Activation-induced deaminase (AID) acts on the immunoglobulin loci in activated B lymphocytes to initiate antibody gene diversification. The abundance of AID in the nucleus appears tightly regulated, with most nuclear AID being either degraded or exported back to the cytoplasm. To gain insight into the mechanisms regulating nuclear AID, we screened for proteins interacting specifically with it. We found that REG-γ, a protein implicated in ubiquitin- and ATP-independent protein degradation, interacts in high stoichiometry with overexpressed nuclear AID as well as with endogenous AID in B cells. REG-γ deficiency results in increased AID accumulation and increased immunoglobulin class switching. A stable stoichiometric AID–REG-γ complex can be recapitulated in co-transformed bacteria, and REG-γ accelerates proteasomal degradation of AID in vitro assays. Thus, REG-γ interacts, likely directly, with nuclear AID and modulates the abundance of this antibody-diversifying but potentially oncogenic enzyme.
RESULTS AND DISCUSSION
REG-γ associates in high stoichiometry with overexpressed nuclear AID
To facilitate purification of AID-interacting proteins, we used a tagged AID derivative fused at its N terminus to two tandem protein G domains followed by a FLAG3 peptide, with the two protein G domains separated from the FLAG3 peptide by a cleavage site for TEV protease (Fig. 1 A). Most AID in B cells is normally found in the cytoplasm. We therefore used a mutant (F193A), in which the phenylalanine residue within AID’s NES had been substituted by alanine, allowing more of the protein to be retained within the nucleus (Fig. 1 B; Geisberger et al., 2009). However, because increased nuclear expression of AID appears to be toxic, we incorporated an additional mutation, a glutamic acid→alanine substitution at AID’s active site (ES8A), which destroys the enzyme’s catalytic activity but does not abolish its ability to coordinate zinc (Fig. S1 A).

Recombinant AID was purified from whole cell extracts of Ramos transfectants that expressed the tagged-nuclear or cytosolic AID (i.e., with or without the F193A NES mutation).

The purification procedure comprised binding Protein G–FLAG3-AID onto IgG-Sepharose, releasing the FLAG3-AID by cleavage with TEV protease and then purifying the FLAG3-AID by absorption onto an anti-FLAG mAb matrix and elution with 3xFLAG peptide. After silver staining of a SDS-PAGE gel, the most striking difference between the nuclear and cytosolic AID samples was the presence, specifically in the nuclear sample, of a band of ~31 kD which was of roughly the same intensity as the putative FLAG3-AID band itself (Fig. 1 C). Mass spectrometric analysis confirmed the identity of the FLAG3-AID band and revealed the 31 kD band to be REG-γ, a nuclear protein (also known as PSME3, PA28γ or 11S regulator) implicated in a pathway of proteasome-dependent but ubiquitin- and ATP-independent protein degradation (Li and Rechsteiner, 2001; Zhou, 2006; Mao et al., 2008). Immuno-fluorescence analysis confirmed that although the F193A-tagged AID mutant is substantially detected in the nucleus of transfected Ramos cells, wild-type AID and REG-γ are largely found in different intracellular compartments in untransfected Ramos cells with REG-γ appearing exclusively nuclear, whereas AID is largely cytosolic (Fig. 1 B).
REG-γ is associated with endogenous nuclear AID

We were obviously concerned to ascertain whether endogenous (as opposed to overexpressed and tagged) AID is also found to associate with endogenous REG-γ. Immunoprecipitation of REG-γ from Ramos cells does indeed bring down endogenous AID (Fig. 1 D). Furthermore, a larger amount of AID is brought down with immunoprecipitated REG-γ if the Ramos cells are pretreated with leptomycin B (LMB; an inhibitor of Crm1-mediated nuclear export) to increase the concentration of AID in the nucleus (Fig. 1 E). Similar observations were made with two other human B lymphoma lines, EL1BL and Daudi. Because Western blot analysis of separated cytosolic and nuclear fractions shows that ~10% of the total AID in Ramos cells is associated with the nuclear fraction (unpublished data), we estimate that in the order of ~5% of the nuclear AID in Ramos cells is brought down by immunoprecipitation with anti-REG-γ antiserum.

Stable direct AID–REG-γ association can be recapitulated in bacteria

We suspected that the interaction between AID and REG-γ might be direct given that REG-γ was brought down in stoichiometric amounts from the Ramos cell transfectants without evidence of other similarly dominant nuclear AID-associated bands (Fig. 1 C). We therefore asked whether we could recapitulate the AID–REG-γ association in bacteria. A fusion protein between AID and maltose-binding protein (MBP; MBP-AID) was coexpressed in E. coli with His-tagged REG-γ. Purification of the MBP-AID on an amylose column revealed that the MBP-AID was indeed complexed with His–REG-γ (Fig. 2 A). The extracts of the MBP-AID–expressing bacterial cells also contain a band migrating slightly faster than full-length MBP-AID. Mass spectrometric and N-terminal sequence analyses revealed that this band was caused by truncation of the MBP-AID at AID residue 116. This MBP-AID[116] truncation protein also binds REG-γ, indicating that REG-γ binds residues located within the N-terminal 60% of AID (Fig. 2 B). The binding of MBP-AID to REG-γ appears to be of high affinity with the complex, remaining stable and displaying a constant ratio of MBP-AID to REG-γ through further chromatographic purification (unpublished data).

REG-γ is a member of family of three structurally homologous proteins. REG-α and REG-β are interferon-inducible proteins that function in the cytoplasm, where they form a heterohexameric complex that binds to the ends of the core 20S proteasome catalytic machinery and is implicated in peptide generation during the MHC class I pathway of antigen processing (Rechsteiner et al., 2000; Sijts and Kloetzel, 2011). In contrast, REG-γ is nuclear and has been shown to bind a small number of intact proteins, including SRC-3 (steroid receptor co-activator 3; a transcriptional co-activator and oncogene; Li et al., 2006) and cell-cycle regulator p21 (a cyclin-dependent kinase inhibitor; Chen et al., 2007; Li et al., 2007). REG-γ has been implicated in their degradation by a pathway that is both ubiquitin and ATP independent. We found that whereas association is readily detected between MBP-AID and His–REG-γ coexpressed in E. coli, no similar association is seen when MBP-AID is coexpressed with REG-α or REG-β (Fig. 2 C).

Effects of REG-γ on AID abundance and turnover

REG-γ has been proposed to mediate the degradation of both SRC3 and p21 by binding them directly and escorting them to the proteasome (Li et al., 2006, 2007; Chen et al., 2007). To assess whether REG-γ could similarly assist the proteasomal degradation of AID, we asked whether coexpression of REG-γ with tagged AID would lead to accelerated in vitro degradation by purified 20S proteasomes. The results revealed that, in the presence of the proteasome, REG-γ causes substantial destabilization of AID and that this destabilization is inhibited by the protease inhibitor MG132 (Fig. 3 A).

![Figure 2](image-url)

**Figure 2.** His6–REG-γ interacts directly with MBP-AID. (A) Lysates of MBP- or MBP-AID–expressing E. coli cells that had or had not been co-transformed with a plasmid directing expression of His6–REG-γ were analyzed by SDS-PAGE before (left four lanes) and after (right four lanes) pulldown by binding to amylose resin and elution with maltose. The gel (representative of three experiments) was stained with Coomassie blue. The asterisk indicates a band migrating slightly faster than full-length MBP-AID. (B) Both MBP-AID and MBP-AID[116] form a stable complex with REG-γ. MBPs from lysates of E. coli transformants coexpressing MBP-AID[116] and His6–REG-γ (as well as lysates from various control cells) were purified on amylose resin and/or Ni-NTA agarose as appropriate. The gel is representative of three experiments. (C) SDS-PAGE analysis of proteins pulled down on amylose resin from lysates of MBP-AID–expressing E. coli cells that had been co-transformed with plasmids encoding His6–tagged REG-α, REG-β, or REG-γ (right three lanes). Whole cell lysates before purification are shown for comparison (left three lanes).
If REG-γ plays a role in the turnover of nuclear AID in normal B cells in vivo, one might expect an increased abundance of AID in its absence. Two groups have described REG-γ-deficient mice and found that they display a very mild phenotype exhibiting a slight growth retardation in one study (Murata et al., 1999) and a small reduction in CD8 T cell numbers in the other (Barton et al., 2004). In most respects, however, the mice resembled their wild-type siblings. To ascertain whether REG-γ deficiency affects AID abundance, we compared AID levels by Western blot analysis in whole cell extracts of (IL4+LPS)-activated splenic B cells from REG-γ+/+, REG-γ−/−, and REG-γ−/− siblings generated by interbreeding of REG-γ+/− heterozygous mice. The results (Fig. 3 B) revealed that B cells from REG-γ−/− animals had a significantly higher abundance of AID than their REG-γ-proficient siblings.

Treatment of (IL-4+LPS)-activated B cells with LMB to inhibit AID nuclear export does indeed lead to AID destabilization (Fig. 3 C). This destabilization of AID is somewhat less rapid in REG-γ-deficient B cells, supporting the proposal that REG-γ plays a role in nuclear AID turnover in vivo. However, even in the absence of REG-γ, LMB treatment still leads to rapid AID degradation, indicating that there must also be a REG-γ-independent pathway of destabilization of nuclear AID. Indeed, enforced expression of AID[F193A] mutants in REG-γ-proficient and –deficient B cells revealed that the REG-γ-independent pathway for degrading overexpressed nuclear AID is dependent on AID’s lysine residues (Fig. 3 D), consistent with previous evidence of a ubiquitin-dependent pathway of AID turnover (Aoufouchi et al., 2008).

Figure 3. REG-γ is implicated in AID degradation. (A) Lysates of E. coli cells that had been induced with IPTG to coexpress His6-AID together with REG-γ (left) or alone (right) were mixed with 20S proteasomes and incubated with 37°C for the indicated times before subjecting to SDS-PAGE. The blots were then probed with anti-AID (EK2 5G9), anti–REG-γ, and anti-20S proteasome antibodies (top). A graph below summarizes the results of two independent such experiments along with controls showing the effects of the inclusion of a proteasome inhibitor (MG132; closed circle) or omission of the 20S proteasome (w/o 20S; open square). Error bars indicate mean ± SEM. (B) 100-µg extracts of purified splenic B cells from REG-γ+/+, REG-γ−/−, and REG-γ−/− siblings that had been activated for 3 d with IL-4 and LPS were subjected to SDS-PAGE and probed after blotting with anti–mouse AID (mAb 94.16), anti–REG-γ, and anti–α-tubulin (tub) antibodies. Two independent experiments (#1 and 2) are shown in the top, with the results of four independent experiments comparing AID protein abundance in B cells from REG-γ-deficient mice relative to littermates presented in the histogram below. The strengths of the AID bands have been quantified with ImageJ (National Institutes of Health) and normalized with respect to α-tubulin. Error bars indicate mean ± SEM. (C) Purified splenic B cells from REG-γ−/− and REG-γ−/− siblings that had been activated for 3 d with IL-4 and LPS and then cultured with 20 ng/ml LMB for 2.5, 5, and 7.5 h. 100 µg LMB-treated extracts was loaded onto SDS-PAGE gels and blots probed with anti-mouse AID, anti–REG-γ, and anti–α-tubulin antibodies (top). Relative AID protein level was quantified with ImageJ and normalized with respect to α-tubulin in each time point. To compare the reduction of AID protein between two genotypes, the level of AID protein at time zero was set to 100%. Pooled data from four independent experiments are plotted below. Error bars indicate mean ± SEM. (D) C-terminally HA-tagged AID[F193A] and two derivatives (KR1 and KR2) in which all eight AID lysine residues had been mutated to arginine (the two derivatives differ in assigned codon usage) were expressed in splenic B cells from REG-γ−/− and REG-γ−/− mice by retroviral transduction using pMX-IRES-GFP vector. The abundance of AID-HA and GFP in the whole cell extracts 2 d after transduction was monitored by Western blotting (top). The graph below summarizes the results of two independent experiments (all AID mutants analyzed carry the F193A mutation). Error bars indicate mean ± SEM.
substantially increased class switching as compared with their littermate controls in each of six independent comparisons; the mean percentage of IgG1+ cells at day 3 of culture increased from 19.6% in REG-γ+/− heterozygous mice to 28.1% in REG-γ−/− deficient siblings (Fig. 4 A). On average, the REG-γ−/− deficient B cells gave 40% more switching on day 3 as compared with their litter-matched controls. However, deficiency in REG-γ does not lead to any detectable change in the abundance of γ1-sterile transcripts (Fig. S2 A). Extending the analysis of switching to a further seven sets of litter-matched siblings revealed that although deficiency in REG-γ does not lead to any detectable change in the rate of B cell proliferation, the REG-γ−/− deficient B cells exhibited a substantially higher proportion of IgG1+ cells compared with the REG-γ+/- proficient controls as analyzed at each cell division (Fig. 4 B). Furthermore, flow cytometric comparison of bone marrow and spleen samples from REG-γ−/− and control siblings did not reveal any changes in B cell development, although it confirmed the previously noted (Barton et al., 2004) very small reduction in the proportion of CD8+ single positive splenic T cells (Fig. S2 C).

Reduced AID degradation in REG-γ−/− mice might also be expected to lead to a more general increase in genomic instability. However, REG-γ−/− mice on a normal background do not show any marked increase in tumor incidence, possibly reflecting p53 and other checkpoints (Jankovic et al., 2010). Only a small increase in the frequency of c-myc-IgH translocations was detected in cultured B cells from REG-γ−/− deficient (as opposed to REG-γ−/− proficient) mice in which AID overexpression had been induced by retroviral transduction (Fig. S3), possibly reflecting the ubiquitin-dependent degradation of such overexpressed AID (Fig. 3 D). Thus, although the results suggest that the dramatically increased class-switching in REG-γ−/− deficient mice is likely to be at least in part a direct consequence of the increased AID abundance, we cannot exclude the possibility that there is an additional contribution from some as yet unidentified effect of REG-γ deficiency (e.g., a perturbation of AID’s interaction with other nuclear partners).

These results identify AID as a novel target of REG-γ regulation. A feature shared by AID with most cellular REG-γ targets identified so far (SRC3, p21, and MDM2–p53; Li et al., 2006, 2007; Chen et al., 2007; Zhang and Zhang, 2008) is that misexpression of these nuclear proteins is potentially oncogenic. With AID, as with the other REG-γ targets, REG-γ contributes to its nuclear degradation but clearly does not provide the sole route to its regulated turnover. Nevertheless, the phenotype of B cells from REG-γ−/− deficient mice suggests that, under normal conditions, REG-γ might well provide a major pathway of nuclear AID destruction, with other pathways taking over in its absence. It is also possible that the ubiquitin– and ATP-independent REG-γ pathway might prove of especial importance under certain conditions, for example in cells with low concentrations of ATP.

Our results show that ∼5% of the endogenous nuclear AID in Ramos B cells (as well as a substantially higher proportion of overexpressed mutant AID) is associated with REG-γ. Although the structural basis is at present unclear, the bacterial coexpression assays indicate that this interaction is likely direct. We suspect that part of the AID surface might exhibit a naturally unfolded conformation that can fold to adopt a structure, which facilitates interaction with REG-γ or other suitable partners. Indeed, a role for such naturally unfolded segments in REG-γ’s clients has already been proposed to account for the ability of REG-γ to mediate the proteasomal degradation of these clients without requirement of ATP (Li and Rechsteiner, 2001; Zhou, 2006). In which case, the ability to interact with REG-γ in vitro might extend to other members of the AID/AP0BEC family. We therefore
performed additional bacterial pulldown experiments and found that, although REG-γ exhibits preferential binding to AID, lesser binding is also obtained with APOBEC1 and APOBEC3 when coexpressed in Escherichia coli but not with APOBEC2 or substantial amounts of endogenous bacterial proteins (Fig. S1 B). We have been unable to reconstitute the REG-γ interaction with fully denatured MBP-AID, but REG-γ recognition of AID does not require the intactness of AID’s Zn-binding motif (Fig. S1 C), although we do not know the effect on AID folding of the loss of critical zinc-coordinating residues. The results are therefore consistent with a model in which AID presents a naturally unfolded face (which might contribute to the stickiness and aggregation of the protein as analyzed in vitro) that could also form part of the site of interaction of AID with several of its bona fide cytoplasmic and nuclear partners. Indeed, the role of REG-γ could be to scavenge unattached AID for degradation in situations when it has been jettisoned by one of its nuclear partners. Alternatively, in view of the apparent high affinity of the AID–REG-γ interaction, REG-γ might serve to actually limit the availability of AID to its physiological or even nonphysiological interactors. Insight into these possibilities might be provided by elucidating the structural basis of the AID–REG-γ interaction, comparing to the interactions that AID displays with other bona fide partners.

MATERIALS AND METHODS

Cell lines. Human AID[ES8A], AID[ES8A; F193A], and DNA polymerase η cDNAs were cloned into plasmid pEAK8-ProteinG-FLAG3. The pEAK8 plasmid was obtained from Edge BioSystems. Protein G−FLAG3 is a tag comprising two tandem Protein G domains (Bürckstümmer et al., 2006) and a FLAG3 epitope separated by a TEV cleavage site. These plasmids were transfected into Ramos cells by electroporation. Stably transfected colonies were picked up after 2 wk of selection with puromycin and expanded in RPMI supplemented with 10% FBS, 0.05 mM 2-mercaptoethanol, and 0.3 µg/ml puromycin.

Purification and mass spectrometric analysis of tagged AID from Ramos cells. Transfected Ramos clones (10⁶ cells) were lysed in 10 ml buffer A (20 mM Hepes, pH 7.9, 10% glycerol, 150 mM NaCl, 2.5 mM MgCl₂, 0.5 mM EDTA) supplemented with 0.2% Triton X-100. Untransfected Ramos cells were stained with goat anti–rabbit IgG-FITC (Southern Bio-Technologies) and permeabilized with 0.2% Triton X-100. Untransfected Ramos cells transfectants were stained with goat anti–rabbit IgG-FITC (Southern Bio-Technologies) and permeabilized with 0.2% Triton X-100. Untransfected Ramos cells were stained with goat anti–rabbit IgG-FITC (Southern Bio-Technologies) and permeabilized with 0.2% Triton X-100. Untransfected Ramos cells. Stably transfected Ramos clones (10⁹ cells) were lysed in 10 ml buffer A (20 mM Hepes, pH 7.9, 10% glycerol, 150 mM NaCl, 2.5 mM MgCl₂, 0.5 mM EDTA, 2 mM DTT, and 2 mM β-mercaptoethanol) and subjected to SDS-PAGE and stained with a SilverQuest staining kit (0.15 µg/ml; Sigma-Aldrich). The samples eluted with 3xFLAG peptide were taken at −8°C. Aliquots of the eluates were subjected to SDS-PAGE and silver stained. The samples were then subjected to SDS-PAGE and stained with a SilverQuest staining kit (Invitrogen). The indicated bands were excised for tryptic digestion. Mass spectrometric analysis was performed with HCT Ultra II with electron transfer dissociation (Bruker Daltonics). Spectra were analyzed with Scaffold 2 proteome software (Proteome Software Inc.).

Immunofluorescence analysis. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Untransfected Ramos cells were stained with rat anti-AID mAb (EK2 SG9; Cell Signaling Technology), followed by anti-rat IgG-488 (Invitrogen) and rabbit anti–REG-γ (Biomol), followed by anti-rabbit IgG-568 (Invitrogen). Protein G−FLAG3 stable transfectants were stained with goat anti–rabbit IgG-FITC (Southern Bio-Technologies) to detect Protein G−containing fusion proteins. Samples were mounted with DAPI-containing mounting medium (VECTASHIELD; Vector Laboratories) and observed using a Radiance 2100 confocal microscope (Bio-Rad Laboratories) with a Plan Apo 60×/1.40 NA oil immersion lens (Nikon) using LaserSharp 2000 acquisition software (Bio-Rad Laboratories).

Immunoprecipitation and Western blot analysis. For analysis of endogenous AID in B cell lines, cells (which had, if required, been preincubated with 20 ng/ml LMB [LC Laboratories] for 4 h at 37°C) were lysed in buffer A supplemented with 0.4 mg/ml lysozyme, 50 µg/ml RNase A, 150 µM benzonase (Novagen), and protease inhibitor cocktail. Clarified lysates were mixed with anti–REG-γ antibody at 4°C for 30 min and incubated with Protein A-Sepharose (GE Healthcare) at 4°C for 30 min. Beads were then washed with buffer A and subjected to SDS-PAGE.

For analysis of endogenous AID in mouse splenic B cells, clarified lysates prepared from cells lysed by 30 min of incubation on ice in 10 mM Hepes, pH 7.9, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 150 µM benzonase, 0.1 mM PMSF, and protease inhibitor cocktail were subjected to SDS-PAGE (100 µg/lane). Protein concentration in lysates was measured with a BCA protein assay kit (Thermo Fisher Scientific). Human AID and REG-γ were detected in Western blots using the same antibodies used for immunofluorescence, whereas mouse AID was detected with mAb 94.16 (provided by Hans-Martin Jäck, Erlangen, Germany) and α-tubulin is a rabbit antiseraum (Abcam). Mutant AIDs containing a C-terminal HA tag were expressed in mouse splenic B cells by retroviral transduction using pMX-IRES-GFP vector as previously described (Geisberger et al., 2009).

Class-switching assays. REG-γ-deficient mice that had been bred onto a C57BL/6 background (Barton et al., 2004) were bred in our animal facility under UK Home Office project license PPL80/2226 against C57BL/6 mice. Heterozygous animals were used to produce age-matched littermates of 8–12 wk for class-switching assays. Splenic B cells that had been enriched by depletion of CD43⁺ cells using anti-CD43–coupled magnetic beads (Miltenyi Biotec) were seeded in 24-well plates (0.5 × 10⁷ per well) in 1 ml RPMI supplemented with 10% FBS, 0.05 mM 2-mercaptoethanol, 25 µg/ml recombinant mouse IL-4 (R&D Systems), and 40 µg/ml LPS (Sigma-Aldrich). On day 3 of stimulation, B cells were stained with PE-conjugated anti-B220 and biotinylated anti-IgG1 (followed by APC-conjugated streptavidin; all from BD). Flow cytometry was performed using an LSR II (BD), excluding dead cells with propidium iodide and analyzing switching data with Flowjo software (Tree Star). For cell division analysis, isolated splenic B cells were stained with 5 mM CFSE (Invitrogen) and stimulated with IL-4 and LPS as described.

Assaying AID−REG-γ association in bacteria. E. coli BL21(DE3) cells that had been transformed with AID/APOBEC cDNAs cloned into pMAL−c2X vector (NEB) and/or N-terminally hexa-His−tagged REG-α/B/γ cDNAs cloned into the p15A origin−containing vector pACYC-T7 (Yasukawa et al., 1995) were induced with 0.3 mM IPTG at 20°C for 16 h. Bacterial pellets were sonicated in buffer B (10 mM Hepes, pH 7.9, 200 mM KCl, 0.1% Triton X-100, and 1 mM DTT) containing 10 µg/ml DNase, 10 µg/ml RNaseA, 1 mM PMSF, and protease inhibitor cocktail. Malate binding proteins were purified from the clarified lysates by binding for 1 h at 4°C on to amylose resin (NEB) and eluted from the washed beads with 50 mM malate in buffer B.

Assaying proteasomal degradation in vitro. E. coli BL21(DE3) was co-transformed with pET30 (Novagen)−His6-AID and either pACYC-T7−REG-γ or unmodified pACYC-T7 control plasmids. Cells were induced with 0.3 mM IPTG at 20°C for 16 h. Bacterial pellets were sonicated in buffer B (10 mM Hepes, pH 7.9, 150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 1 mM DTT, and 140 nM 20S proteasomes (BostonBiotech) at 37°C for the indicated times. MG132 (Sigma-Aldrich), where required, was included at 10 µM.

Online supplemental material. Fig. S1 shows that the ES8A and truncation mutants of AID retain the ability to coordinate zinc and that, after
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**Figure S1. Zn and REG-γ binding by MBP-AID and MBP-APOBEC proteins.** (A) The Zn binding by recombinant MBP-AID, MBP-AID[E58A], MBP-AID[116], and MBP proteins that had been purified on amylose resin from the extracts of BL21(DE3) E. coli transformants was measured by incubating different quantities of the purified proteins (as specified on the x-axis) in 20 mM Hepes, pH 7.9, 200 mM NaCl, and 1 mM DTT with 50 µg/ml proteinase K (56°C, 30 min) and then adding 4-(2-pyridylazo) resorcinol (Sigma-Aldrich) to 1 mM and measuring the absorbance at 492 nm. Error bars indicate ± SEM. (B) Lysates of E. coli cells expressing His6–REG-γ together with MBP, MBP-AID, or various MBP-APOBEC fusion proteins were analyzed by SDS-PAGE before (left six lanes) and after pulldown on amylose resin and elution with maltose (right six lanes). (C) MBP-AID mutants carrying amino acid substitutions in the catalytic site still bind REG-γ in bacterial coexpression assays. Lysates of E. coli cells coexpressing His6–REG-γ together with MBP, MBP-AID, and MBP-AID variants carrying the indicated substitution mutations were analyzed by SDS-PAGE after purification of MBPs by binding onto amylose resin and elution with maltose.
Figure S2. Class switching and lymphoid subpopulations in REG-γ-deficient mice. (A) Real-time RT-PCR of γ1-sterile transcripts in (IL-4+LPS)-activated splenic B cells from REG-γ^{+/+} and REG-γ^{-/-} mice were compared with REG-γ^{+/+} mice at day 3 of culture. Each symbol corresponds to B cells isolated from an individual mouse with bars indicating the mean ± SEM. For RT-PCR, RNA was extracted with TRIzol (Invitrogen) and converted to cDNA with the high capacity cDNA reverse transcription kit (Applied Biosystems). Synthesized cDNA was mixed with SYBR GreenER qPCR SuperMix Universal (Invitrogen) and the primers for γ1-sterile (5’-TCGAGAAGCCTGAGGAATGTG-3’ and 5’-ATGGAGTTAGTTGCGAGCA-3’) and GAPDH (5’-GGAAGGTGGAAGAGTGGGAG-3’; Reina-San-Martin et al., 2003). Reaction mixtures are amplified with 7900 HT Fast real-time PCR system (Applied Biosystems) and each sample was normalized to GAPDH expression. Error bars indicate ± SEM. (B) Representative examples of cell division analysis of (IL-4+LPS)-stimulated switching to IgG1 of the experiment summarized in Fig. 4 B. The graph shows the number of cells (top) and percentage of IgG1-positive cells (bottom) on the y-axis plotted against the number of cell divisions undergone (x-axis) in a representative pair of REG-γ^{+/+} and REG-γ^{-/-} littermates. (C) Flow cytometric analysis of lymphoid subpopulations in REG-γ^{+/+} and REG-γ^{-/-} siblings. Representative flow cytometric analyses (of two independent sets of comparisons) of cells from bone marrow (i and ii) and spleens (iii, iv, and v) from 8-wk-old REG-γ^{+/+} and REG-γ^{-/-} mice. The bone marrow cells analyzed were gated on being CD43^{+} (i) or IgM^{+} (ii). The spleen cells analyzed were gated on being B220^{+} (iii), on both B220^{+} and CD19^{+} (iv), and on being CD3^{+} B220^{-} (v). All antibodies used for flow cytometry were from BD except APC-conjugated anti-B220 (Invitrogen) and APC-conjugated anti-IgM and PECY7-conjugated anti-CD3 (eBioscience).
Figure S3. c-myc-Igh translocations in B cells from REG-γ⁺/⁻ and REG-γ⁻/⁻ mice. Chromosomal translocations between c-myc and IgH in vitro cultured REG-γ-proficient and REG-γ-deficient splenic B cells. Splenic B cells were transduced with pMX-AID-IRES-GFP for 3 d and GFP⁺ cells were sorted by flow cytometry. Specifically PCR-amplified fragments were analyzed by Southern blotting as described by Ramiro et al. (2006). The experiment shown here is based on analyses of 48 pools of 25,000 cells for each set but with a similar low frequency of translocations (around two to four translocations per 10⁶ cells) observed in two independent experiments.

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