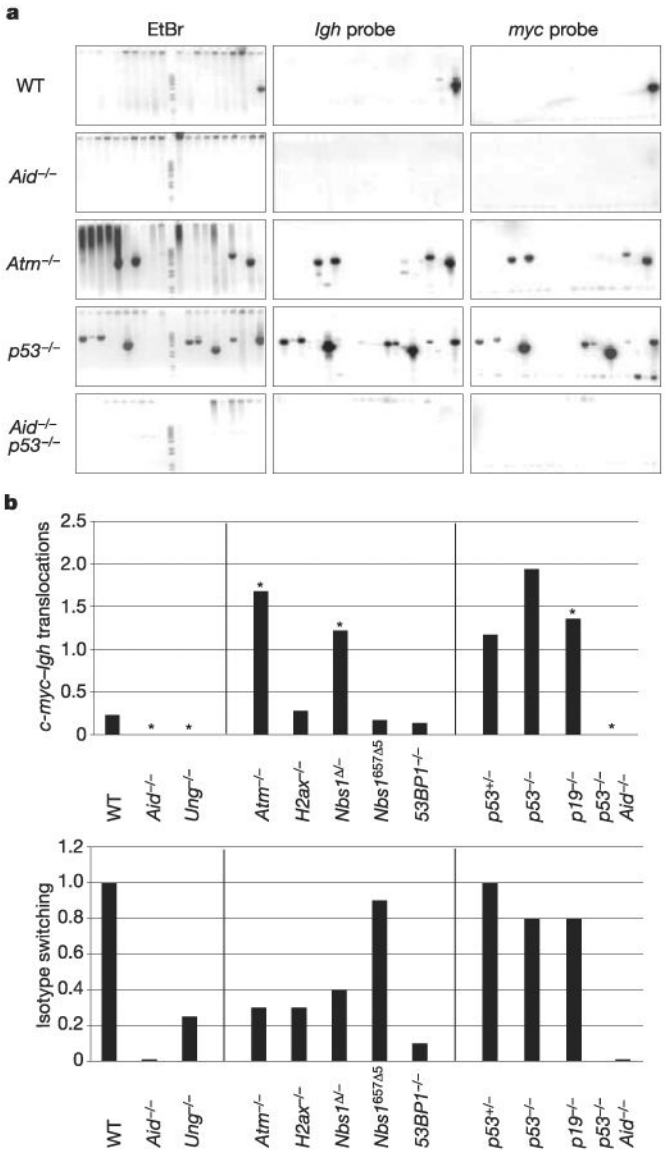

Figure 2. *c-myc-Igh* translocations in mutant B cells

a, *c-myc–Igh* translocations. B cells were cultured with LPS and IL-4 and assayed by PCR after 72h. Representative agarose gels (left) and Southern blots with *Igh* (middle) and *myc* (right) probes are shown. **b,** Translocation frequency and isotype switching. The upper graph shows the number of *c-myc–Igh* translocations per 10^7 cells: $Aid^{-/-}$ ($P = 0.0097$ versus wild type), $Ung^{-/-}$ ($P = 0.0097$ versus wild type), $Atm^{-/-}$ ($P < 0.0001$ versus wild type), $H2ax^{-/-}$ ($P = 0.487$ versus wild type), $Nbs1^{\Delta/-}$ ($P = 0.0001$ versus wild type), $Nbs1^{657\Delta5}$ (hypomorph) ($P = 0.611$ versus wild type), $53BP1^{-/-}$ ($P = 0.427$ versus wild type), $p53^{-/-}$ ($P < 0.0001$ versus wild type), $p53^{+/-}$ ($P = 0.004$ versus wild type), $p19^{-/-}$ ($P < 0.0001$ versus wild type) and $p53^{-/-}$ $Aid^{-/-}$ ($P < 0.0001$ versus $p53^{-/-}$, $P = 0.0152$ versus wild type, $P = 0.611$ versus $Aid^{-/-}$) mutants. Samples with significant *P*-values versus the wild-type value are labelled with an asterisk. The lower graph shows the efficiency of isotype switching to IgG1 relative to wild type.

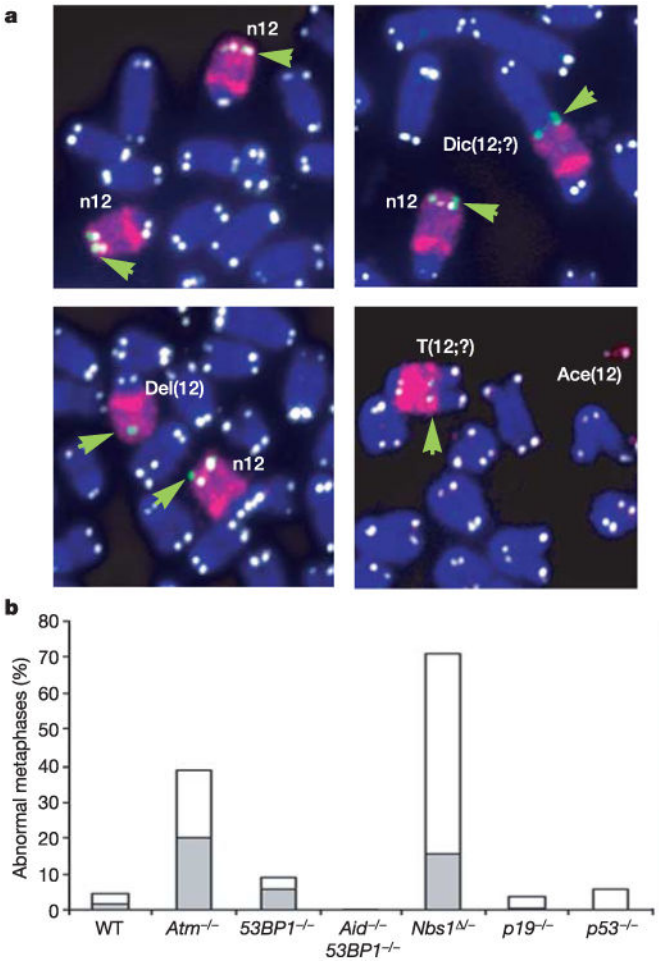

Figure 3. Chromosomal instability

Metaphase spreads were prepared at 72 h after culture with LPS and IL-4 and hybridized with a combination of painting probes for chromosome 12 (pink), IgH Cα (green) and telomere-specific PNA probes (white), and counterstained with DAPI (blue). **a,** Examples of chromosome-12 associated lesions in mutant B cells include dicentrics (Dic), translocations (T), deletions (Del) and acentric fragments (Ace). Normal 12 (n12) is indicated. **b,** Percentage of metaphases with abnormalities specifically associated with chromosome 12 (filled bar, including chromatid and chromosome breaks and fusions) and with all other chromosomes (open bar).

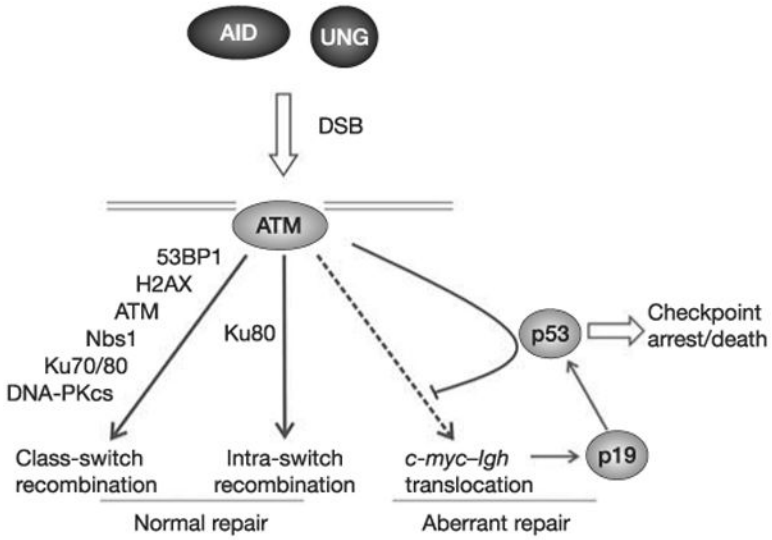

Figure 4. Distinct pathways mediate class switching and chromosome translocations

AID/UNG-induced DSBs at the IgH locus are normally resolved by 53BP1/Nbs1/ATM/γ-H2AX and NHEJ (Ku70, Ku80 and DNA-PKcs), leading to class switching or intra-switch recombination, which frequently leads to internal Sμ deletions (53BP1/Nbs1/ATM/γ-H2AX-independent and Ku80-dependent)[24]. Alternatively, DSBs may be resolved by translocations, which are suppressed by ATM- and/or p19-dependent activation of p53.