Activation-Induced Cytidine Deaminase does not impact on murine meiotic recombination

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Running title: **AID and meiotic recombination**

Keywords (5): AID; meiotic recombination; germline; cytidine deaminase; double-strand breaks.

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ABSTRACT

Activation-Induced Cytidine Deaminase (AID) was first described as the triggering enzyme of the B-cell-specific reactions that edit the immunoglobulin genes, namely somatic hypermutation, gene conversion and class switch recombination. Over the years, AID was also detected in cells other than lymphocytes, and it has been assigned additional roles in the innate defense against transforming retroviruses, in retrotransposition restriction and in DNA demethylation. Notably, AID expression was found in germline tissues, and in heterologous systems it can induce the double-strand breaks required for the initiation of meiotic recombination and proper gamete formation. However, since AID deficient mice are fully fertile, the molecule is not essential for meiosis. Thus, the remaining question that we addressed here is whether AID influences the frequency of meiotic recombination in mice. We measured the recombination events in the meiosis of male and female mice F1 hybrids of C57BL/6J and BALB/c, in Aidca+/+ and Aidca−/− background using a panel of SNPs that distinguishes C57BL/6J from BALB/c genome across the 19 autosomes. In agreement with the literature, we found that the frequency of recombination in the female germline was higher than in male germline, both in the Aidca+/+ and the Aidca−/− backgrounds. No statistical difference was found in the average recombination events between Aidca+/+ and Aidca−/− animals, either in females or males. In addition, the recombination frequency between SNPs flanking the IgH and Igκ loci was also not different. We conclude that AID has a minor impact, if any, on the overall frequency of meiotic recombination.
INTRODUCTION

Genomes can be diversified through the recombination of genetic material. One example is the meiotic recombination that occurs during the process in which a diploid cell gives rise to haploid gametes (Gerton and Hawley 2005). In meiosis, two consecutive divisions segregate first the homologous chromosomes and then the sister chromatids. The events leading to a crossover start in prophase I, with the pairing of the homologous chromosomes in leptotene, which progresses through zygotene and is completed in pachytene. Subsequently, synapsis between the chromosomal axes of the paired homologous chromosomes is achieved by the formation of the synaptonemal complex. The critical enzyme for meiotic recombination is a topoisomerase II-like enzyme, Spo11, which introduces double-strand breaks (DSBs) throughout the genomic DNA in the leptotene to zygotene transition (Keeney et al. 1999; Romanienko and Camerini-Otero 1999; Inagaki et al. 2010) and in mammals is also involved in the formation of the synapsis (Baudat et al. 2000; Romanienko and Camerini-Otero 2000). Meiotic DSBs are not distributed at random in the genome but rather concentrated in hotspots (Smagulova et al. 2011).

In vertebrates, additional examples of genome diversification through recombination are found in the antigen receptor loci of lymphocytes, and most notably in the immunoglobulin genes of B-cells. During V(D)J recombination in developing lymphocytes, the RAG1/RAG2 complex assembles different sequence modules to produce a rearranged immunoglobulin, thus creating cells with unique specificities (Tonegawa 1983). When activated, these cells can then change the constant region of the antibody and its effector function, through a reaction termed class switch recombination. The enzyme that triggers such recombination is Activation-Induced Cytidine Deaminase (AID or Aicda gene), a molecule that is also responsible for further editing of the rearranged immunoglobulin genes through somatic hypermutation and gene conversion (Muramatsu et al. 2000; Revy et al. 2000; Arakawa et al. 2002; Harris et al. 2002). The triggering
event in all AID-dependent reactions is the deamination of cytidines in the exposed single strands of DNA of the transcription bubble, which become uracils and will eventually lead to the fixation of point mutations (somatic hypermutation), the introduction of a germline-encoded patch of nucleotides (gene conversion) or to the generation of DSBs (class switch recombination) (Petersen-Mahrt et al. 2002).

While the physiological role of AID in the context of antibody formation and also its oncogenic potential have been thoroughly described, some reports suggested that the role of this enzyme is not confined to adaptive immunity (reviewed in Barreto and Magor 2011). AID transcripts were found in cells other than lymphocytes, most notably in mouse primordial germ cells and unfertilized oocytes (Morgan et al. 2004), and in testicular tissue from human biopsies (Schreck et al. 2006). It has been proposed that AID shares a role in retrotransposition restriction with other members of the AID/APOBEC family, but it has not been shown that AID plays such a role in the germline or early embryogenesis (MacDuff et al. 2009). It has also been observed that Aicda−/− mice have increased levels of methylated DNA in the primordial germ cells (Popp et al. 2010). This is consistent with the original idea of Morgan et al. that AID may function as a demethylase (Morgan et al. 2004) and with other reports on AID-dependent DNA demethylation (Rai et al. 2008; Bhutani et al. 2010; Guo et al. 2011), although 5-methylcytosine is a poor substrate for AID in vitro (Larijani et al. 2005; Wijesinghe and Bhagwat 2012). Finally, based on the observation that the expression of AID in S. pombe and C. elegans partially rescues the rec12Δ and Spo11 deficient phenotypes, respectively, it has been proposed that AID may contribute to meiotic recombination (Pauklin et al. 2009), but this hypothesis has not been tested in vivo.

Besides the rescue experiment suggesting that AID may introduce DSBs that lead to crossover events between homologous chromosomes, additional arguments strengthen the hypothesis that this cytidine deaminase could have a role in meiotic recombination: i) AID was shown to mutate
other regions of the genome besides the antigen receptor loci, making it a potential contributor to the overall frequency of meiotic recombination (Chiarle et al. 2011; Klein et al. 2011); ii) AID is more easily detected in oocytes than in the male germline (Morgan et al. 2004) and the AID-dependent demethylation in the primordial germ cells is stronger in females (Popp et al. 2010), which correlates with the known observation that meiotic recombination levels are higher in female mice than in males (Shifman et al. 2006); iii) the reactions involving AID and Spo11 share a number of features, from the H3k4me3 mark in the sequences where the DSB will be introduced to the subsequent recruitment of DNA repair pathways (Begum and Honjo 2012). However, given that meiotic recombination is an unavoidable step in gamete formation and that, unlike Spo11−/− mice (Romanienko and Camerini-Otero 2000), Aicda−/− mice are fully fertile, AID does not have a fundamental role in meiosis. Thus, the question that we explore here is whether AID functions as a modifier that influences the frequency of meiotic recombination in mice.
MATERIALS AND METHODS

Mice: *Aicda*<sup>-/-</sup> mice in C57BL/6J genetic background (B6.Aicda<sup>-/-</sup>, courtesy of Dr. Cristina Rada), *Aicda*<sup>-/-</sup> in BALB/c genetic background (BA.Aicda<sup>-/-</sup>, courtesy of Dr. Almudena Ramiro), F1 offspring and C57BL/6J (received from the Jackson Laboratory and bred in the Instituto Gulbenkian de Ciência (IGC) mouse facility) were housed and handled in accordance with the IGC ethical committee.

Meiotic recombination frequency detection and analysis

Sample selection: B6.Aicda<sup>-/-</sup> were crossed with BA.Aicda<sup>-/-</sup> to generate F1.Aicda<sup>-/-</sup> and F1.Aicda<sup>-/+</sup> littermates, confirmed by standard genotyping PCR. From these animals, 18 stable reciprocal matings were set, by crossing F1.Aicda<sup>-/-</sup> or F1.Aicda<sup>-/+</sup> with C57BL/6J (Supplementary Fig.S1 A). From the offspring of these reciprocal matings, tail biopsies were frozen for DNA extraction. Of these, 700 samples of DNA were extracted by isopropanol (Sigma, USA) precipitation and the origin and quality of DNA was confirmed by regular genotyping PCR. DNA from original mating pairs B6.Aicda<sup>-/-</sup> and BA.Aicda<sup>-/-</sup> were used as controls for Single Nucleotide Polymorphism (SNP) genotyping (B6.Aicda<sup>-/-</sup> has 98% of C57BL/6J genetic background (n=8); and BA.Aicda<sup>-/-</sup> has 97.4% of BALB/c genetic background (n=6)). After SNP genotyping, only samples with over 90% of detectable genotype signal (call) were considered for analysis.

SNP selection and genotyping: A panel of 148 Single Nucleotide Polymorphisms (SNPs) that distinguish between C57BL/6J and BALB/c genetic backgrounds was selected across the 19 autosomes to be evenly spaced with a physical distance of approximately 17 Mbp (based on the NCBI build36) between 2 consecutive SNPs on the same chromosome, using online tools (NCBI, MGI and Applied Biosystems), regardless of function (Supplementary Table S1, Supplementary Fig.S1 B). Multiplex SNP assays were designed using the Sequenom Platform Assay Design (SNP
primers were ordered from Metabion, Germany) and SNP genotyping was performed on the Sequenom™ massARRAY iPLEX platform (Sequenom, San Diego, CA) using multiplexed amplification followed by mass-spectrometric product separation.

From the initial panel, only SNPs with over 95% of detectable genotype signal (call) and SNPs with no distortion from the expected 50% inheritance of each allele on a 99% confidence interval were considered for analysis (Supplementary Table S1). 17 SNP/sample pairs passed the above filters but had no detectable genotype signal. To control for the effect of this absent data, we considered two extreme scenarios: recombination in all locations versus no recombination in all locations; and overall recombination frequencies were calculated for the two extreme scenarios (Supplementary Fig.S2). Given that the statistical differences were similar in both, the values considered for further analysis were the ones substituted in a conservative manner (no recombination in that location). After applying these filters, 130 SNPs were used to distinguish between C57BL/6J from BALB/c genome, across the 19 autosomes with an average distance of 18.57 Mbp between 2 consecutive SNPs on the same chromosome (range 6.93 Mbp – 74.98 Mbp) and a minimum resolution power of 34.62 Mbp (Table 1).

**Meiotic recombination frequencies:** Meiotic recombination events occurring during meiosis of F1.Aicda−/− or F1.Aicda+/+ were measured in the DNA of their offspring by crossing either F1 with C57BL/6J. In this way, for the expected signal per SNP genotype, one of the alleles always referred to the C57BL/6J parent and the other allele to the F1, which could either be of C57BL/6J or BALB/c origin. Thus, recombination events were defined as a change of SNP genotype signal from homozygous C57BL/6J to heterozygous or vice-versa between two consecutive SNPs on the same chromosome. Meiotic recombination frequencies were calculated using the genotyping results from 130 SNPs in 314 samples divided in four analysis groups corresponding to the offspring of the following matings: females F1.Aicda−/− with male C57BL/6J (group termed FKO;
n=79); females F1.Aicda+/+ with male C57BL/6J (group termed FWT; n=79); females C57BL/6J with males F1.Aicda+/− (group termed MKO; n=78) and females C57BL/6J with males F1.Aicda+/+ (group termed MWT; n=78).

For a more detailed approach, six randomly chosen SNP pairs, two SNP pairs (in chromosome 19 for female WT vs KO analysis) for which in the first analysis the statistical difference was relatively high but not significant, and one SNP pair (in chromosome 17 for male WT vs KO analysis) for which the statistical analysis indicated a significant difference were used for genotyping an increased sample size. Thus an additional 97 samples of DNA from FKO, 97 from FWT, 91 from MKO and 97 from MWT were used for SNP genotyping (Supplementary Table S2).

**VNTR sequencing:** To test meiotic recombination frequencies flanking the IgH locus in chromosome 12, recombination frequencies were calculated using genotyping results from SNPs approximately at position 108 Mbp and using PCR results from a Variable Number Tandem Repeat (VNTR) sequence that distinguishes C57Bl6/J from BALB/C genome approximately at position 116 Mbp, assayed for offspring of FWT (n=68); FKO (n=67); MWT (n=68) and MKO (n=67). For this, PCR standard procedures with primers D12Mit134 (DNA segment, Chr 12, Massachusetts Institute of Technology 134)_F: 5’-CTATCTACAACAACTTCTCCTGGG-3’ and D12Mit134_R: 5’-ACTCAGTCCAAACATATAACATGC-3’ were used.

**Sorting of subpopulations of cells according to DNA content in spermatogenesis**

**Sample preparation and staining:** Mouse testes were surgically removed from 8 to 12 weeks old B6.Aicda+/+ (n=3) and B6.Aicda+/− (n=3). For testicular cell suspension, as previously described (BASTOS et al. 2005), testes were meshed and seminiferous tubules were dissociated by collagenase I digestion (100U/ml; Invitrogen, USA) for 30 min at 32° in Solution-1 (HBSS (Invitrogen, USA) supplemented with 20 mM HEPES (pH 7.2), 1.2 mM MgSO₄.7H₂O, 1.3 mM CaCl₂.2H₂O, 6.6 mM sodium pyruvate, and 0.05% lactate). The cell suspension was then filtered
through a 70 µm nylon mesh, centrifuged for 10min at 1500rpm, 4º and the pellet was carefully ressuspended in Solution-2 (Solution-1 with L-glutamine (Invitrogen, USA), and 1% fetal bovine serum (FBS; PAA, Austria)) and prepared for sorting. From the same mice, epididymis and vas deferens were removed to a Petri dish with Solution-1. Using forceps, the epididymis and vas deferens were pressed and meshed to release mature sperm cells. Cells were then washed, ressuspended in Solution-2, counted and processed for RNA extraction. In order to discriminate and sort subpopulations of testicular sperm cells according to DNA content, Hoechst 33342 (Ho; Invitrogen, USA) - a vital dye that binds to DNA - was used as previously described (BASTOS et al. 2005). For the staining, 20 million cells were incubated with 5µg/ml Ho in 10ml of Solution-2 for 90mins at 32º. Cells were then put on ice for at least 5min, centrifuged for 15min at 1500 rpm, 4º and the pellet was carefully ressuspended in Solution-2 with Propidium Iodide (PI; 1µg/ml; Invitrogen, USA) to exclude dead cells.

**Analysis and sorting:** Analysis and sorting was performed on a MoFlo (Beckman Coulter, USA) equipped with a Coherent Innova I90C argon laser tuned to multiline UV (330-360nm, 60mW output) to excite Ho and a 488 nm Coherent Sapphire 488-200 CDRH laser (140 mW output) for Forward (FSC) and Side Scatter (SSC) as well as for PI excitation. A 505 nm long pass dichroic mirror (505DCXR, Chroma) was used to separate blue from red Ho fluorescence and to direct emitted light to different detectors. Ho blue was collected using a 440/20 nm band pass filter (Z440/20X, Chroma) and Ho red fluorescence with a 645 nm long pass filter (HQ645LP, Chroma). Fluorescence from PI was detected using a 670/40 nm band pass filter (D670/40, Chroma). Sorting was performed with a 70 µm nozzle at 414 kPa (60 psi) and with a ~96 kHz Drop Drive frequency. Purity of sorted populations was accessed by analysis of sorted populations by flow cytometry immediately after the sort and also by analysis of DNA content in fixed sorted cells by PI incorporation. Briefly, samples of different sperm cell populations were fixed with 80% ethanol and incubated with PI (30µg/ml) and RNAase (30µg/ml; Sigma, USA) in
phosphate buffered saline (PBS; Invitrogen, USA) to check DNA content (Supplementary Fig.S4A and B). Flow cytometric analysis was performed on a FACScan (Becton Dickinson). Analysis was done using FlowJo software (Tree Star Inc, USA). After sorting, testicular sperm cells 4N, 2N, N and also unsorted testicular sperm cells were immediately processed for RNA extraction.

**Isolation of oocytes:** B6.Aicda+/+ and B6.Aicda−/− females were given i.p. Pregnant Mare Serum Gonadotropin (PMSG; 5IU; Sigma, USA) and 48h later were given i.p. Human Chorionic Gonadotropin (HCG; 5IU; Sigma, USA) for super-ovulation. The next day oviducts were surgically removed, separated and placed in a drop of M2 (Sigma, USA). The ampullae (part of the oviduct) were tore using needles to release the super-ovulated oocyte structures that include the germ-cell surrounded by layers of granulosa cells. For “complete oocyte” samples, the oocytes with granulosa were briefly washed in M2 and mouth-pipetted to a different Petri dish with M2 and set aside for processing. For oocyte “granulosa free” samples, the oocytes with granulosa were incubated in M2 with hyaluronidase (150µg/ml; Sigma, USA) for 5mins at room temperature. Single oocytes were washed 3 times and set aside for processing and the remaining cells, considered “oocyte free” granulosa cells, were carefully pipetted to a different Petri dish with M2, washed 3 times and set aside for processing. As control, ovaries from the super-ovulated females (“empty” ovaries) and from non super-ovulated females were surgically removed, washed in M2 and meshed. All samples were immediately processed for RNA or protein extraction.

**Splenocytes isolation and culture:** As controls for expression of Aicda, B-cells from spleens of B6.Aicda+/+ and B6.Aicda−/− were stimulated in culture to upregulate Aicda expression. Briefly, homogenized single-cell splenocyte suspensions from 8 to 12 weeks old mice were depleted of red blood cells by lysis with ACK buffer (0.15M NH₄Cl, 10.0mM KHCO₃ and 0.1mM EDTA), washed 2 times, counted and 1.5x10⁶ cells/ml were plated in 24-well plates in complete RPMI
1640 with Glutamax (Invitrogen, USA) with 10%FBS penicillin and streptomycin (100mM, Invitrogen, USA), and 2-mercaptoethanol (50µM, Invitrogen, USA) supplemented with Lipopolysacharide 20mg/ml (LPS; Sigma, USA) and Interleukin-4 (IL-4 supernatant from hybridoma clone X63, in-house production). After 3 days in culture cells were washed, incubated for 5min in TriPure Isolation Reagent (Tryzol; Roche, USA) and stored at -80°C.

**Preparation of DNA for SNP genotyping and for regular PCR:** DNA was extracted from tail biopsies by proteinase K (Novagen, Germany) digestion and isopropanol (Sigma, USA) precipitation. Mice were genotyped for Aicda locus using standard PCR procedures with primers FR312 5’-CCTAGTGCCAAGGTGCAGT-3’ and FR313 5’-TCAGGCTGAGGTTAGGGTCC-3’ for the wild-type allele and primers FR310 5’-GGCCAGCTCATTCCTCCACT-3’ and FR311 5’-CACTGAGGTCACTGAGTCC-3’ for the knockout allele.

**RNA extraction, cDNA synthesis and real-time PCR:** All sorted and unsorted cell populations were further processed for RNA extraction using a commercial kit specific for small cell samples (Quick-RNA MicroPrep, Zymo Research, USA) or for cells stored in Trizol (Direct-zol RNA MiniPrep, Zymo Research, USA). Single stranded cDNA was produced using random primers and SuperScript II reverse transcriptase (Invitrogen, USA). cDNA from stimulated B-cells of B6.Aicda+/+ and B6.Aicda−/− were used as controls for the real-time PCR. Aicda mRNA was quantified using a SYBR green assay (Applied Biosystems). Beta-actin amplifications were used as normalization controls. The primers used were as follows: F_5-CCTAAGACTTTGAGGGA-3 and R_5-CAGGTAGCAGAGGTAGG-3 for Aicda and F_5-AGCTGTGCTATGTTGCTCTAGACTT-3 and R_5-CACACTTCATGATGGAATTGAATGTAG-3 for beta-actin. Quantitative PCRs were performed on 7900HT fast real-time PCR system (Applied Biosystems, USA) using the following cycling program: 2min at 50°, 10min at 90° and 45 cycles of 95° for 15sec and 60° for 1min. Expression
data of real-time PCR was averaged between triplicate replicas, calculated using Pfaffl ratio (Pfaﬄ 2001) and beta-actin as internal control.

Western Blot: Protein extraction was performed using NP40 Cell Lysis Buffer (Invitrogen, USA) supplemented with protease inhibitor cocktail (Complete, Roche, USA). Samples were run on a 4-12% SDS-PAGE gel using the NuPAGE system (Life technologies, USA) and semi-dry transferred to Imobilon-P membrane (Millipore). Membranes were blocked in 5% Bovine Serum Albumin (BSA, Sigma, USA) in Tris-Buffered Saline, 0.05% Tween-20 (TBS-T), stained with rabbit anti-AID serum (1:500 dilution, courtesy of Dr. Kevin McBride) and α-tubulin was used as loading control (anti-α-tubulin, 1:5000 dilution, Sigma, USA). Signal was developed using Horseradish Peroxidase conjugated antibodies (1:5000 dilution, SantaCruz Biotech, USA) and Super-Signal West Pico Chemiluminescent Substrate Kit per manufacturer instructions (Thermo Scientific, USA).

Statistical analysis: For the analysis of frequency of meiotic recombination between different groups, a Mann-Whitney test was used and statistical significance threshold is referred for each case. For the analysis of the proportion of recombination events for each SNP pair, the confidence intervals were calculated using the Agresti-Coull method and differences between wild type and knockout data were tested using a Fisher exact test. Standard Bonferroni correction on the p values was applied for multiple testing. All statistic tests were performed using GraphPad Prism (v5.00 Windows, GraphPad Software, San Diego California USA).
RESULTS

AID does not affect global levels of recombination

The documented expression of AID in gametes, reproductive organs and pluripotent tissues, (Morgan et al. 2004; Schreck et al. 2006; MacDuff et al. 2009; Bhutani et al. 2010), its activity in primordial germ cells (Popp et al. 2010), and the observation that the heterologous expression of AID can partially rescue meiotic recombination in *S. pombe* and *C. Elegans* (Paekin et al. 2009) raised the question of whether normal levels of endogenous AID can influence the frequency of meiotic recombination in mice. To test this, recombination frequencies in the germline of male or female *Aicda*+/+ or *Aicda*−/− (C57BL/6JxBALB/c)F1 genetic background were inferred by SNP genotyping of genomic DNA from the offspring of reciprocal matings of F1.Aicda+/+ or F1.Aicda−/− with C57BL/6J (Supplementary Fig.S1 A). For the expected signal per SNP genotype, one of the alleles referred to the C57BL/6J parent and the other allele to the F1, which could either be of C57BL/6J or BALB/c origin. Recombination events were then defined as a change of genotype signal (from homozygous to heterozygous or vice-versa) between two consecutive SNPs on the same chromosome. We found a total of 3629 recombinations across the 19 autosomes of the 314 animals included in the four analysis groups. Since the detection of virtually all crossovers would require a 10 Mbp-resolution (Paagen et al. 2008) and our analysis has a 34 Mbp-resolution, we first compared the number of recombination events calculated for F1.Aicda+/+ with the one described in larger studies. The estimated total number of sex-averaged recombination events per meiosis according to data from Shifman et al. (2006) and Paagen et al. (2008) is 13,9 and we observe 11,5 sex-averaged recombination events per meiosis. Thus, a small collection of SNPs is sufficient to detect around 83% of all recombination events, most likely due to the positive crossover interference in the murine meiosis (Broman et al. 2002). The comparison of the frequencies of recombination per chromosome we observed with those from a larger study...
(Shifman et al. 2006) shows that we are detecting the vast majority of recombination events in some chromosomes (Supplementary Table S3), which is confirmed by a similar distribution of the number of recombination events in chromosome 1 \((p=0.88\) for females; \(p=0.13\) for males by \(\chi^2\)) when we compare our data with data from Paigen et al. (2008). We then asked whether detecting the majority of crossover events is sufficient to capture a number of general features of meiotic recombination, such as the differences due to the gender of the transmitting parent, chromosomal location and the physical size of the chromosome (Jensen-Seaman et al. 2004). The average number of recombination events in all of the 19 autosomes, averaged per mouse, showed that the frequency of recombination in the female germline was higher than in male germline \((12.29\pm0.35\) for female and \(10.63\pm0.27\) for male, \(p=0.0007\), Fig. 1A, Table 2), which is consistent with the literature (Jensen-Seaman et al. 2004; Lynn et al. 2005; Shifman et al. 2006) and it is also observed for the F1.Aicda\(^{-/}\) study groups \((12.29\pm0.30\) for female and \(11.00\pm0.34\) for male, \(p=0.0008\)). We then compared recombination frequencies according to position in the chromosome, and, although the differences are not statistically significant, we found the expected trends towards higher recombination frequencies in the male germline near the telomeres and the inverse for the recombination frequencies near the centromeres (Fig. 1B) (Shifman et al. 2006). Finally, when we pooled the female and male recombination frequencies between chromosome 1 \((\text{size 197Mbp})\) and 19 \((\text{size 61Mbp})\) we found at least a two-fold increase in the smallest chromosome compared to the biggest, an inverse correlation between chromosomal size and average recombination frequencies in the mouse genome that is well-documented (Shifman et al. 2006), (Fig. 1C). Overall, these observations indicate that, although we miss 17% of the recombination events reported in larger studies, detecting the majority of the crossovers is sufficient to capture global features of meiotic recombination.

We then compared the overall recombination frequency between Aicda\(^{-/}\) and Aicda\(^{+/+}\) and found no statistical difference in the female \((p=0.6155)\) or male \((p=0.8192)\) groups (Fig. 1A). The same
analysis including only chromosomes for which we detect the vast majority of recombination events, leads to loss of statistical significance in the female versus male comparison and no difference in the recombination frequency of Aicda−/− (data not shown). In conclusion, our results show that AID does not have a major impact on the global frequency of meiotic recombination.

**Recombination frequencies per chromosome and SNP pair**

The recombination frequencies were analyzed per chromosome and plotted in an (x,y) scatter plot for comparison, between Aicda+/+ versus Aicda−/− in female and male groups (Supplementary Fig. S3). A bisector line (represented as a dashed line) corresponds to equal recombination frequencies between the FWT and FKO or MWT and MKO analysis groups and deviations from this line, even if not significant, were observed for females in Chromosomes 12, 13 and 16 and for males in Chromosome 17. To further analyze this, recombination frequencies were compared per SNP pair (Fig.2) for the analysis groups of both female and male parents, Aicda+/+ versus Aicda−/−, and no statistical significant differences were found, except for the comparison between MWT vs MKO in Chromosome 17 (SNP pair in approximate position 68 Mbp and 86 Mbp), where there is a statistically significant increase in the recombination frequency in MKO compared to MWT. This SNP pair, two SNP pairs (in chromosome 19 for female WT vs KO analysis) for which in the first analysis the statistical difference was relatively high but not significant, and six randomly chosen SNP pairs, were then used to genotype an additional set of samples. For the SNP pairs in chromosomes 17 and 19 (as for the other repeated SNP genotyping), results show that increasing the sample size does not lead to statistically significant differences and that the statistically significant difference from the previous set of samples is now lost (Supplementary Table S2). Finally, as SNP pairs with low number of recombination events are less likely to include two recombination events in the same region than SNP pairs with high number of recombination events, which would not have been quantified in our system, we repeated the
overall analysis using only SNP pairs with proportions of recombination events below a cut off of 10% or 5%, but this did not reveal any increase in the frequency of meiotic recombination in Aicda<sup>+/+</sup> compared to Aicda<sup>−/−</sup> (data not shown).

**Recombination frequencies of SNPs flanking Immunoglobulin (Ig) genes:** We looked for the average recombination frequencies of the natural AID targets. AID-dependent mutations are found in the Ig loci at a frequency at least two orders of magnitude higher than in the remainder of the genome. In the IgH locus, which is the most complex of the antigen receptor loci, as it is a target for both somatic hypermutation and class switch recombination, recombination frequencies were calculated using genotyping results from SNPs approximately at position 108 Mb and using PCR results from a Variable Number Tandem Repeat (VNTR) sequence that distinguishes C57Bl6/J from BALB/c genome approximately at position 116 Mb and no differences in recombination frequencies were found (Fig. 3). For the Igκ (in Chromosome 6), using flanking SNPs, no difference in recombination frequencies was detected. Other putative targets of AID mutagenic potential such as c-myc (chromosome 15) were also included in the analysis and no difference in recombination frequencies was found (Fig 2).

**Aicda expression in murine germline cells**

Since we found no interference of AID activity in meiotic recombination, we decided to readdress whether Aicda transcripts can be detected in the germline. Morgan et al. reported no Aicda expression in mouse testes but high expression in ovaries and even higher in unfertilized oocytes (Morgan et al. 2004), however another study suggests that the expression of an Aicda reporter is observed only in somatic cells surrounding the oocyte (Qin et al. 2011) and Schreck et al. reported expression in testis tissue from human biopsies (Schreck et al. 2006). These discrepancies may result from the methods used and/or reflect species differences between
mouse and humans (Morgan et al. 2004; Schreck et al. 2006; Qin et al. 2011). We focused on the expression of Aicda in sorted populations of sperm cells, including the cells undergoing meiosis, and in isolated (post-meiotic) oocytes from adults and additional cells from the ovary.

As a consequence of ongoing spermatogenesis, the germinal population in the testis is highly heterogeneous with premeiotic, meiotic and haploid postmeiotic cells and using the whole organ for expression studies may not reflect the true Aicda expression in homogeneous populations of cells at different stages of differentiation. Thus, testicular sperm cells were sorted based on DNA content (4N, 2N and N) and Aicda transcripts were quantified by real-time PCR (Fig. 4A; Supplementary Fig. S4 A and B). In the haploid (N) or diploid (2N) cell populations, these being the most prevalent in testis tissue, no Aicda expression was detected. In addition, no Aicda expression was detectable in non-sorted total testis tissue and in maturing sperm removed from the epididymis. In the 4N cell population, which is enriched in gametocytes that are in prophase I of meiosis, after chromosome duplication, Aicda expression was detectable, but at vestigial levels when compared to Aicda expression in activated B-cells (Fig. 4A).

For female gametes, we did not look at the stage and tissues during embryonic development when meiosis takes place, but simply investigated whether AID is expressed in the germline of adult mice. Oocytes were manually separated from the granulosa cells upon hyaluronidase digestion, to discriminate populations. Aicda expression was then tested in isolated oocytes, granulosa cells (“oocyte free”), oocytes with granulosa (follicles) and, as controls, whole ovaries post-super-ovulation (“empty”) and whole ovaries from animals that did not undergo super-ovulation. In isolated oocytes, Aicda expression was similar to activated B-cells and in the samples of “complete oocytes” and granulosa only (oocytes free) Aicda expression was around four-fold that of the activated B-cells; the lower proportion of RNA coming from oocytes compared to that of granulosa in the combined sample probably explain the combined sample
not having a lower expression of *Aicda* than the granulosa alone (Fig. 4B). The transcript in whole ovaries was almost undetectable both in super-ovulated and non-super-ovulated samples. The effect of the hormones used to induce super-ovulation on the expression of AID was also tested under different activation conditions of splenic B-cells from super-ovulated and non-super-ovulated animals, and under the absence or presence of PMSG and HCG in the culture medium. Super-ovulation did not prime B-cells for higher *Aicda* expression upon activation and the effect of the hormones in culture was not always consistent, although we cannot exclude that certain conditions may enhance *Aicda* by two-fold (Supplementary Fig. S5). In any case, levels of *Aicda* transcripts are higher in the granulosa cells than in oocytes and, although in oocytes the transcript levels are similar to those of activated B-cells, the protein was only detected in the granulosa (Fig. 4C).
DISCUSSION

Here we show that AID does not have a major impact in the frequency of meiotic recombination in mice, despite being detected in the germline (Morgan et al. 2004; Schreck et al. 2006) and prior observations that, when ectopically expressed in heterologous systems, this molecule produces DNA lesions that partially compensate for the absence of the natural DSB inducer in meiosis, Spo11 (in C. elegans) or Rec12 (S. pombe) (Pauklin et al. 2009). Our sample of genotyped animals and the collection of SNPs used are of modest size, however the results for Aicda+/+ capture a number of essential features of a larger dataset (Jensen-Seaman et al. 2004; Shifman et al. 2006) : i) we detect the sex-specific differences in the frequency of meiotic recombination; ii) we find an inverse relation between chromosome size and frequency of recombination iii) we reproduce the finding that recombination rates are higher near the centromeres for females and towards the telomeres for males. Thus, although we cannot exclude that the inclusion of more animals and/or more SNPs in the analysis could reveal a role for AID in meiotic recombination, based on our data we estimate that its contribution, if any, is small. In addition, we cannot formally exclude that increasing the number of SNPs would not bring to view regions where AID has an impact, but it is worth noticing that when we increased the size of the sample for the pairs of SNPs that had the most pronounced differences, the statistical significance dropped. Finally, our data clearly show that AID does not determine the sex-specific differences in the recombination frequency. Although a number of causes for this difference were already proposed (e.g. Lercher and Hurst 2003; Lenormand and Dutheil 2005; Petkov et al. 2007), an additional role for AID was a possibility, given the differences in the AID expression levels detected in the male and female gametes, which are consistent with a slightly more pronounced increase in the methylation levels of the genome from primordial germ cells in Aicda−/− females than in the males (Popp et al. 2010).
At first glance, arguing for a role of AID in the germline is counterintuitive, as this molecule is a potent mutator of immunoglobulin genes in the context of the immune response. However, error-prone repair of the AID-induced lesions seems to be characteristic of these target genes, as the bulk of the off-target lesions are faithfully removed by the combined action of mismatch and base excision repair (Liu et al. 2008). How this is achieved is not fully understood, but it is reasonable to assume that, in the germline, high-fidelity repair would deal with most of AID-induced lesions. Interestingly, homologous recombination, the repair pathway involved in meiotic crossover, is also actively protecting the genome of lymphocytes against the activity of AID (Hasham et al. 2010). Furthermore, the AID-dependent demethylation observed in murine primordial germ cells argues for an activity of the molecule in the germline (Popp et al. 2010). Thus, knowing from the work of Pauklin et al. (Pauklin et al. 2009) that the AID-induced lesions can be intermediates in the reaction leading to crossovers, why is AID not making a sizable contribution to meiotic recombination in mice? The expression level of the molecule during meiosis and the availability of the target sequences are the most trivial explanations.

Physiological levels of AID in the germline may not be sufficient to drive detectable meiotic recombination. It is not known whether AID is expressed in cells undergoing meiosis in female mice, but we have sorted different sperm cell populations and we were unable to detect AID expression. The only signal we found was in primary spermatocytes, which is consistent with what has been reported for the human testis (Schreck et al. 2006), but the levels we detected by real-time PCR are extremely low when compared to the expression levels in activated B-cells.

In addition, it is possible that the cell-cycle regulation of AID activity and AID’s specific requirement for transcription limit the impact of this molecule in meiotic recombination. AID-induced DSBs and Nbs1/gamma-H2AX foci in the switch regions of the IgH locus are observed in the G1 phase of cells undergoing class switch recombination (Petersen et al. 2001; Schrader et
al. 2007), similarly to point mutations in the V region (FAILI et al. 2002). Thus, AID lesions are introduced and repaired in G1, although for the lesions that evade the G1/S checkpoint, repair occurs in S phase (HASHAM et al. 2012). In any case, there are no reports of AID-induced lesions in G2 or during mitosis and, unless the cell-cycle regulation of AID in the germline is peculiar, probably AID is not active in prophase I, when recombination takes place. Furthermore, it is known that AID only acts on single stranded DNA, which can be exposed during transcription (CHAUDHURI et al. 2003), but transcription is low in the early stages of the first meiotic prophase (PAGE et al. 2012). This does not imply that AID could not have access to target sequences exposed as single-stranded DNA in some transcription-independent manner, it simply suggests that the level of potential targets at that particular stage is probably lower than in a G1 cell.

Investigating whether AID-dependent crossovers can occur with the low frequency of the off-target AID-dependent DSBs found in activated B-cells by high throughput methods (CHIARLE et al. 2011; KLEIN et al. 2011) does not seem fruitful. Thus, we conclude that AID has no sizable contribution for meiotic recombination. This finding shows that Spo11 and AID have non-overlapping functions, since Spo11−/− mice also show normal immunoglobulin somatic hypermutation and class switch (KLEIN et al. 2002).

In agreement with a study using a lineage tracer reporter (QIN et al. 2011), we detected for the first time that the AID protein is expressed in the granulosa cells but not in the oocyte, in spite of being highly transcribed in both cell types. These findings suggest the possibility of two non-essential and largely unaddressed roles played by AID. First, AID transcripts may be stored in the oocyte for translation after fertilization, which could associate this gene to a maternal-effect. Second, given the intercellular communication between these cell types, the allocation of the AID protein expression to the surrounding granulosa cells may allow the enclosed oocyte to be
protected against exogenous DNA and endogenous retroelements without being exposed to the direct mutagenic effects of AID.

ACKNOWLEDGEMENTS

The authors would like to thank J. Carneiro, C.P. Gonçalves and R. Masilamani for comments on the manuscript, R.Gardner, T. Lopes and J.Bom for their contribution to the cell sorting, J. Costa for the SNP genotyping, P. Almeida for help with data analysis, M. Rebelo and F. Marta for animal handling. This work was funded by a Marie Curie Reintegration Grant (PIRG03-GA-2008-230967). CSC is funded by the FCT (SFRH/BPD/66824/2009).
REFERENCES


Petersen-Mahrt S. K., Harris R. S., Neuberger M. S., 2002  AID mutates E. coli suggesting a DNA


Figure Legends:

**Figure 1.** (A) Recombination events for all studied groups. The numbers of recombination events were calculated for all 19 autosomes per offspring of females F1.Aicda+/+ (FWT, n=79); females F1. Aicda−/− (FKO, n=79); males F1. Aicda+/+ (MWT, n=78) and males F1. Aicda−/− (MKO, n=78). Each dot represents a sample, lines represent Mean±SEM. A Mann-Whitney test was used to compare sample groups using a Bonferroni correction for multiple testing, *p < 0.0125; n.s. is non-significant. (B) Average recombination events for centromeric and telomeric regions. For these analyses, only the SNP pairs closer to the regions were included. Thus, for the centromeric region only the recombination frequencies of the first SNP pair in chromosomes 1, 7, 8, 11, 18 and 19 (n=6) were used in the comparison between progeny of the female (FWT) and the male (MWT) F1.Aicda+/+. For the telomeric regions only the recombination frequencies of the last SNP pair in chromosomes 1, 2, 3, 4, 5, 8, 9, 11, 14, 16, 17 and 19 (n=12) were used in the comparison between the progeny of the female (FWT) and the male (MWT) F1.Aicda+/+. Bar and bar error represent Mean±SEM. A Mann-Whitney test was used to compare sample groups using a Bonferroni correction for multiple testing, *p < 0.0125; n.s. is non-significant. (C) Average recombination events per physical size (Mbp/cM) for Chromosomes 1 and 19 for F1.Aicda+/+ progeny. Bar and bar error represent Mean±SEM. A Mann-Whitney test was used to compare FWT vs MWT groups in each Chromosome, *p < 0.05.

**Figure 2.** Proportions of recombination events per SNP pair for all 19 autosomes calculated for offspring of females F1.Aicda+/+ (FWT, n=79, white bars); females F1.Aicda−/− (FKO, n=79, white bars with stripes) males F1.Aicda+/+ (MWT, n=78, grey bars) and males F1.Aicda−/− (MKO, n=78, grey bars with stripes). In the x-axis is the order of the SNP pairs. Error bars correspond to 95%
confidence intervals as estimated by the Agresti-Coull method. A Fisher-exact test was used to compare sample groups using a Bonferroni correction for multiple testing. *p<0.000225.

Figure 3. Average recombination frequencies of the IgH locus (chromosome 12), a putative AID target. Recombination frequencies were calculated using genotyping results from SNP in approximate position 108 Mbp and PCR results from Variable Number Tandem Repeat (VNTR) sequence that distinguishes C57Bl6/J from BALB/C genome in approximate position 116 Mbp calculated for offspring of FWT (n=68); FKO (n=67); MWT (n=68) and MKO (n=67). Bar and bar error represent Mean±SEM. A Mann-Whitney test was used to compare sample groups using a Bonferroni correction for multiple testing, * p < 0.0125; n.s. is non-significant.

Figure 4. (A) Aicda/beta-actin expression levels measured by real-time PCR in activated B-cells of B6.Aicda+/+ and B6.Aicda−/−; B6.Aicda+/+ sorted 4N, 2N and N (WT 4N, 2N and N, respectively) spermatocytes, unsorted testicular (WT T) and epididymal (WT E) sperm cells. (B) Aicda/beta-actin expression levels from two independent experiments and western-blot analysis of activated B-cells of B6.Aicda+/+ and B6.Aicda−/−; B6.Aicda+/+ ovaries from non super-ovulated females (WT ovary-non-super-ovulated), “complete oocyte” samples (WT oocytes + granulosa cells); only cells of the granulosa “oocyte free” (WT granulosa cells) and isolated oocytes (WT oocytes). Error bars correspond two independent experiments in which the tissues were pooled from 16-20 females per sample. (C) Western-blot analysis of activated B-cells of B6.Aicda+/+ and B6.Aicda−/−, B6.Aicda+/+ ovaries from non super-ovulated females (WT ovary-non-super-ovulated), “complete oocyte” samples (WT oocytes + granulosa cells); only cells of the granulosa “oocyte free” (WT granulosa cells) and isolated oocytes (WT oocytes).
**Supplementary figure legends**

**Figure S1.** (A) Schematics of the mating strategy: matings of B6.Aicda+/- and BA.Aicda+/-; F1 were genotyped for *Aicda* locus. F1.Aicda-/- and F1.Aicda+/+ were reciprocally mated with C57BL/6J and offspring was genotyped for SNPs that distinguish C57BL/6J from BALB/c genetic background. (B) Schematics of the SNP relative positions on the 19 autosomes in black. In Chromosome 6, the light grey area represents the portion that contains the *Aicda* locus and that is still of 129 genome. In medium grey is represented the relative position of the Variable Number Tandem Repeat (VNTR) in chromosome 12.

**Figure S2.** For the 17 SNP/sample pairs that passed the selection filters but had no detectable genotype signal, two extreme scenarios (recombination in all locations *versus* no recombination in all locations) were considered and overall recombination frequencies were calculated for the two extreme scenarios.

**Figure S3.** Representation of the average recombination frequency per chromosome of FWT vs FKO and MWT vs MKO study groups. Each dot is a chromosome identified by its number. A bisector line (represented as a grey dashed line) corresponds to equal recombination frequencies between the FWT and FKO or MWT and MKO analysis groups and deviations from this line, even if not significant, were observed for females in Chromosomes 12, 13 and 16 and for males in Chromosome 17 (arrows).

**Figure S4.** (A) Sorting strategy for subpopulations of testicular sperm cells according to DNA content using Hoechst 33342 - a vital dye that binds to DNA – as previously described (BASTOS et...
al. 2005). In the 2 top panels, are represented dot-plots with gates on the cell subpopulations to sort and in the bottom panel is represented the FSC-SSC profile of the subpopulations, which is easily identifiable. (B) Purity of the sorted populations, measured by acquisition of the subpopulations after sorting (left) and by DNA content measured by PI incorporation (right) and in the table the percentages of purity achieved for all the samples used in the real-time PCR.

**Figure S5.** To control for the effect of the hormone stimulation on AID expression, splenic B-cells from super-ovulated and from non-super-ovulated C57BL/6J were stimulated in culture with LPS and under different IL4 concentrations (++ is double concentration of +), with or without PMSG (2.5 and 0.5 IU/ml) and HCG (2.5 and 0.5 IU/ml) or both in the culture medium. AID expression was measured by real-time PCR. Error bars correspond to technical triplicates.
Table 1. Panel of 130 Single Nucleotide Polymorphisms (SNPs) across the 19 autosome.

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a. Reported Chromosome size in NCBI.
b. Distance between two consecutive SNPs, averaged for all SNPs per Chromosome.
c. Minimal distance between two consecutive SNP per Chromosome.
d. Maximal distance between two consecutive SNP per Chromosome.
e. The distance in Mbp between the first and the last SNP.
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\(^a\) Sum of the recombinations per chromosome.
\(^b\) Recombination frequency divided by the physical length (see Table 1).