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AID is required to initiate Nbs1/ γ -H2AX focus formation and mutations at sites of class switching

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Class switch recombination (CSR) is a region-specific DNA recombination reaction that replaces one immunoglobulin heavy-chain constant region (C_H) gene with another. This enables a single variable (V) region gene to be used in conjunction with

different downstream C_H genes, each having a unique biological activity. The molecular mechanisms that mediate CSR have not been defined, but activation-induced cytidine deaminase (AID), a putative RNA-editing enzyme, is required for this reaction¹. Here we report that the Nijmegen breakage syndrome protein (Nbs1) and phosphorylated H2A histone family member X (γ -H2AX, also known as γ -H2afx), which facilitate DNA double-strand break (DSB) repair^{2–4}, form nuclear foci at the C_H region in the G1 phase of the cell cycle in cells undergoing CSR, and that switching is impaired in H2AX^{-/-} mice. Localization of Nbs1 and γ -H2AX to the I_{gH} locus during CSR is dependent on AID. In addition, AID is required for induction of switch region (μ)-specific DNA lesions that precede CSR. These results place AID function upstream of the DNA modifications that initiate CSR.

AID, a recently characterized protein belonging to the RNA-editing cytidine deaminase family, is expressed specifically in B lymphocytes undergoing CSR and somatic hypermutation¹. Human and mouse B cells lacking AID appear to develop normally and respond to challenge with antigens; however, they fail to hypermutate their immunoglobulin V genes or undergo CSR^{1,5}. CSR joins immunoglobulin switch regions by looping out and excision of intervening DNA. However, the mechanisms of DNA cleavage, switch region synapsis, and DNA repair after cleavage are not well defined.

To determine whether DNA repair factors associate with DSBs at the switch regions, we first examined the intracellular localization of γ -H2AX, Nbs1, Rad51 and Brca1 in activated B cells by immunofluorescence. Brca1 and Rad51 are required for homologous recombination, the Mre11–Rad50–Nbs1 complex has been implicated in both homologous recombination and non-homologous end-joining (NHEJ), and γ -H2AX is critical for recruiting these repair factors to DSBs⁶ and facilitates NHEJ in *Saccharomyces cerevisiae*⁴. All four proteins showed diffuse nuclear staining in most of the resting B cells from C57BL/6 wild-type mice. High local concentrations of these factors (nuclear foci) were detected in a very small percentage of cells (<5%), which increased significantly when the cells were stimulated to undergo CSR *in vitro* with lipopolysaccharide (LPS) and interleukin (IL)-4 (Fig. 1a). After 3 days of stimulation, 37% of the B cells contained discrete Brca1 foci (12 ± 6 per cell) and 43% contained Rad51 foci (7 ± 3 per cell), consistent with previous results⁷; the remaining cells exhibited a weak, diffuse pattern of nuclear staining (Fig. 1a). Many of the stimulated B cells also formed Nbs1 foci (32% contained, on average, 3 ± 2 per cell) and γ -H2AX foci (40% contained, on average, 4.5 ± 3 per cell). To determine which of these repair factors are co-localized in activated B cells, we performed two colour immunofluorescence experiments (Fig. 1b). Only 20% of the cells that contained Rad51 and Nbs1 (n = 687) or Brca1 and Nbs1 foci (n = 431) exhibited co-localization. In contrast, γ -H2AX foci co-localized with Nbs1 in 79% of the cells (n = 354). Thus, DNA-repair focus formation is induced in B cells by LPS and IL-4, and most of the Nbs1 foci co-localize with γ -H2AX, but less frequently with Brca1 or Rad51 foci.

The Nbs1/ γ -H2AX/Brca1/Rad51 nuclear foci observed in activated B cells may represent repair-complex storage sites, replication-associated DNA damage⁸, telomeric complexes⁹, or a specific response to CSR-induced breaks. To determine whether Nbs1/ γ -H2AX/Brca1/Rad51 foci are associated with sites of CSR, we performed immunocytochemistry staining followed by fluorescence *in situ* hybridization (ICC-FISH) to simultaneously visualize DNA (I_{gH}, TCR α , or immunoglobulin light chain (I_g κ) loci) and protein (Nbs1, γ -H2AX, Brca1 or Rad51) in lymphocytes stimulated with LPS and IL-4 (Fig. 1c)¹⁰. Approximately 15% of cells in a given optical section contained at least one Nbs1 or γ -H2AX focus. Coincidence of either signal with one or both I_{gH} alleles was detected in 69% of the cells with Nbs1 foci and 76% of the cells with γ -H2AX foci (Fig. 1c and Table 1). This co-localization was specific as only 3–5% of the stimulated B cells showed co-localiza-

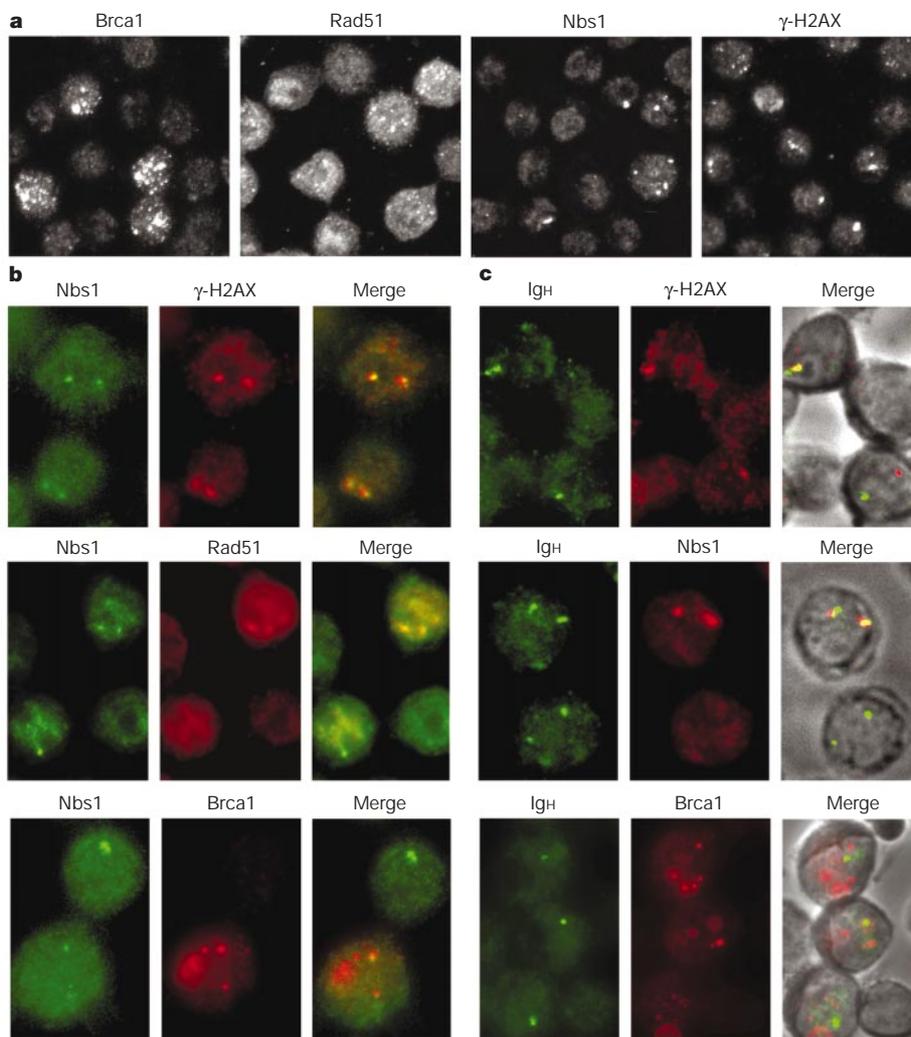


Figure 1 DNA repair foci in wild-type B lymphocytes after stimulation with LPS and IL-4 for 72 h. **a**, Distribution of Brca1, Rad51, Nbs1 and γ -H2AX in activated wild-type B cells. Confocal images were optically sectioned at 0.5- μ m intervals and merged into a maximum projection. **b**, Double staining with Nbs1 (green) together with γ -H2AX (red), Rad51 (red) or Brca1 (red). The images were merged to determine co-localization (yellow). **c**, Co-localization of DNA repair foci with the IgH locus. B cells were stained with anti- γ -H2AX, anti-Nbs1, or anti-Brca1 antibodies (ICC (red)) followed by DNA FISH (green) detection of the C_H region. Cells were visualized by phase-contrast microscopy and the images were merged to determine co-localization (yellow). Fluorescence images in **b** and **c** represent a single optical section.

tion of Nbs1 or γ -H2AX with TCR α or Ig κ (Table 1). In contrast, neither Rad51 nor Brca1 foci significantly co-localized with IgH (Table 1), although the average number of these foci per cell was greater than the number of Nbs1 or γ -H2AX foci. DNA DSBs arising from immunoglobulin V gene somatic hypermutation^{11–13} could potentially recruit DNA repair factors, but there was no significant immunoglobulin gene mutation detected in B cells stimulated under these same conditions (two changes in 10,620 V κ nucleotides were found in activated B cells carrying a pre-arranged light chain gene). These findings indicate that Nbs1 and γ -H2AX, but not Brca1 or Rad51, are recruited to DNA lesions created during CSR.

In vertebrates, DNA repair by NHEJ occurs primarily during the G1 phase of the cell cycle, whereas homologous recombination is restricted to S/G2 phases. To determine in which phase of the cell cycle CSR occurs, live B cells were electronically sorted 2 days after stimulation into G1/early S and late S/G₂/M phases, and then examined by ICC-FISH for foci associated with C_H (Fig. 2a). Most of the cells (81%) that formed γ -H2AX or Nbs1 foci at the IgH locus were in G1 (Fig. 2a, b). Thus, DNA repair factors are recruited to the CSR sites in the G1 phase of the cell cycle, a result that is consistent with NHEJ as the repair pathway for CSR^{14–16}.

DNA lesions associated with CSR would be expected to form after initiation of germline transcription and before expression of mature switch transcripts^{17,18}. To determine when IgH-associated Nbs1/ γ -H2AX foci appear relative to these events, we assayed germline I γ 1 transcription (I γ 1-C γ 1), mature IgG1 transcripts, S μ -S γ 1 DNA rearrangements, and surface IgG1 expression (Fig. 3a, b). Germline I γ 1 transcription was present 24 h after stimulation. S μ -S γ 1 DNA, mature IgG1 transcripts and surface IgG1 expression

Table 1 Co-localization of protein foci with genomic loci

Genotype	Antibody staining	Cells with foci that co-localize with IgH (%)	Cells with foci that co-localize with TCR α (%)	Cells with foci that co-localize with Ig κ (%)
Wild type	Nbs1	69	3.3	3.7
Wild type	γ -H2AX	76	5.2	3
Wild type	Brca1	8.4	11	5.1
Wild type	Rad51	0	ND	ND
<i>AID</i> ^{-/-}	Nbs1	3.7	2	ND
<i>AID</i> ^{-/-}	γ -H2AX	7	1	ND
<i>AID</i> ^{-/-}	Brca1	5.5	5.9	ND

Co-localization values were measured in B cells after 72 h of LPS and IL-4 culture. For each genotype, all hybridizations were performed in parallel and repeated up to four times. A minimum of 100 cells with foci were examined after each hybridization. ND, not done.

were barely detectable at 48 h, but were clearly present after 72 h. IgH-associated Nbs1 foci began accumulating in B cells 48 h after stimulation (Fig. 3b), after the detection of immunoglobulin germ-line transcripts but before high levels of completed recombination, indicating that focus formation is coincident with initiation of CSR.

To determine the functional significance of γ -H2AX focus formation at sites of CSR, we examined the effects of H2AX ablation on switch recombination. B cells that were isolated from $H2AX^{-/-}$ mice (Supplementary Information Fig. 1) were stimulated with LPS and IL-4 under conditions identical to those used for ICC-FISH analysis. CSR was measured by flow cytometry. B cells from $H2ax^{-/-}$ mice ($n = 8$) exhibited impaired switching as indicated by a 50–86% reduction in surface IgG1 levels relative to littermate controls (Fig. 3c). This deficiency was not due to a gross difference in proliferation as measured by cell number, ^3H -thymidine incorporation ($H2AX^{+/+}$, $2,120 \pm 244$ c.p.m.; $H2AX^{-/-}$, $1,910 \pm 102$ c.p.m. at day 3 of culture), or cell cycle distribution (Fig. 3c).

Furthermore, sterile switch transcripts were induced at similar levels in B cells of $H2AX^{-/-}$ and $H2AX^{+/+}$ mice, but mature IgG1 transcripts and DNA recombination were decreased in the knock-out mice in a manner consistent with the flow cytometric analysis (Fig. 3d). We conclude that H2AX promotes efficient CSR, but it is not essential for the reaction, suggesting that H2AX-independent DNA repair pathways can also process switch DNA lesions.

To determine whether AID is required for switch-region-associated, DNA-repair focus formation, we assayed for co-localization of C_H with Nbs1, γ -H2AX, or Brca1 protein in stimulated B cells from $AID^{-/-}$ mice. In contrast to wild-type B cells, in which a significant fraction of Nbs1/ γ -H2AX foci were detected at the IgH locus, only 4–7% of the cells containing C_H signals and Nbs1 or γ -H2AX foci showed co-localization (Fig. 4c and Table 1). This coincidence was similar to the background frequency observed with the TCR α probe (Table 1). In $AID^{-/-}$ mutant cells, as in wild-type cells, all DNA probes failed to significantly co-localize with Brca1 (Table 1). $AID^{-/-}$ B cells stimulated with LPS and IL-4 were

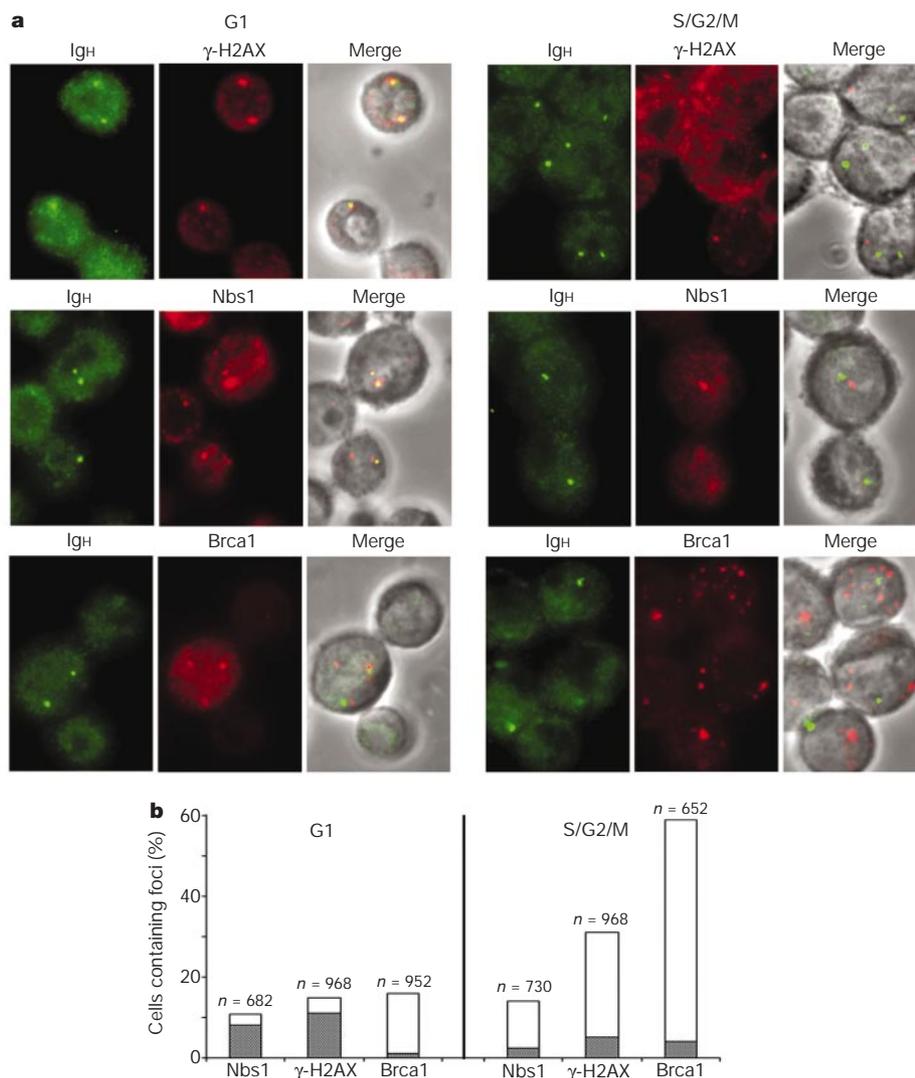


Figure 2 DNA repair foci associated with CSR form predominantly in the G1 phase of the cell cycle. **a**, Representative ICC-FISH images of G1 and S/G2/M sorted cells. Wild-type B cells stimulated for 48 h with LPS and IL-4 were labelled with Hoechst and live cells were electronically separated into G1 (purity >95%) and S/G2/M (purity >80%) cell cycle fractions. Sorted populations were analysed by ICC-FISH for co-localization (yellow) of γ -H2AX, Nbs1 and Brca1 with C_H . **b**, Percentage of sorted cells in a given optical section

that contain Nbs1, γ -H2AX and Brca1 foci (whole bar), and percentage of sorted cells in which DNA repair foci co-localize with C_H (hatched section). Brca1 and γ -H2AX foci were found disproportionately in a greater fraction of S/G2/M cells relative to G1 (γ -H2AX, 31% compared with 15%; Brca1, 59% compared with 16%, respectively), whereas the percentage of G1 cells (11%) and S/G2/M (14%) cells with Nbs1 foci was similar. More than 1,000 cells were analysed.

indistinguishable from those of wild type in terms of the percentage of cells with foci (Nbs1, γ -H2AX, Brca1, or Rad51), the average number of foci per cell and co-localization of these proteins with each other (Fig. 4a, b and data not shown). γ -irradiation of wild-type and *AID*^{-/-} B cells caused a similar increase in the number of foci per cell and the percentage of cells with foci (not shown). This indicates that AID is required specifically for CSR-associated Nbs1/ γ -H2AX focus formation but not for the recruitment of DNA repair factors to DSBs.

To determine whether AID is required to produce DNA lesions in the switch region, we cloned the germline S μ region from *AID*^{-/-} and control B cells that were stimulated with LPS and IL-4. To amplify the unrearranged, highly repetitive and (G+C)-rich S μ region, we used primers that flank germline S μ and Long-Expand Taq polymerase¹⁹. We analysed the first 450 base pairs (bp) of the germline S μ clones, which is a region 5' of the S μ core repeats (starting at nucleotide 4,600; GenBank accession number J00440). We focused on this region because it is frequently involved in CSR²⁰ and might show evidence of aberrant repair of DNA lesions that precede the CSR reaction²¹. Small numbers of mutations, consistent with Long-Expand Taq polymerase error¹⁹, were found in the I γ H enhancer (E μ), S μ and the I γ μ constant region (C μ) cloned from resting B cells (Fig. 5a). In contrast, sequence analysis revealed a specific increase in mutation in the S μ region in wild-type B cells stimulated with LPS and IL-4 (Fig. 5a; *P* = 0.0006 for S μ mutation in

B cells stimulated with LPS and IL-4 for 72 h compared with resting B cells (52 mutations/18,660 bp versus 10 mutations/16,542 bp, respectively); *P* = 0.0003 for S μ mutation in stimulated B cells at 72 h compared with E μ plus C μ mutation in B cells stimulated under the same conditions (33 mutations/59,196 bp for E μ plus C μ); *P*-values were determined by a two-tailed *t*-test assuming unequal variance; the frequency of mutation in the 5' region of S μ was 5×10^{-4} when measured with a proofreading polymerase). Consistent with previous reports, we did not find deletions in this part of S μ ²². The induced mutations were AID dependent because no significant increase in mutation over background was observed in S μ in *AID*^{-/-} B cells cultured with LPS and IL-4 (Fig. 5a). These changes in nucleotide sequence were not found on stimulation with CpG (Fig. 5b)—a synthetic oligodeoxyribonucleotide that induces B-cell proliferation but not CSR. The mutations induced in S μ resembled V gene hypermutation in that they were predominantly single-base changes, and 60% of the changes were in sequence motifs corresponding to hypermutational hot spots²³ (Fig. 5c; *P* = 0.0015, χ^2 test, 31 mutations in 166 hot-spot nucleotides compared with 21 mutations in 284 non-hot-spot nucleotides). Hypermutation has also been reported in *BCL-6* (refs 24, 25) and in areas adjoining switch joints²⁶, but the relationship of these mutations to the switch recombination reaction has never been determined. Our experiments show that the S μ lesions are inducible, that they are S μ specific because they are not found in

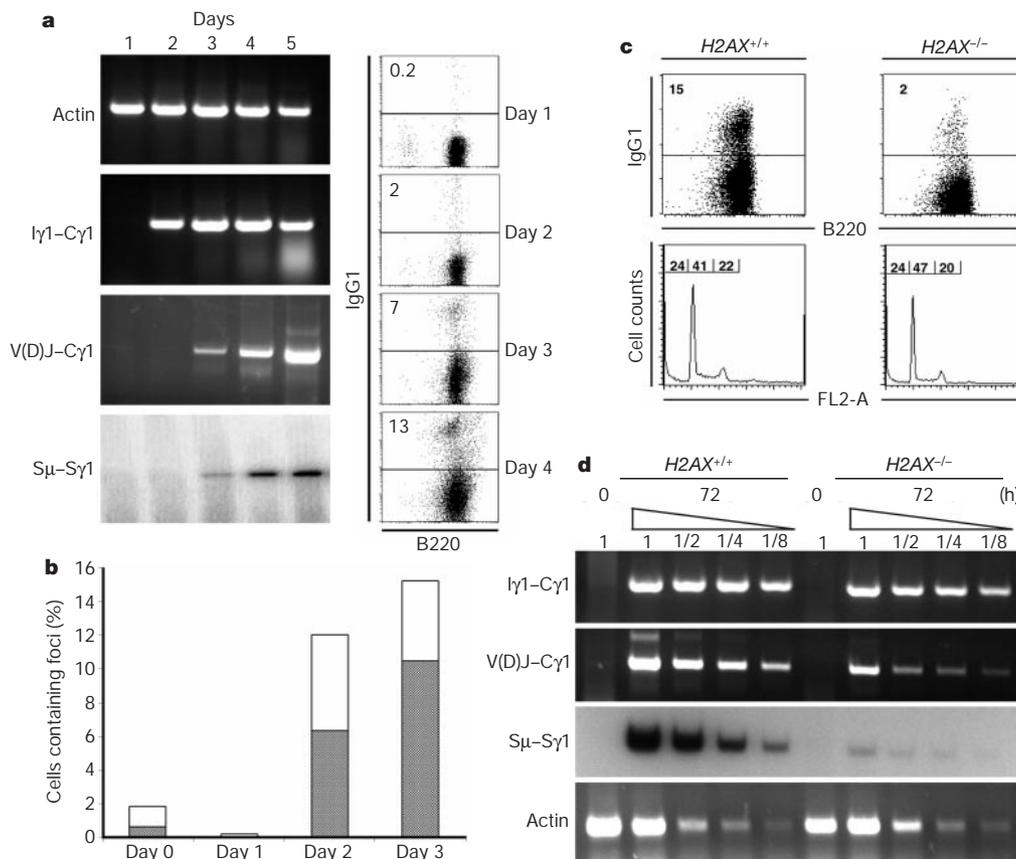


Figure 3 Kinetics of CH-associated foci accumulation in wild-type mice and impaired switching in *H2AX*^{-/-} mice. **a**, Actin control, germline sterile I γ 1-C γ 1 transcripts, mature switch V(D)J-C γ 1 transcripts (assayed by PCR with reverse transcription (RT-PCR)), and S μ -S γ 1 DNA rearrangements assayed by digestion-circularization PCR (DC-PCR) at indicated times of LPS and IL-4 culture. Cell surface expression of IgG1 was detected by flow cytometry. Percentages from total lymphocyte-gated populations are indicated. RT-PCR, DC-PCR and FACS analysis were performed as described¹⁴. **b**, Analysis by ICC-FISH for co-localization of Nbs1 foci with CH, quantified as in Fig. 2b. **c**, Cell surface

expression of IgG1 in B cells from *H2AX*^{-/-} mice and littermates assayed 72 h after stimulation with LPS and IL-4. An aliquot of the samples (shown below) was used to simultaneously measure cell cycle distribution at 72 h. Numbers indicate the percentage of sub-G1, G1 and S/G2/M cells, respectively. **d**, I γ 1-C γ 1 transcripts, mature switch V(D)J-C γ 1 transcripts, and S μ -S γ 1 DNA rearrangements in B cells of *H2AX*^{-/-} and *H2AX*^{+/+} mice assayed at 0 and 72 h after LPS and IL-4 culture. DNA and RNA samples taken at 72 h were diluted as indicated.

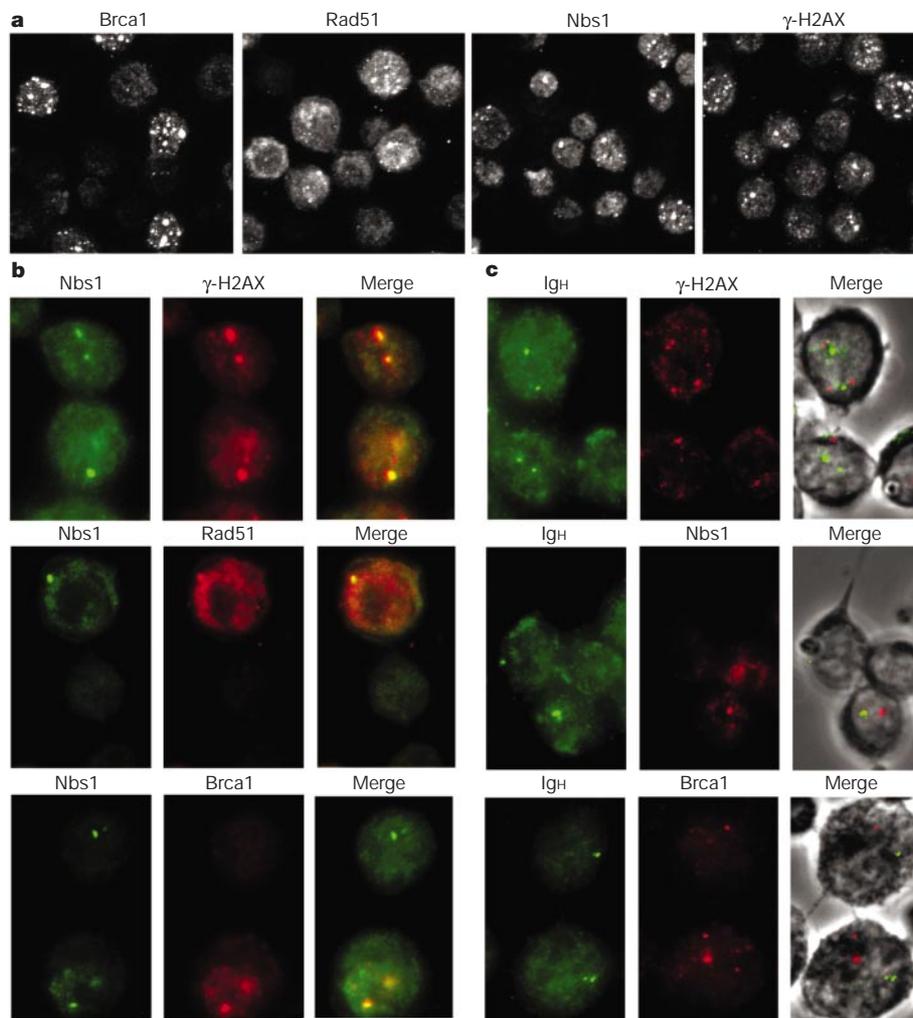


Figure 4 Co-localization of Nbs1 and γ -H2AX at the *Igh* locus is dependent on AID. **a**, *AID*^{-/-} B cells were stimulated for 72 h with LPS and IL-4 and the intracellular localization of Brca1, Rad51, Nbs1 and γ -H2AX was determined by immunofluorescence as in Fig. 1a. **b**, Double staining with Nbs1 (green) together with either γ -H2AX (red),

Brca1 (red) or Rad51 (red) in *AID*^{-/-} cultures. The images were merged to determine co-localization (yellow). **c**, Co-localization of DNA repair foci with *Igh* locus in activated *AID*^{-/-} B cells, analysed by ICC-FISH as in Fig. 1c.

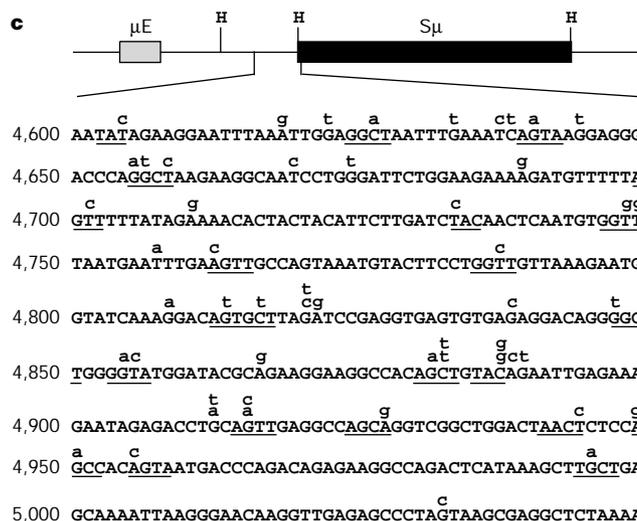
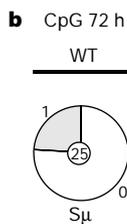
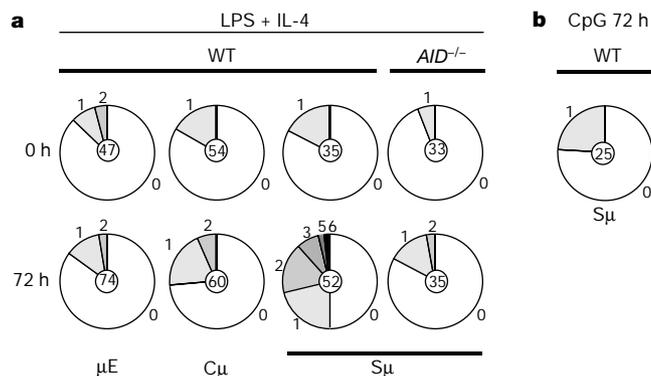


Figure 5 AID-dependent *Sμ* mutation induced by LPS and IL-4. **a**, Proportion of *Eμ*, *Cμ* and *Sμ* sequences carrying different numbers of mutations before and after stimulation with LPS and IL-4 for 72 h. Segment sizes in the pie charts are proportional to the number of sequences carrying the number of mutations indicated in the periphery of the charts. The total number of independent sequences analysed is indicated in the centre of each chart. **b**, Proportion of *Sμ* sequences carrying different numbers of mutations

72 h after stimulation with CpG. **c**, Distribution of point mutations. The region sequenced is indicated with the first base corresponding to position 4,600 in the μ region germline sequence (GenBank accession number J00440). H, position of *Hind*III restriction enzyme sites. Lower-case letters above the line indicate mutations found among the sequenced clones. Each lower-case letter represents an independent mutational event. Hot spots containing mutations are underlined.

adjacent DNA, that the lesions precede CSR, and that they are AID dependent.

It has been proposed that AID initiates somatic hypermutation and CSR by activating an endonuclease that produces lesions in either *V* genes or switch regions²¹. In this model, CSR breaks would be generated by means of nicking at staggered positions on both strands²¹. We propose that such lesions become associated with Nbs1/ γ -H2AX foci in the G1 phase of the cell cycle and that repair proceeds by NHEJ^{14–16}. By contrast, DNA breaks associated with hypermutation in *V* genes are found in the S/G2 phase of the cell cycle¹¹ and are repaired by pathways independent of DNA-dependent protein kinase catalytic subunit (DNA-PKcs)²⁷. The lesions in *V* genes can lead to either gene conversion or somatic hypermutation depending on the mechanism of DNA repair²⁸. In addition to CSR, LPS and IL-4 induce AID-dependent mutations in $S\mu$. By analogy to *V* mutation, two alternative pathways might also repair AID-dependent lesions in $S\mu$. Lesions in $S\mu$ might be processed by an error-prone polymerase, thereby giving rise to mutation²⁹; alternatively, staggered DSBs could be processed by the NHEJ pathway, leading to CSR^{14–16}. Our observations suggest that AID is either involved directly in producing the switch lesion or activating the switch endonuclease, thereby placing AID upstream of $S\mu$ mutation, H2AX phosphorylation, Nbs1 recruitment to sites of CSR, and DNA repair. □

Methods

Lymphocyte culture and sorting

B lymphocytes from wild-type (C57BL/6), AID^{-/-} (ref. 1), I κ k knock-in³⁰ and H2AX^{-/-} mice (Supplementary Information Fig. 1) were isolated from spleen using CD19 microbeads (Miltenyi Biotec). A total of 1×10^6 cells ml⁻¹ was cultured with LPS and IL-4, or 10 nM CpG (MWG biotech, ODN number 1638) for 3 days before isolation. For viable cell DNA staining, 2.5 μ g ml⁻¹ Hoechst 33342 (Molecular Probes) was added to actively growing cells for 2 h, and propidium iodide (0.5 μ g ml⁻¹) was added immediately before laser excitation at 350 nm and 488 nm. Live (propidium-iodide-negative) cells with a DNA content of $2n$ and $>2n$ were electronically sorted using a Vantage SE flow cytometer (Becton Dickinson Biosciences). An aliquot of each of the sorted fractions was fixed with ethanol, stained with propidium iodide (50 μ g ml⁻¹) and re-analysed for DNA content by flow cytometry.

ICC and ICC-FISH analysis

After stimulation, 1×10^6 B cells were spun onto 24-well plates containing 12-mm coverslips (Fisher) coated with 150 μ g ml⁻¹ poly-L-lysine (Sigma). We performed ICC detection essentially as described¹⁰. Briefly, cells were fixed with methanol (-20 °C), blocked with 5% goat serum/1% bovine serum albumin (BSA)/1 \times PBS overnight, incubated with either Nbs1 (ref. 10) (1:1,000 dilution), γ -H2AX³ (1:500), Brca1 (provided by S. Ganesan; 1:500) or Rad51 (Oncogene; 1:200) polyclonal antibodies, washed and then stained with Alexa-568 conjugated goat anti-rabbit antibody (Molecular Probes). Double staining for Nbs1 with γ -H2AX, Brca1, or Rad51 was performed as described using directly conjugated Nbs1 Alexa-488 antibody¹⁰. For ICC-FISH, antibodies were cross-linked using 50 mM ethylene glycol bis(succinimidyl succinate) for 30 min at 37 °C followed by RNase (100 μ g ml⁻¹) treatment for 60 min at 37 °C. Chromosomal DNA was denatured in 0.07 M NaOH (pH 13.0) for 2 min followed by immersion in cold PBS. The biotin-labelled DNA probe was hybridized at 37 °C overnight, followed by standard FISH washes. DNA probes were detected with avidin-fluorescein isothiocyanate (FITC), and then amplified with biotinylated goat anti-avidin and avidin-FITC (all antibodies diluted 1:200 in 3% BSA/4 \times SSC/0.05% Tween 20; Vector Laboratories).

Mutation analysis

Genomic DNA was amplified by polymerase chain reaction (PCR) from 5,000 cell equivalents in four independent reactions using Expand Long Taq¹⁹ (Roche). This polymerase has a higher error rate than Pfu¹⁹, but the germline $S\mu$ could not be amplified with Pfu polymerase. We estimate the background error rate of this polymerase to be 0.4×10^{-3} under our amplification conditions based on time 0 values for all sequences analysed (40 mutations in 96,440 bp). For the $S\mu$, $E\mu$ and $C\mu$ regions, amplification conditions were 10 cycles at 94 °C (10 s), 60 °C (30 s), 68 °C (3 min), and 20 cycles at 94 °C (10 s), 60 °C (30 s), 68 °C (3 min and 20 s per cycle). The primers that we used were: μ switch region $5\mu.3$ (5'-AATGGATACCTCAGTGGTTTTAATGGTGGGTTTA-3') and $3\mu.2$ (5'-AGAGGCTAGATCTGGCTTCTCAAGTAG-3'); μ intronic enhancer $5\mu.E2$ (5'-ATTTTAAATGAATTGAGCAATGTTGAGTTGGAGT-3') and $3\mu.EA$ (5'-GGCAACTTCAAATTCATTAACACAT-3'); and the I κ k constant region $C\mu.1$ (5'-AGCCCTCCACCTCCACCTACCTATTAC-3') and $C\mu.B$ (5'-ATTCAGGGTTTCATAGTTGGCCAGGTTT-3'). PCR products were cloned using TOPO-TA cloning kit (Invitrogen) and

sequenced using M13 universal primers. Sequence alignment was performed using Sequence manager II software (DNASTAR).

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Competing interests statement

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