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Mismatch repair proteins and AID deaminase activity are required for the dominant negative function of C terminally-deleted AID in class switching

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Abstract

Activation-induced cytidine deaminase (AID) is essential for class switch recombination (CSR) and somatic hypermutation (SHM) of Ig genes. The AID C terminus is required for CSR but not for S region DNA DSBs during CSR, and it is not required for SHM. AID lacking the C terminus (AID^{ΔC}) is a dominant negative (DN) mutant, as human patients heterozygous for this mutant fail to undergo CSR. In agreement, we show that AID^{ΔC} is a DN mutant when expressed in AID-sufficient mouse splenic B cells. In order to have DN function, AID^{ΔC} must have deaminase activity, suggesting that its ability to induce DSBs is important for the DN function. Supporting this hypothesis, Msh2-Msh6 have previously been shown to contribute to DSB formation in S regions, and here we find that Msh2 is required for the DN activity, as AID^{ΔC} is not a DN mutant in *msh2*^{-/-} cells. Our results suggest that the DNA DSBs induced by AID^{ΔC} are unable to participate in CSR, and might interfere with the ability of full-length AID to participate in CSR. We propose that AID^{ΔC} is impaired in its ability to recruit non-homologous end joining (NHEJ) repair factors, resulting in accumulation of DSBs that undergo aberrant resection. Supporting this hypothesis, we find that the S-S junctions induced by AID^{ΔC} have longer microhomologies than those induced by full-length AID. In addition, our data suggest that AID binds S_μ regions in vivo as a monomer.

Introduction

AID initiates antibody gene diversification after immunization or infection by deamination of cytosines in Ig S regions, leading to DSBs and CSR, and also in recombined V(D)J gene segments, leading to SHM (1–4). It has been known for several years that the C terminus of AID is required for CSR, although it does not appear to have a role during SHM of antibody genes (5–7). Some Hyper-IgM (HIGM) human patients, who cannot undergo CSR, are

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heterozygous for mutant AID proteins lacking 8 or more amino acids at the C terminus of AID (AID^{ΔC}), indicating that AID^{ΔC} is a dominant negative (DN) mutant (5, 8). Although it is unknown why AID^{ΔC} is a DN mutant, it has been suggested that AID functions as a dimer or tetramer (5, 9–12), and perhaps the full-length AID and AID^{ΔC} heterodimerize and fail to perform a function, or fail to interact with a protein essential for CSR (5). However, dimerization of full-length AID and AID^{ΔC} would not explain the very low level of CSR in human HIGM patients, since both proteins are likely to be simultaneously induced, and therefore some dimers of full-length AID should be present.

Mouse and human AID have a deaminase domain between amino acids (aa) 56 and 94, a C terminal domain reported to be required for CSR but not for SHM between aa 182 and 198(2, 5–7), and a nuclear export signal also located at the C-terminus between aa 190 and 198 (13, 14). AID predominantly resides in the cytoplasm, and nuclear AID undergoes rapid ubiquitin-mediated proteasomal degradation, causing the half-life of nuclear AID to be 3 times shorter than that of cytoplasmic AID (15). However, nuclear export is not required for CSR, as mouse AID^{F198A} is not exported from nuclei, and yet CSR and SHM are only modestly reduced in cells expressing this mutant (14). Also, some C terminus substitution mutants that retain a functional nuclear export signal cannot potentiate CSR (16).

A relevant difference between CSR and SHM is that the latter does not require DSBs or recombination. During CSR, the dUs in S regions generated by AID are converted into DSBs in the donor (S_μ) and acceptor S_x regions (17). The dU bases are excised by uracil-*N*-glycosylase (UNG) and UNG deficiency causes a dramatic reduction in S region DSBs and CSR (18–20). Apurinic/aprimidinic endonuclease 1 and 2 (APE1/2) can nick the abasic sites generated by UNG and are important for creating S region DSBs during CSR (21). When the single-strand breaks (SSBs) created by APE activity on opposite DNA strands within S_μ are sufficiently near, they can form a DSB, which can recombine with a DSB in a downstream S region. When the SSBs created by AID-UNG-APE activities are too far apart to spontaneously form a DSB, mismatch repair (MMR) proteins are thought to convert these distal SSBs into DSBs during CSR, and in MMR-deficient B cells S region DSBs are decreased by 50–80% (17, 22–24). The DSBs in two different S regions are recombined by non-homologous end joining (NHEJ), resulting in CSR.

Retrovirally transduced AID tagged at the C terminus with the estrogen receptor (ER) binds to S regions, as assayed by chromatin immunoprecipitation (ChIP) in *aid*^{-/-} splenic B cells (25). In that study (25), we reported that AID associated poorly with S region DNA. However, our current ChIP results, now obtained many times, show that AID associates with S_μ as well as full-length AID. We cannot repeat the previous result and do not understand the basis of this discrepancy. We have published a correction (26). All other results reported previously are reproducible. The new finding that AID binds S_μ is in better agreement with the fact that AID is competent for introducing mutations and DSBs at the S_μ and S_{γ3} regions (7, 25, 27, 28).

AID-ER introduces as many mutations in unrearranged (germline) S_μ in B cells induced to undergo CSR as does AID-ER (7); thus, AID can produce the DNA substrate for UNG and Msh2-Msh6 (MutS_α). However, these repair proteins bind poorly to S_μ in cells expressing

AID, indicating the importance of the C terminus for their binding (25, 29). Consistent with the dependence on the C terminus for the binding of UNG, there is an increase in the proportion of transition mutations at G:C bp in the S μ region in cells expressing AID, as expected if UNG does not readily access the mutations (7). CSR is reduced 2–3 fold in Msh2- or Msh6-deficient B cells relative to MutS α -sufficient cells (30–33), and this effect is reproduced in our retroviral system when full-length RV-AID is expressed in Msh2- or Msh6-deficient *aid*^{-/-} B cells (25). However, when AID is expressed, the small amount of CSR induced (~10% of full-length AID) is not *further* reduced in MutS α -deficient cells. This indicates that MMR does not contribute to CSR efficiency in cells expressing AID, consistent with the poor binding of Msh2 and Msh6 to S μ in cells expressing AID (25).

It is not understood why the AID C terminus is required for CSR, nor is it understood how its deletion creates a DN mutation. *Aid*^{-/-} B cells transduced with AID-ER have S region DSBs (25, 27), indicating that the AID C terminus is important for events after DSB formation. However, it is possible that S region DSBs are aberrantly processed and/or inefficiently introduced in cells expressing AID, and that they accumulate due to lack of repair and/or inefficient S-S recombination. Thus, the C terminus might be required for recruiting proteins involved in producing non-resected DSBs that are appropriate for NHEJ and/or for recruiting proteins involved in the end-joining process itself, as previously suggested (29, 34, 35). If AID functions as a dimer, it is possible that a heterodimer of AID and AID cannot recruit these proteins. Numerous proteins have been demonstrated to associate with AID (36), and several require the AID C terminus for their association (14, 37, 38). These proteins have been shown to help export AID from the nucleus (14, 39), or help maintain it in the cytoplasm (38), or help recruit AID to S regions (37, 40).

In this report, we show that the DN function of AID observed in humans is also found in mouse B cells, and we also show that AID lacking deaminase activity does not have DN function and does not associate stably with S μ in *aid*^{+/+} cells. We also find that the DN phenotype of AID depends upon the MMR protein Msh2. Our results suggest that the DN function depends upon the ability of AID to bind S μ and to induce DSBs in S regions, and also indicate that AID-induced DSBs are not recombined by NHEJ. It is possible that the inability of these DSBs to recombine by NHEJ results in inefficient S-S recombination and generation of aberrantly resected DSBs that interfere with the ability of full-length AID to induce normal DSBs that can be recombined properly during CSR.

Materials and Methods

Mice

Mice were extensively (8 generations) backcrossed to C75BL/6. AID-deficient mice were obtained from T. Honjo (Kyoto University, Kyoto, Japan) (1). Msh2-deficient mice were obtained from T. Mak, Univ. of Toronto CA (41). Mlh1-deficient mice were obtained from R.M. Liskay, Oregon Health Sciences University, Portland OR (42). UNG-deficient mice were obtained from D. Barnes and T. Lindahl, London Research Institute, London UK (43). Msh6-deficient mice were obtained from W. Edelmann, Albert Einstein Medical College, NY (44). For each experiment, splenic B cells were isolated from littermates. Mice were housed in the IACUC-approved specific pathogen-free facility at the University of

Massachusetts Medical School; mice were used according to the guidelines from University of Massachusetts Medical School IACUC.

Antibodies

Antibodies to ER (sc-8002X), GAPDH (sc-25778), growth factor receptor-bound protein 2 (Grb2) (sc-255), and Msh6 (sc-10798) were purchased from Santa Cruz, and antibody for Lamin A/C was from Cell Signaling (#2032). Rabbit antibodies to mouse AID (20) and UNG (23) were previously described. For ChIP experiments using untagged AID, we used rabbit AID antibody provided by J. Chaudhuri (10).

Production of retroviruses in Phoenix-E cells

pMX-PIE-AID-FLAG-ER-IRES-GFP-*puro* and pMX-PIE- AID-FLAG-ER-IRES-GFP-*puro*(7) were received from Drs V. Barretto and M. Nussenzweig (The Rockefeller University, NY). The control retrovirus, pMX-PIE-ER-IRES-GFP, was constructed and viruses were prepared as previously described (25). To create the AID^{H56R/E58Q} and AID^{H56R/E58Q} mutants, the AID-ER gene was subcloned into Bluescript (Stratagene), mutated using Quik-Change (Stratagene), sequenced, and then reinserted into pMX-PIE. pMIG-AID and pMIG (45) were received from J. Chaudhuri, Rockefeller University. pMIG-AID was created by converting amino acid position 189 to a nonsense codon.

Chromatin immunoprecipitation (ChIP)

Live cells were isolated by flotation on Lympholyte M (Cedar Lane, Ontario Canada) 24 hrs after retroviral transduction. After recovery and washing twice, $\sim 2 \times 10^7$ live cells were resuspended in SBSS, and were cross-linked with formaldehyde at a final concentration of 1% for 5 min at 37°C. Cross-linking was stopped by adjustment to 125 mM glycine, and incubation for 5 min at room temp. Cross-linked cells were intermittently sonicated at 4°C for 60 min total. Samples were filtered through glass wool. 2×10^6 cell equivalents were incubated overnight with antibody at 4°C. On the following day, Protein G or Protein A Dynabeads (Invitrogen) were added to the samples and incubated for 2 h at 4°C. The beads were then washed 5 times for 10 min and reverse crosslinking was performed at 65°C in the presence of RNase A. The samples were treated with Proteinase K at 55°C and DNA was recovered using phenol-chloroform extraction followed by ethanol precipitation. ChIP results were assayed by real time PCR using Sybr Green. Significance was calculated by a paired two-tailed T test. Primers for S μ were forward primer DK99: AACTAGGCTGGCTTAACCGAGATG and reverse primer DK100: GTCCAGTGTAGGCAGTAGAGTTTA. Primers for *mb-1/CD79a* were: mb-1FW: CCACGCACTAGAGAGAGACTCAA and mb-1REV: CCGCCTCACTTCTGTTCAGCCG. Primers for C μ were: forward primer CuPG-F: TCTGACAGGAGGCAAGAAGACAGATTCTTA and reverse primer: CuPG-R: GCCACCAGATTCTTATCAGACAGGGG (46), except for the experiment shown in Fig 5C, in which C μ primers previously described were used (25).

B cell purification and cultures

Mouse splenic B cells were isolated by T cell depletion with antibody and complement (21). B cells were cultured at 10^5 cells/ml. LPS (25 μ g/ml), anti-IgD dextran (0.3 ng/ml)(Fina Biosolutions) +/- IL-4 (20 ng/ml) were used to induce class switch recombination to IgG1 and IgG3, respectively. Human BLyS/BAFF (50 ng/ml)(Human Genome Sciences) was included in all cultures. Retroviral infection and assay of CSR by FACS was performed as previously described (25). Briefly, cells were activated for 2 days, and then infected. One day later they were harvested for all experiments. The one exception is noted in the Results section. Statistical difference in CSR between different cultures was determined by a 2-tailed T test.

Western blotting

Preparation of extracts and western blots were previously described (21).

RT-PCR

RNA was isolated using Trizol (Ambion) from splenic B cells after 2 days of activation under IgG3 switching conditions. The RNA was treated with DNase I twice (DNA-free Kit, Ambion), and cDNA synthesis was performed using oligop (dT)₁₀ and Super Script II reverse transcriptase (Invitrogen). Primers to amplify endogenous AID mRNA were located in the 4th exon (forward primer: AACTTCGGCGCATCCTTTTG) and in the 3' untranslated region (reverse primer: CGTGTGACATTCCAGGAGGT). Primers to amplify RV-AID-ER were the same forward primer as for endogenous mRNA and the reverse primer was located in the ER tag (GGTTGGCAGCTCTCATGTCT).

Ligation-mediated PCR (LM-PCR)

After culture for 2 days, viable cells were isolated by flotation on Ficoll/Hypaque gradients (=1.09), or Lympholyte (Cedar Lane, Ontario, Canada); cells were imbedded in low melt agarose plugs, and DNA isolated as described (20). For linker ligation, 50 μ L 1 \times ligase buffer was added to the plugs which were then heated to 68°C to melt the agarose. 20 μ L DNA (about 10,000 cell equivalents) was added to 2 μ L T4 DNA Ligase (2 Weiss units, MBI Fermentas, Hanover, MD), 10 μ L ds annealed linker in 1 \times ligase buffer, 3 μ L 10 \times Ligase buffer, 5 μ L 50% PEG 4000 and 25 μ L dH₂O and incubated overnight at 16°C. Linker was prepared by annealing 5 nmoles each of LMPCR.1 (5'-GCGGTGACCCGGGAGATCTGAATTC-3') and LMPCR.2 (5'-GAATTCAGATC-3') in 300 μ L 1 \times ligase buffer, which results in a dsoligo with a 14 ntss overhang that can only ligate unidirectionally. Ligated DNA samples were heat inactivated at 70°C for 10 min, diluted 3 \times in dH₂O, and then heated to 70°C for an additional 20 min. This sample was then assayed for *mb1* DNA (primer sequences same as used for CHIP) by PCR to adjust DNA input prior to LM-PCR. The primer 5'S μ (5'-GCAGAAAATTTAGATAAAATGGATACCTCAGTGG-3') was used in conjunction with linker primer (LMPCR.1) to amplify DNA breaks. Three-fold dilutions of input DNA (0.5, 1.5 and 4.5 μ L) were amplified by HotStarTaq (Qiagen) using a touchdown PCR program. PCR products were electrophoresed on 1.25% agarose gels and blotted onto nylon membranes. Blots were hybridized with an S μ -specific oligonucleotide probe (μ probe5':

AGGGACCCAGGCTAAGAAGGCAAT for 5' S μ LM-PCR; end-labeled with [γ 32P]-ATP at 37°C overnight and washed at 50°C with 2 \times SSC/0.1% SDS.

Amplification and sequencing of S μ -Sa junctions

Genomic DNA (100ng) from RV-transduced cells induced to switch to IgA (47) is amplified (in 12 reactions per genotype) using 2 nested PCRs (Expand Long Template system, Roche), using the same program for each PCR. Primers for the first round were: 5u3 – AATGGATACCTCAGTGGTTTTTAATGGTGGGTTTA and SaR3 – CCCATCCCATCCCATCCCATC and for the second round were: u3H3 – AACAAAGCTTGGCTTAACCGAGATGAGCC and SaR2 – CCAGCCCAGCTCAGGCCATTT. The products were cloned using the Topo TA cloning kit (Invitrogen) and sequenced by Macrogen.

Results

AID-ER is a DN mutant in *aid*^{+/+} cells

To study the DN effect of AID, we transduced RV constructs encoding full-length AID-ER, AID-ER, or the ER tag alone into WT mouse splenic B cells two days after activation to induce CSR. The cultures were treated with tamoxifen (OHT) at the time of retroviral transduction to induce nuclear localization of ER-tagged proteins, allowing AID to reach its target. We harvested the cells one day after transduction, because we found this was optimal for their viability (25). We detected no consistent difference in cell viability and recovery from cultures expressing full-length AID, AID or the ER tag alone at this time point; it averaged ~90%. Two days after transduction, cell viability was reduced to ~70% among all cultures. To compare expression of the AID constructs, we prepared extracts of transduced WT cells for western blot analysis of AID and AID expression. Full-length AID-ER and AID-ER are expressed at similar levels in cytoplasmic and nuclear extracts from WT cells with similar GFP expression (Fig 1A: 3 left-most lanes). Note that the ER-tagged constructs have two forms of protein, which probably are monomers and dimers, although their apparent molecular weights do not have a clear 2-fold relationship on these gradient polyacrylamide gels. To determine if the transduced AID-ER or AID-ER affects endogenous AID levels, we also assayed endogenous AID in total cell extracts, and did not find a difference between cells transduced with the three retroviruses (Fig 1B). This indicates that expression of AID does not cause degradation of endogenous AID, ruling out one possible explanation for the mechanism of its DN effect. As our AID antibody is specific for the C terminus of AID, which is where the ER tag is located, we cannot detect transduced AID with this antibody. To compare the levels of transduced AID-ER and endogenous AID, we compared their relative mRNA levels by qRT-PCR. As shown in Fig 1C, the levels of mRNAs for endogenous and transduced AID are similar. Taken together, the data in Fig 1 indicate that endogenous AID, AID-ER and AID-ER are expressed at similar levels in *aid*^{+/+} cells one day after transduction, and that AID-ER does not cause degradation of endogenous AID.

To determine whether AID-ER has a DN effect on CSR when expressed in WT mouse splenic B cells, we performed a dose-response assay to compare IgG1 and IgG3 CSR as a

function of GFP mean fluorescence intensity (MFI), which is an indicator of AID and AID expression levels. Increasing expression of full-length AID-ER results in increased IgG1 CSR (Fig 2A, C), as previously reported (48), whereas increasing doses of AID results in decreasing CSR to IgG1. CSR to IgG3 increases with increasing expression of AID in WT B cells, but decreases with increasing expression of AID (Fig 2B, D). These data demonstrate that AID has a DN effect on CSR in WT mouse B cells. The weaker DN effect we observe relative to the nearly complete inhibition of CSR found in most HIGM patients with the heterozygous C terminal AID deletion (5, 8) is likely due to the fact that in our experiments endogenous full-length AID is expressed prior to retroviral transduction. Endogenous AID is already highly expressed in mouse splenic B cells two days after treatment with switch inducers (20), which is the day of retroviral transduction and one day prior to harvesting.

Untagged AID has DN activity

To better compare with endogenous AID in HIGM patients, we tested whether an untagged AID expressed in the pMIG retrovirus would show DN function in RV transduction experiments. Fig 3A shows that CSR to IgG3 and to IgG1 is significantly lower in WT cells expressing AID than in cells expressing the empty pMIG, demonstrating the DN effect is not dependent upon the ER tag. In these experiments we did not add tamoxifen to the cultures, and we infected the cells one day after activation and harvested two days later. The increased duration of infection used in experiments with untagged AID (2 days instead of 1 day) was required to obtain the DN effect with these constructs, probably because unlike ER-tagged AID, the untagged AID appears to be poorly expressed, as reported previously for human AID (29, 39). This is consistent with the facts that AID lacks a nuclear export signal, and AID is degraded more rapidly in nuclei than in cytoplasm (15). We could not examine the expression of AID directly, as our AID antibody is directed against the C terminus. We tested the two commercially available antibodies that were reported to be against different epitopes in mouse AID, but, unlike our AID antibody, they did not detect mouse AID in our western blots. Again, we did not detect consistent differences in cell viability or recovery after infection with the different viruses, although the cells were less viable when harvested two days after infection (~70%) than when harvested after one day (~90%), independent of the particular AID expression construct. Although it has been speculated that the reason untagged AID poorly induces CSR is due to its low expression, the finding that both AID-ER and untagged AID poorly induce CSR, and both have DN effects on CSR in WT cells indicate that the phenotype of AID is not solely due to its low expression.

As untagged AID might be less stable than AID-ER, we asked if untagged AID might destabilize endogenous AID. As shown in Fig 3D, the levels of endogenous AID in WT cells expressing AID are not reduced relative to untransduced WT cells or cells transduced with pMIG, similar to the results obtained with AID-ER, and confirming that AID does not exert a DN effect by causing degradation of endogenous AID.

We used LM-PCR to determine if untagged AID induces DSBs in the S μ region in *aid*^{-/-} cells, and found that although AID induces S μ DSBs, there are many fewer than in cells expressing full-length AID (Fig 3B). This result differs from what we obtain in cells

transduced with AID-ER, in which the DSB frequency is similar to that observed in cells transduced with AID-ER (Fig 3C) and (25). This difference is consistent with the likely reduced expression of untagged AID relative to that of AID-ER. Note that LM-PCR only detects DSBs that are blunt, or if T4 DNA Polymerase is added prior to ligation, staggered DSBs can be detected. However, addition of T4 Pol does not change the relative frequency of DSBs induced by AID-ER or AID-ER (25). If AID induces extensively end-resected DSBs, as suggested by the results of Zahn et al (29), these DSBs would not be detected by this assay.

To determine if AID might affect $S\mu$ DSBs detected in WT cells, we performed LM-PCR experiments in WT cells transduced with untagged and tagged AID viruses, using T4 Pol to fill in staggered ends. In WT cells, we detect similar numbers of DSBs in cells expressing AID as in cells expressing full-length AID, whether AID is untagged (Fig 4A) or tagged (Fig 4B). As the intensity of the signals is slightly greater in cells expressing full-length AID, it is possible that these DSBs are slightly more numerous or more efficiently amplified. Similar results were obtained in the absence of T4 Pol (not depicted). Again, extensively end-resected DSBs would not be detected. As pointed out previously (25, 27), the role of the C terminus of AID appears to manifest itself subsequent to DSB formation.

ChIP assays show that AID binds $S\mu$ in WT cells

To further explore the mechanism of the DN effect, we asked whether AID-ER binds $S\mu$ in WT cells expressing endogenous AID, or whether endogenous AID might compete and thereby inhibit its binding. WT B cells transduced with ER were used as negative controls. As shown in Fig 5A, AID-ER is recruited at least as well as AID-ER to the $S\mu$ region in AID-sufficient cells, similar to results in *aid*^{-/-} cells (Fig 5B). Binding of AID and AID was not detected at the $C\mu$ gene in WT *oraid*^{-/-} cells.

AID lacking deaminase activity is not a DN mutant and does not bind $S\mu$ in WT cells

We previously showed that AID lacking deaminase activity due to mutations in the catalytic domain, AID^{H56R/E58Q} (AID^{RQ}) (10, 49), does not bind to $S\mu$ and $S\gamma3$ in our ChIP assays in *aid*^{-/-} cells (25) (see also Fig 5B). This result differs from that reported by Vuong et al (50) who found that AID^{RQ} expressed without a tag binds similarly as WT AID to $S\mu$, so we asked whether the difference was due to the ER tag. However, we found that untagged AID^{RQ} is also unable to bind $S\mu$ under our ChIP conditions (Fig 5C). There are several differences between the ChIP conditions in our lab compared to Vuong et al (50), and we do not know which ones are important. Taken together, the data from our lab and the Chaudhuri lab (50) suggest that AID^{RQ} binds $S\mu$ but less stably than WT AID.

We now asked whether AID^{RQ}-ER would have DN function by analyzing CSR in WT cells transduced with AID^{RQ} in comparison with ER and full-length AID^{RQ}, and AID. Fig 5D shows the combined CSR data, normalized to CSR in cells expressing ER alone. Again, CSR in cells expressing AID is significantly lower than in cells expressing ER. However, there is no DN effect observed in cells expressing AID^{RQ} or AID^{RQ}. This is consistent with our finding that AID^{RQ} or AID^{RQ} do not stably associate with $S\mu$ in WT cells (Fig 5A). As shown in the western blot in Fig 5E, the AID^{RQ}-ER proteins are

expressed at similar levels to AID-ER in nuclei, cytoplasm and in whole cell extracts, ruling out the possibility that AID^{RQ} does not have a DN effect due to lack of expression. These data suggest that the DN effect depends upon the ability of AID to bind S μ , and/or the ability of AID to deaminate dC and to induce S μ DSBs.

If AID^{RQ} and AID^{RQ} heterodimerize with endogenous AID, they might be expected to bind S μ in WT cells. Although AID and AID^{RQ} are detected at S μ in WT cells, neither AID^{RQ} nor AID^{RQ} bind (Fig 5A), suggesting that a functional deaminase domain must be present in both partners of the heterodimer for detectable binding to S μ , or that endogenous WT AID does not heterodimerize with catalytically inactive AID, or that AID functions as a monomer.

S μ -S α junctions show increased microhomology in cells expressing AID

Because the deaminase activity of AID is essential for S regions DNA breaks, we hypothesize that the DN function of AID depends upon its ability to induce S region breaks. Perhaps the breaks are aberrantly processed, and cannot participate in S-S recombination by NHEJ. They might interfere with normal S-S recombination induced by full-length AID. To begin to address this possibility, we examined the S-S junctions in *aid*^{-/-} cells expressing AID to determine if they showed increased amounts of microhomology as would be expected if they were not recombined by NHEJ, which is the predominant mechanism for recombining S-S junctions. We examined S μ -S α junctions because S α has the greatest amount of homology to S μ of any S region, thus increasing the sensitivity of the assay (51). Indeed, we found that S μ -S α junctions showed an average of 2.3-fold greater lengths of microhomology in *aid*^{-/-} cells expressing AID-ER compared to cells expressing AID-ER (Fig 6C), similar to observations in human HIGM patients expressing both C terminally deleted AID and full-length AID (35), and recent reports studying a human AID with a 17 amino acid C terminal deletion in mouse *aid*^{-/-} B cells (29, 52). We also examined junctions in cells expressing untagged AID, and found a 2.8-fold increase in microhomology lengths in comparison to cells expressing full-length untagged AID (Fig 6C). There were also significant differences in the distribution of microhomology lengths between cells expressing AID and AID, whether it was tagged or untagged (Fig 6A and B, respectively). Note that in cells expressing AID, we observed junctions with up to 15 and 27 bp of microhomology. These data suggest that the AID C terminus is involved in directing S region DSBs toward recombination by NHEJ, as suggested previously (29, 35, 52).

The DN effect of AID depends on the presence of Msh2

Msh2 and Msh6 are required for optimal CSR in B cells expressing full-length AID-ER but do not contribute to CSR in B cells expressing AID-ER (25). It is clear that at least one of the roles of MMR in CSR is to increase S region DSBs, most likely by converting distal SSBs to DSBs (17, 22–24). Furthermore, in the absence of the MMR proteins Mlh1 or Pms2, S-S junctions show increased microhomology (53–55), similar to the junctions in cells expressing AID. To determine if the DN effect depends on MMR, we expressed AID-ER and AID-ER in AID-sufficient *msh2*^{-/-} or *mlh1*^{-/-} B cells and analyzed CSR by flow cytometry. Fig 7A shows the gating strategy used in these experiments. Figs 7B,D provide

examples of the switching results in the GFP⁺ transduced cells, and Figs 7C,E present the compiled results. Demonstrating the DN effect, CSR is significantly lower in WT cells expressing AID than in cells expressing ER (Fig 7C,E). However, AID does not have a DN effect in *msh2*^{-/-} cells, as CSR is not significantly lower in AID-expressing *msh2*^{-/-} cells than in ER-expressing cells (Figs 7B,C). The levels of RV-AID proteins or endogenous AID in the transduced cells are not affected by lack of the MMR protein Msh2, as shown in Figs 1A, B. In *mlh1*^{-/-} cells the DN effect borders on significance (Fig 7E), perhaps due to the presence of Msh2-Msh6 in these cells. MMR is partially functional in the absence of Mlh1-Pms2 (17, 56). These results suggest that the DN effect of AID in AID-sufficient cells depends upon a function that Msh2 provides. At first glance, this seems surprising because we have previously shown that MMR does not contribute to CSR induced by AID, and because the AID C terminus is important for recruiting MMR proteins to S regions (25). However, endogenous AID can recruit MMR proteins in cells expressing AID, and thus it is possible that the ability of MMR to increase S region DSBs is important for the DN effect.

Discussion

AID has been found to function as a DN mutant in HIGM patients that retain one allele encoding WT AID (5), and here we address the mechanism of this DN effect. We show that the DN effect of AID requires Msh2, and that AID must have deaminase activity. The deaminase activity is required for binding of AID-ER, Msh2-Msh6, and UNG to S_μ in our CHIP assays (25)(and data herein). However, deaminase activity is also essential for AID to induce DNA breaks, suggesting that the DN effect might require the induction of DNA breaks. Consistent with this hypothesis, MMR proteins are important for creating DSBs during CSR (23).

We have shown that Msh2 and Msh6 are recruited to S_μ, dependent upon both the C terminus of AID and deaminase activity (25). Thus, it seemed surprising that the DN effect of AID depends upon Msh2, as AID poorly recruits Msh2 to S_μ. However, full-length endogenous AID would recruit Msh2-Msh6 in cells expressing AID, which could increase S region DSBs. Thus, the dependence of the DN effect upon Msh2 supports the hypothesis that DSBs are required for the DN effect. MMR is normally involved in post-replicative repair during S phase, whereas CSR occurs during G1 phase (23, 57), so it is possible that MMR must be specifically recruited in order to increase DSBs during CSR. As AID does not recruit UNG to S_μ as well as does full-length AID (25, 29, 52), it is also likely that

AID inefficiently induces SSBs. Thus, it is possible that in cells expressing haploid amounts of both AID and AID that DSBs might be even more dependent upon MMR than in cells expressing only full-length AID. MMR appears to help generate DSBs that can be recombined by NHEJ, or perhaps contributes to recruitment of NHEJ proteins, as S-S junctions in cells lacking Mlh1 or Pms2 show increased microhomology similar to those in cells lacking NHEJ proteins (53, 54, 58, 59).

It is clear that the DSBs induced in *aid*^{-/-} cells expressing AID cannot undergo efficient S-S recombination by NHEJ, as CSR is 90% reduced compared to cells expressing FL-AID, and the S-S junctional microhomology is increased. It is possible that AID, but not AID, recruits NHEJ proteins, which normally perform S-S recombination. Thus, in cells

expressing AID, DSBs might undergo extensive end-resection. We hypothesize that the reason the numbers of DSBs in S regions in both *aid*^{-/-} and WT cells expressing AID-ER appear similar in LM-PCR experiments to those in cells expressing AID-ER, is that, while DSBs are created less efficiently due to poor recruitment of UNG and MMR, those that are made are also repaired less efficiently due to poor recruitment of NHEJ. Also, AID has been reported to have higher deaminase activity than full-length AID (28, 29). To explain how the putative inability of AID to recruit NHEJ proteins could have a DN effect, it is possible that aberrantly resected DSBs predominate over breaks induced by endogenous full-length AID, due to their inability to recombine efficiently, and these DSBs interfere with recombination by NHEJ. Figure 8 presents an outline model of this hypothesis.

UNG and Msh2 do not appear to bind AID directly, and we only detect their interaction with AID by ChIP, dependent upon the C terminus of AID (25). The fact that deaminase activity is required for binding of Msh2 and UNG to S μ in our ChIP experiments (25) suggests that UNG and MutS α require their DNA substrates, dU and U:G mismatches, respectively, for binding to DNA. It is possible that NHEJ proteins might also be part of a complex that is recruited by AID, dependent upon the C terminus and deaminase activity. DNA-PKcs, a DNA-dependent kinase, is very important for NHEJ (60). It has been reported to bind to the AID C terminus, but only in the presence of DNA (34). Consistent with this, DNA-PKcs has been shown to ChIP with S μ in CH12 B lymphoma cells expressing full-length AID but not human AID (52). Also, KU70 has recently been shown to ChIP at S μ in mouse splenic B cells expressing human AID but not AID (29). Recent evidence indicates that APE1 and AID interact directly, dependent upon phosphorylation of AID at S38 (50). Thus, NHEJ proteins could be recruited as part of a complex that converts AID-induced dUs to DSBs and then forms S-S junctions.

If AID functions as a dimer, then AID lacking deaminase activity should be able to heterodimerize with endogenous WT AID, as the catalytic site is not located in regions thought to be involved in AID dimerization (11, 12) or in DNA binding (61). However, as do not detect binding of AID lacking deaminase activity (AID^{RQ}) to S regions in WT cells, one possible explanation for our results is that both subunits of the putative AID dimer must have deaminase activity in order for AID to bind detectably to S μ . If heterodimers of AID^{RQ} and AID cannot bind DNA, they should not interfere with the function of WT AID, as transduced AID is not expressed in excess over endogenous AID in our experiments. Alternatively, our results could be interpreted to indicate that AID functions as a monomer. Although several reports suggest that AID functions as a dimer or tetramer (5, 9, 62), another report indicated that purified AID functions preferentially as a monomer in vitro acting on single strand DNA and also on a small loop in dsDNA (63). Thus, our data suggest either that an AID- AID^{RQ} heterodimer does not form or if it forms, it cannot bind DNA, perhaps due to an inability to recruit UNG and/or MutS α , or alternatively, that AID functions as a monomer.

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Abbreviations

AID	Activation-induced cytidine deaminase
aa	Amino acid
anti-δdex	anti-IgD dextran
APE1/2	Apurinic/pyrimidinic endonuclease 1 and 2
ChIP	chromatin immunoprecipitation
ER	estrogen receptor
HIGM	Hyper-IgM
CSR	Class switch recombination
AID^{RQ}	AID ^{H56R/E58Q} (AID lacking deaminase activity)
DN	Dominant negative
Mismatch repair	MMR; proteins in this pathway are Msh2, Msh6, Mlh1, Pms2, Exonuclease I
MH	Microhomology
NHEJ	non-homologous end joining
RV	Retroviral
SHM	Somatic hypermutation
UNG	Uracil-N-glycosylase

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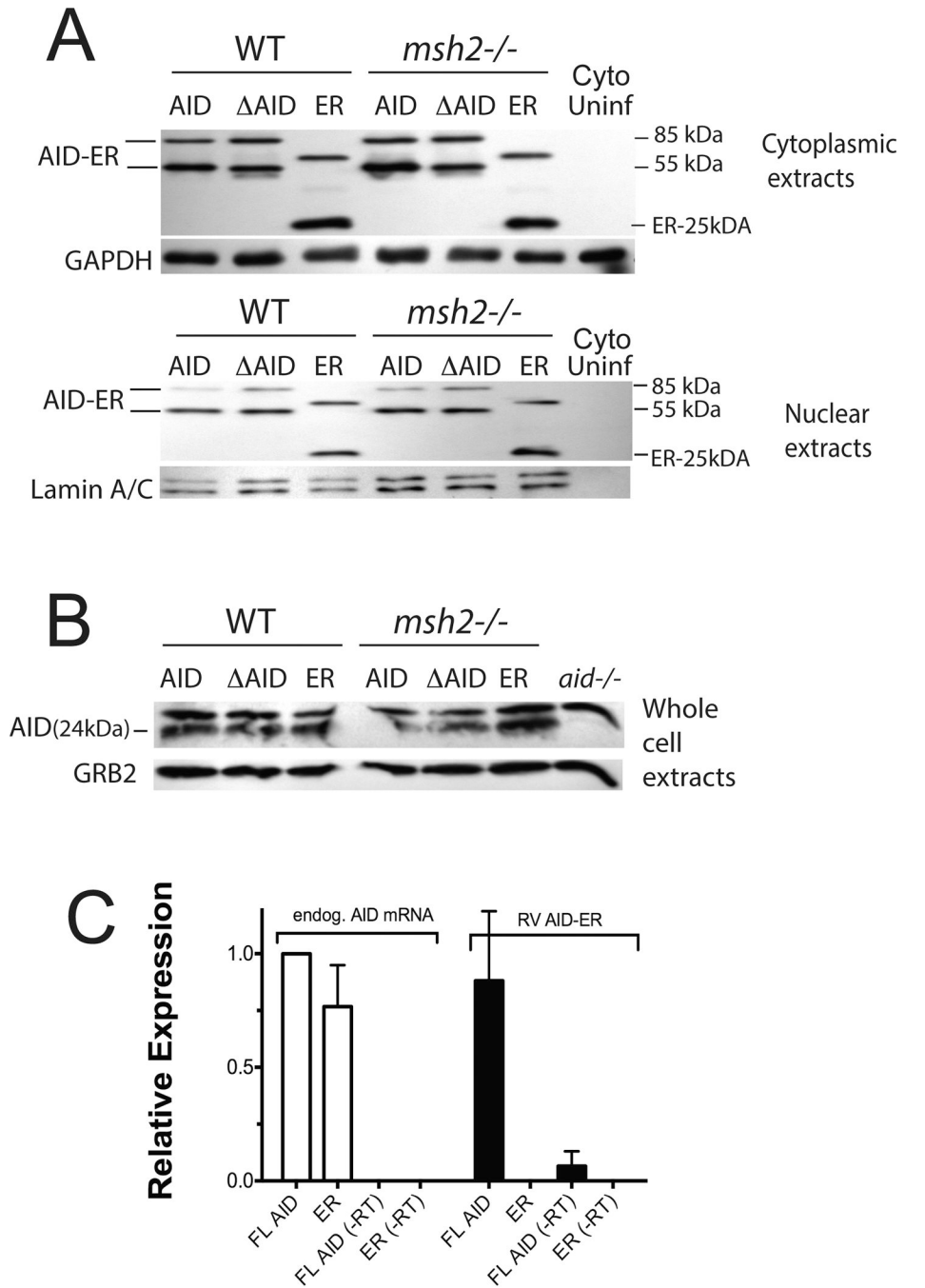


Figure 1. Quantitation of transduced AID-ER and endogenous AID levels in splenic B cells induced to switch to IgG3
 (A) Western blots of cytoplasmic and nuclear extracts (20 μg) from RV-infected splenic B cells one day after viral transduction were probed with anti-ER antibody, and with antibodies to GAPDH or Lamin A/C for loading controls. (B) Western blot of total cell extracts (75 μg) from the RV-infected or uninfected splenic B cells (from the same experiment shown in A) probed with antibody to mouse AID to detect endogenous AID. (C) qRT-PCR analysis shows that endogenous AID mRNA and AID mRNA transcribed from RV-AID-ER are present at similar levels in B cells activated to switch, one day after

transduction with RV-AID-ER or RV-ER. Results of 2 independent experiments (2 mice) are normalized to endogenous AID mRNA in RV-AID-ER transduced cells. (-RT) indicates samples to which reverse transcriptase was not added.

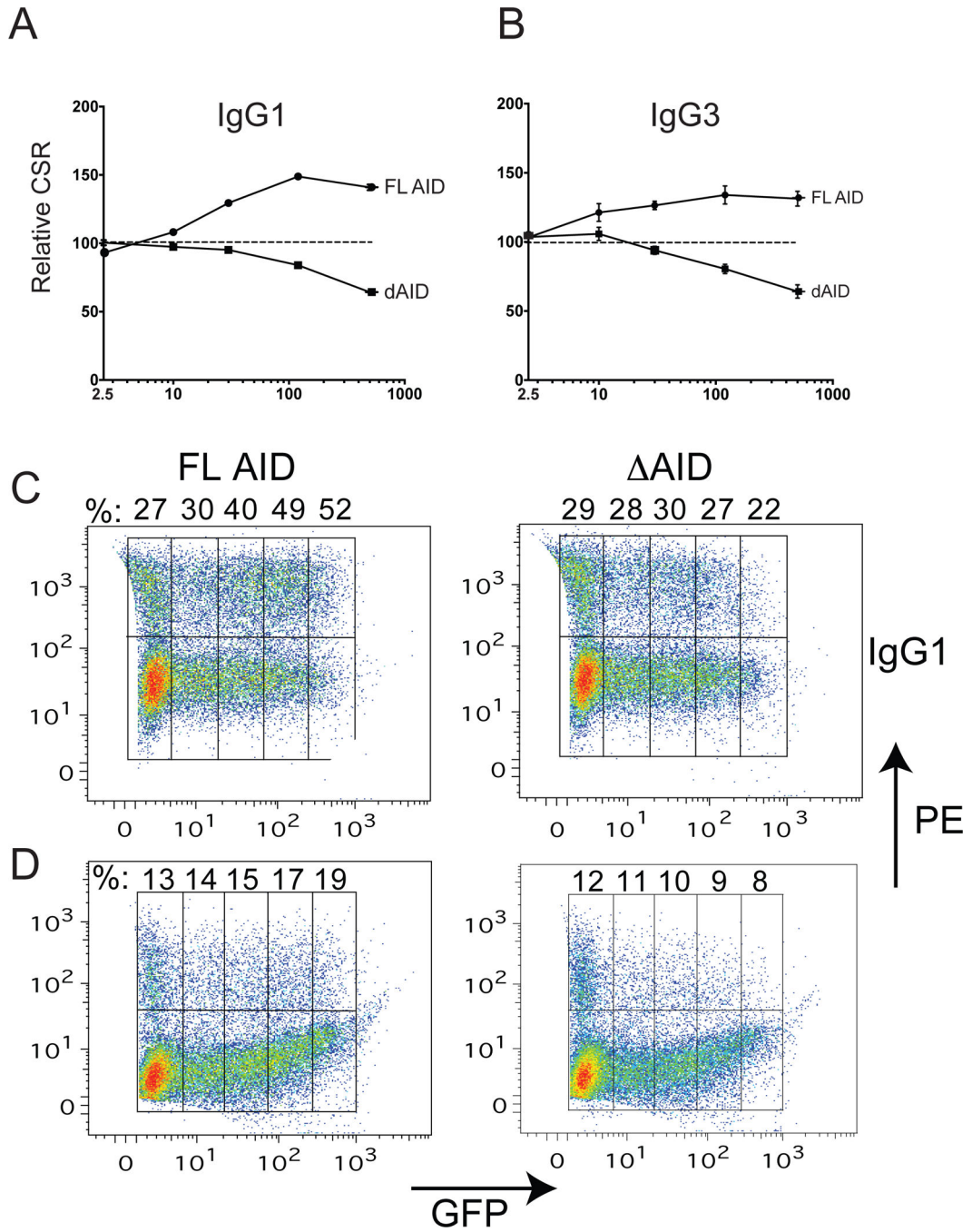


Figure 2. Dose-response assays of IgG1 (A, C) and IgG3 (B, D) switching in *aid*^{+/+} mouse splenic B cells expressing increasing levels of AID-ER or Δ AID-ER, as assayed by GFP expression (MFI) Each data point on the curves in A and B represent the average%IgG1 or %IgG3 (\pm SEM) at the indicated MFI (binned as shown in C and D) of 3 cultures from 1 mouse transduced with the indicated retrovirus relative to %CSR in cells transduced with the empty ER retrovirus. Data points on the Y-axis (MFI=2.5) in A and B represent CSR in GFP-negative cells, expressing only endogenous AID. CSR in the GFP-negative cells are shown in the left most boxes of the FACS dot plots in C and D. The procedure for gating is illustrated in Fig 7A.

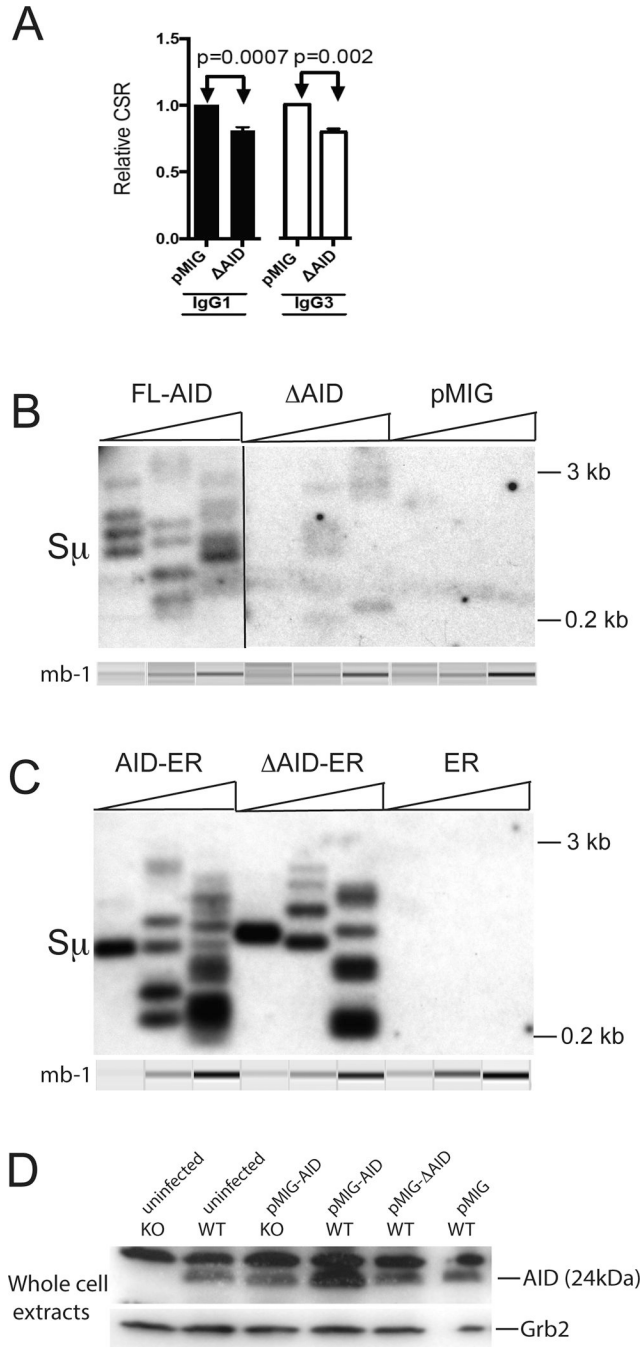
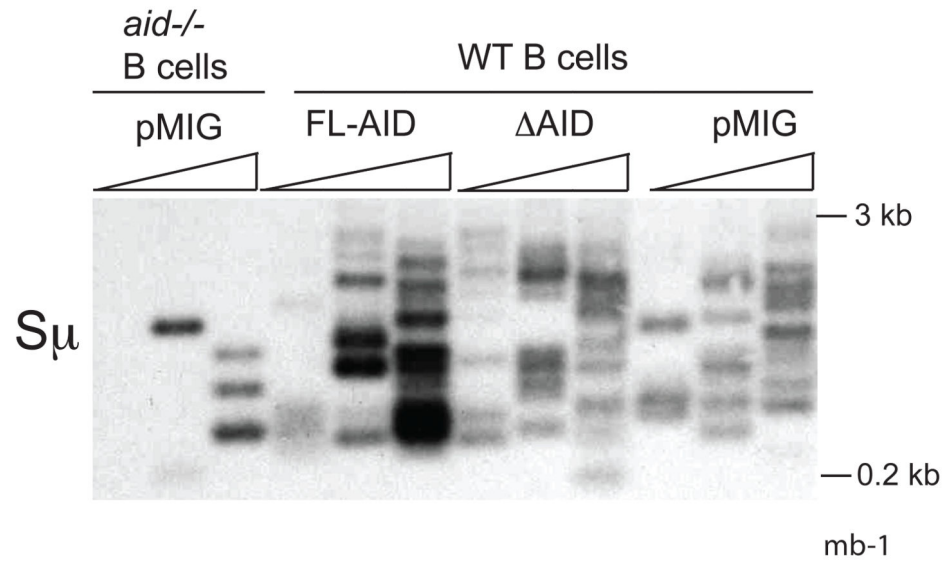


Figure 3. Untagged AID has DN activity, induces very few S μ DSBs, and does not cause degradation of endogenous AID

(A) Compilation of IgG1 and IgG3 CSR results (+SEM) for the untagged DAID in WT cells relative to CSR in cells expressing pMIG. The control retrovirus pMIG does not affect CSR significantly, as GFP-negative cells switched similarly to cells transduced with pMIG (not depicted). In these experiments, cells were activated to switch for 24 hrs, transduced and harvested 2 days later. The procedure for gating is illustrated in Fig 7A. In addition, we gated on the brightest GFP⁺ cells (~50% of the cells). Results from two independent experiments (2 mice) with two cultures each are shown. (B) LM-PCR assay of S μ DSBs in

aid^{-/-} cells induced to switch to IgG3 transduced with untagged AID, AID or the empty retrovirus pMIG. Transduction was performed on day 2 after activation, and cells harvested on day 3. The line indicates where the image was cut to remove irrelevant lanes. Three fold titrations of input DNA were performed, and the *mb-1* gene was amplified as an internal control for template input. The *mb-1* PCR bands shown were obtained by electrophoretic analysis on a QIAxcel Advanced instrument, which subjects each sample to electrophoresis in a capillary, and provides an image and quantitation of each lane. (C) LM-PCR assay of Sμ DSBs in *aid*^{-/-} cells induced to switch to IgG3 transduced with AID-ER, AID-ER or the control retrovirus ER. Methods similar to B. (D) Westerns blot of 80 μg of total cell extracts of *aid*^{-/-} and *aid*^{+/+} cells transduced with untagged AID and AID expressed in the retrovirus pMIG, and then probed with antibody to AID and to Grb2 for loading controls. Cells were cultured and transduced as in A, but similar results were observed if cells were cultured for 2 days prior to transduction, and harvested one day later.

A



B

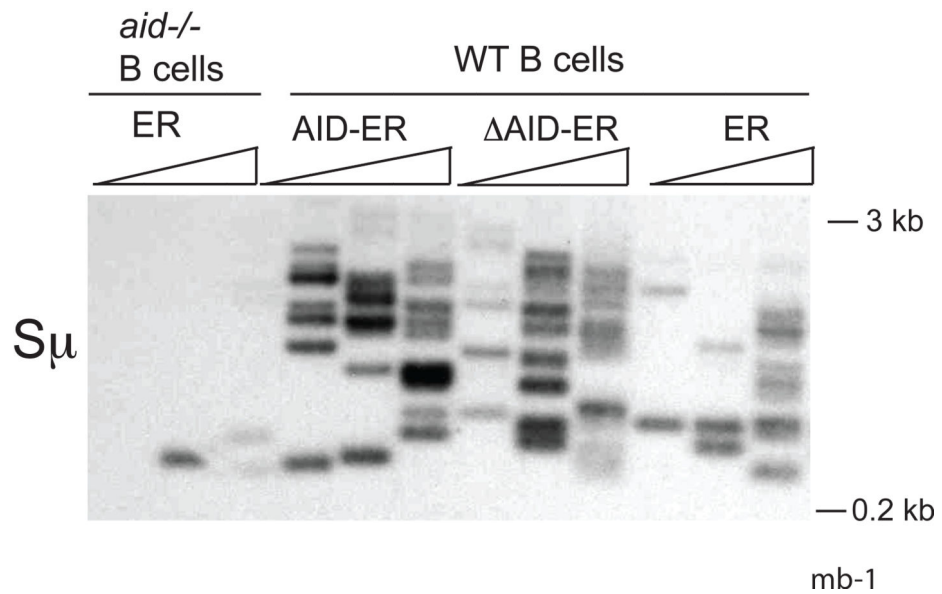


Figure 4. LM-PCR assays of S μ DSBs in WT (*aid*^{+/+}) or *aid*^{-/-} cells induced to switch to IgG3 transduced with pMIG expressing untagged AID constructs (A) or pMXpie expressing ER-tagged AID constructs (B)

Methods are described in Fig 3.

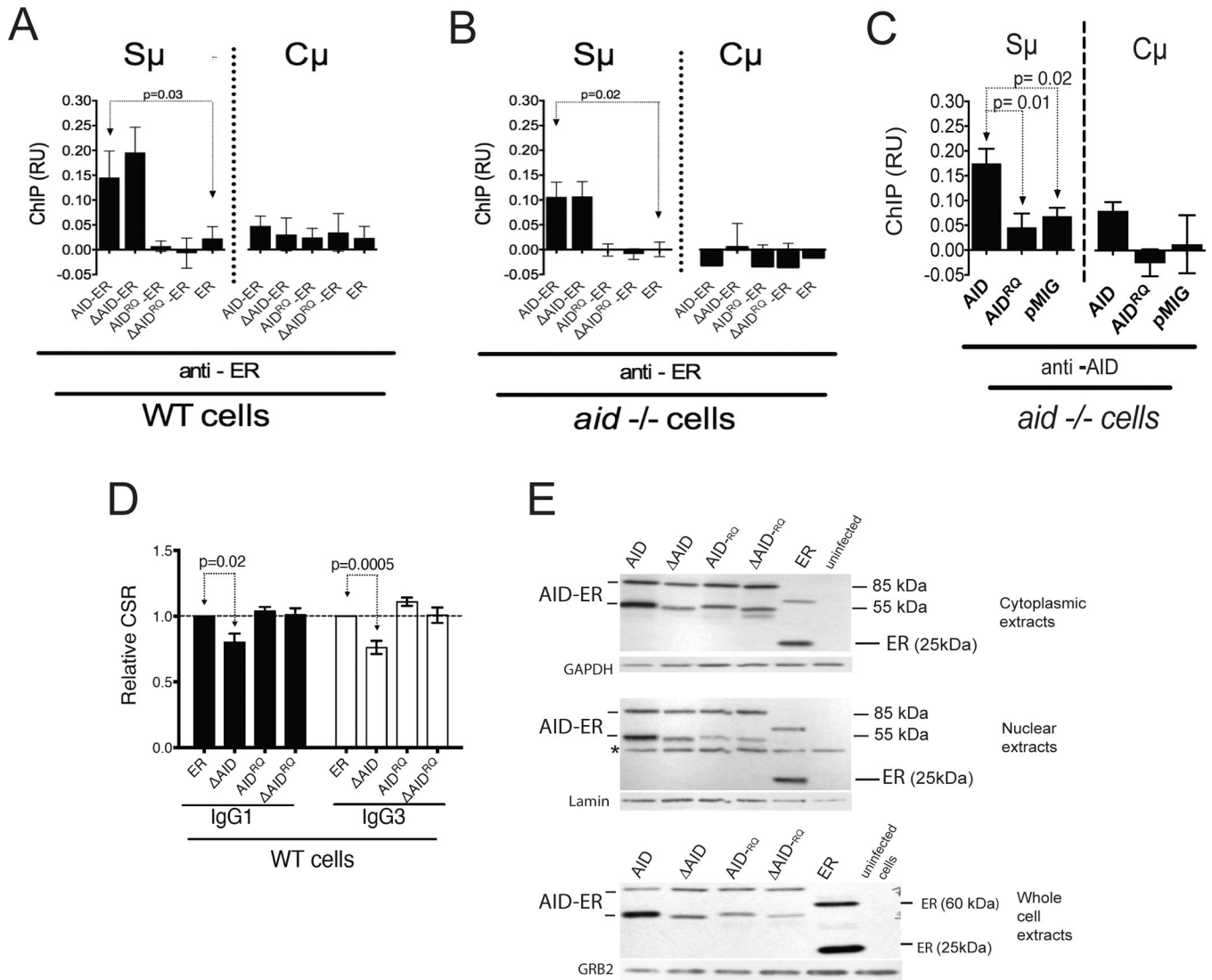


Figure 5. Both the DN effect and association of AID-ER with S μ depend upon deaminase activity of AID-ER

(A) ChIP of the AID-ER proteins at S μ and C μ in WT cells relative to input DNA, using anti-ER antibody. Error bars indicate SEM. 5 ChIPs (2 independent experiments, 2 mice) were performed, except for the no antibody control for FL-AID, where 4 were performed. ChIPs were analyzed by qPCR; % input was calculated, and % input in absence of antibody was subtracted. (B) ChIP of the AID-ER proteins at S μ and C μ in *aid*^{-/-} cells relative to input DNA. 6 ChIPs for AID, Δ AID, and ER and 3 ChIPs for AID^{RQ} and Δ AID^{RQ} were performed (2 mice). Analysis as in A. (C) ChIP experiments performed with *aid*^{-/-} cells transduced with untagged AID pMIG constructs, immunoprecipitated with anti-AID antibody (10). Error bars indicate SEM. 5–6 ChIPs (2 independent experiments, 2 mice) were performed. ChIPs were analyzed by qPCR; % input was calculated, and % input in absence of antibody was subtracted. P values determined by the two-tailed T test. (D) Compilation of IgG1 and IgG3 CSR results (+SEM) for the indicated RV-AID constructs in GFP-hi WT splenic B cells. Results are normalized to AID-ER results in one of the cultures

each for IgG1 and IgG3. Six cultures (3 mice, 2 cultures each) were performed. CSR is plotted for GFP-hi cells, which are ~50% of the total GFP+ cells. Cells transduced with ER switch similarly to GFP-negative cells in the same cultures (not depicted). (E) Western blots of AID-ER in nuclear, cytoplasmic, and whole cell extracts from transduced WT splenic B cells. 20 μ g protein was loaded in each lane. *Unknown irrelevant protein.

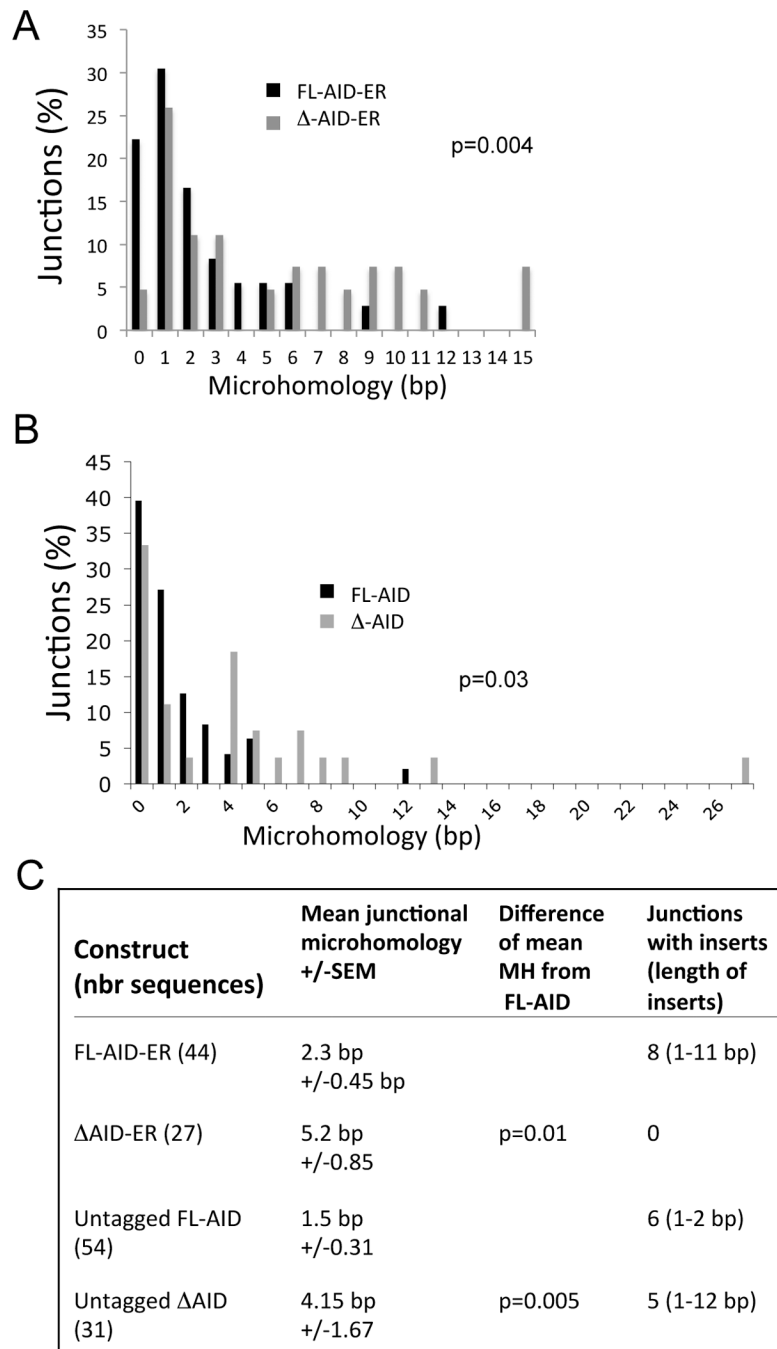


Figure 6. Microhomology (MH) at Sp-Sα junctions in *aid*^{-/-} cells transduced with AID-ER, AID-ER, and untagged AID and ΔAID

(A) Graph indicates the percent of junctions with the indicated lengths of microhomology cloned from *aid*^{-/-} cells transduced with AID-ER and ΔAID-ER retroviruses. Junctions with inserts are not included. (B) Same as A except junctions from cells transduced with untagged AID and ΔAID. The indicated p values show differences in distributions of MH between AID and ΔAID, determined by a Mann-Whitney 2-tailed test. (C) Compiled mean junctional MH +/- SEM for the ER tagged and untagged AID and ΔAID, as indicated. Sequences with inserts are not included in the calculations of MH.

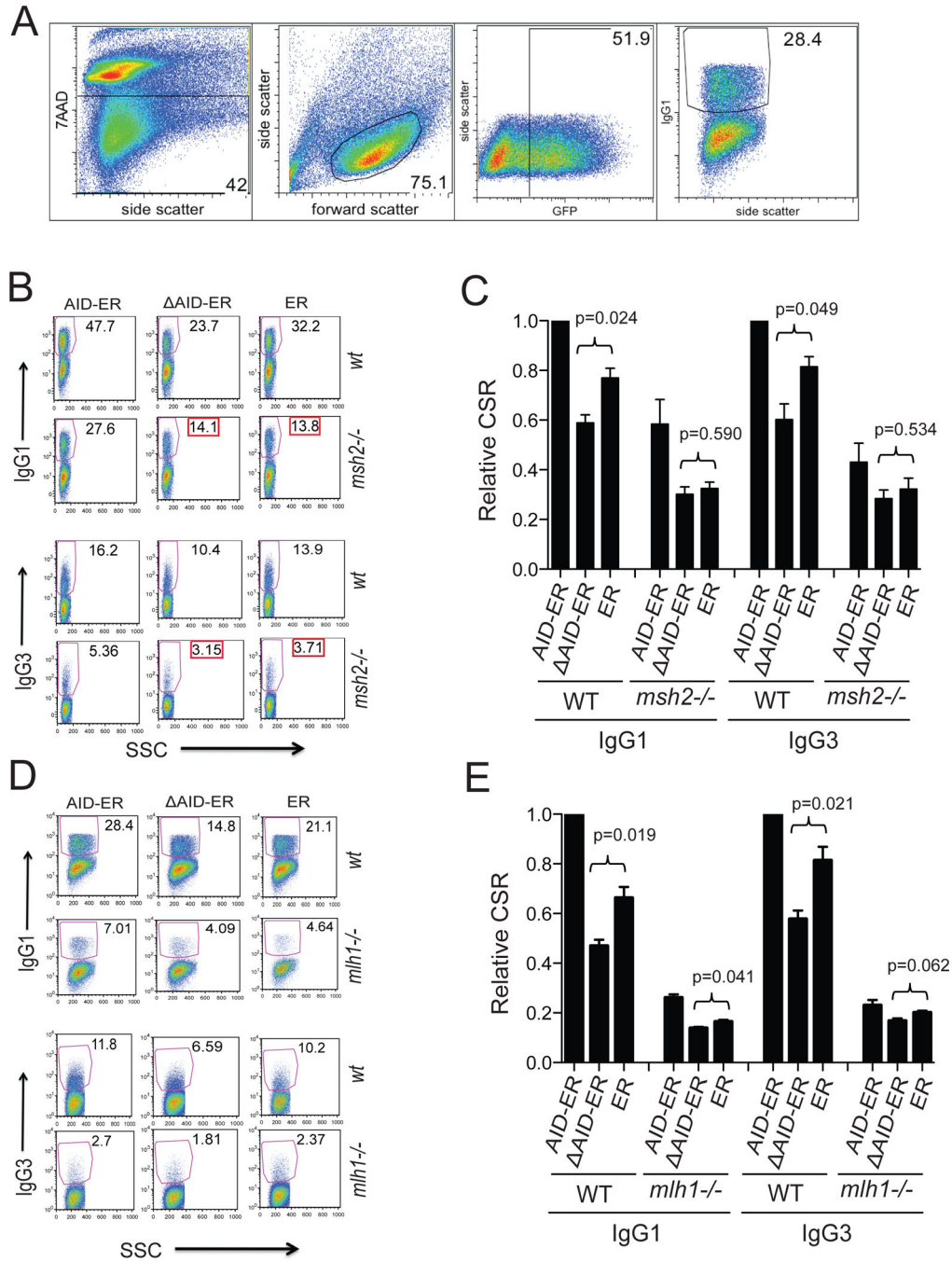
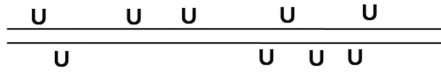


Figure 7. FACS analyses of CSR in WT, *msh2*^{-/-}, and *mlh1*^{-/-} cells demonstrates that the DN effect of AID-ER requires the presence of Msh2
 (A) Gating strategy used for all FACS experiments in this manuscript, proceeding from left to right. Example is from panel D: WT cells transduced with AID-ER transduced and induced to switch to IgG1. Retrovirally-infected cultured cells were stained with 7-AAD to detect dying/dead cells, and with antibody (Fab'2) to IgG1 or IgG3 conjugated to PE. The percent of viable (7AAD-neg), retrovirally-infected (GFP+) cells that were IgG1+ or IgG3+ was determined as shown. Compensation and gating were performed using FlowJo software (Treestar). (B) FACS results for one representative CSR experiment comparing the DN

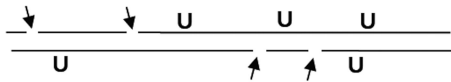
effect of AID-ER in WT and *msh2*^{-/-} B cells (expressing endogenous AID). The FACS plots show only viable and GFP⁺ cells; the gates represent PE-IgG1/PE-IgG3 positive cells within the GFP⁺ populations as indicated. The entire GFP⁺ population was analyzed in these experiments. Side scatter (SSC) is plotted on the X-axis. **(C)** Compilation of IgG1 and IgG3 CSR results (+SEM) for the indicated constructs in WT and *msh2*^{-/-} cells relative to CSR in WT cells expressing AID-ER. Two independent experiments (2 mice, 3 cultures each) were performed. IgG1 and IgG3 CSR are significantly increased in WT cells expressing AID-ER relative to ER (p=0.004 and 0.011, respectively). IgG1 and IgG3 CSR in *msh2*^{-/-} cells expressing AID-ER relative to ER were not significantly increased (p=0.065 and 0.284, respectively). **(D)** FACS results for one experiment comparing the DN effect of AID-ER in WT and *mlh1*^{-/-} B cells. **(E)** Compilation of IgG1 and IgG3 CSR results (+SEM) for the indicated constructs in WT and *mlh1*^{-/-} cells relative to CSR in WT cells expressing AID-ER. Three independent experiments (3 mice, 3 cultures each) were performed. IgG1 and IgG3 CSR were significantly increased in WT cells expressing AID-ER relative to ER (p=0.002 and 0.027, respectively). IgG1 CSR in *mlh1*^{-/-} cells expressing AID-ER relative to ER was significantly increased (p=0.004) but not IgG3 CSR (p=0.256).

A

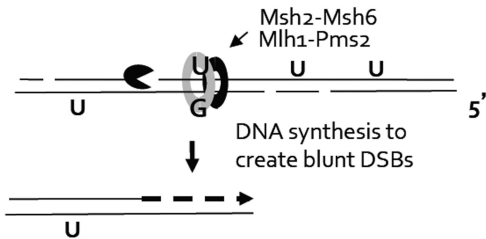
WT AID deaminates several dCs in S region



UNG, APE convert some of the dUs to SSBs



MMR recruited to help convert SSBs to DSBs;
Excision and displacement DNA synthesis

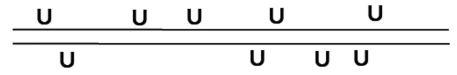


NHEJ proteins are recruited, perhaps as part of a complex; they bind DSBs and perform S-S recombination.

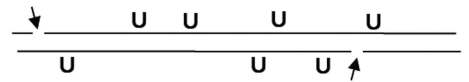


B

Δ AID deaminates several dCs in S region



UNG is poorly recruited, therefore few SSBs



MMR is poorly recruited, so fewer DSBs form

NHEJ proteins are not recruited, so S-S recombination is inefficient.

Unrepaired DSBs accumulate and are aberrantly processed/resected.

Accumulated DSBs with long ss tails attempt to use alternative end-joining for S-S recombination.



Figure 8. Outline model for role of the AID C terminus in the introduction of S region DSBs and S-S recombination