SAMHD1 enhances immunoglobulin hypermutation by promoting transversion mutation

Eddy Sanchai Thientosapol\textsuperscript{a,b}, Daniel Bosnjak\textsuperscript{a,b}, Timothy Durack\textsuperscript{a,b}, Igor Stevanovski\textsuperscript{a,b}, Michelle van Geldermalsen\textsuperscript{a,b}, Jeff Holst\textsuperscript{a,b}, Zeenat Jahan\textsuperscript{a,b}, Caitlin Shepard\textsuperscript{c}, Wolfgang Weninger\textsuperscript{a,b}, Baek Kim\textsuperscript{d}, Robert Brink\textsuperscript{e}, and Christopher J. Jolly\textsuperscript{a,b,1}

\textsuperscript{a}Centenary Institute, Camperdown NSW 2050, Australia; \textsuperscript{b}Sydney Medical School, The University of Sydney, Sydney, NSW 2006, Australia; \textsuperscript{c}Emory School of Medicine, Emory University, Atlanta, GA 30322; \textsuperscript{d}School of Pharmacy, Kyung Hee University, 02447 Seoul, South Korea; and \textsuperscript{e}Garvan Institute of Medical Research, Darlinghurst, Sydney, NSW 2010, Australia

Activation-induced deaminase (AID) initiates hypermutation of Ig genes in activated B cells by converting C\textsuperscript{G} into U\textsuperscript{G} base pairs. G\textsubscript{1}-phase variants of uracil base excision repair (BER) and mismatch repair (MMR) then deploy translesion polymerases including REV1 and Pol \eta, which exacerbates mutation. dNTP paucity may contribute to hypermutation, because dNTP levels are reduced in G\textsubscript{1} phase to inhibit viral replication. To derestrict G\textsubscript{1}-phase dNTP supply, we CRISPR-inactivated SAMHD1 (which degrades dNTPs) in germline activated B cells. SAMHD1 inactivation increased B cell virus susceptibility, increased transition mutations at C\textsuperscript{G} base pairs, and substantially decreased transversion mutations at A\textsuperscript{T} and C\textsuperscript{G} base pairs in both strands. We conclude that SAMHD1’s restriction of dNTP supply enhances AID’s mutagenicity and that the evolution of Ig hypermutation included the repurposing of antiviral mechanisms based on dNTP starvation.

B cells | mutation | DNA repair | dNTPs | deamination

Somatic hypermutation, in combination with clonal selection, creates high-affinity antibodies (Ig) in response to viral and bacterial infection. Activation-induced deaminase (AID, gene \textit{Aicda}) initiates mutation by deaminating genomic cytosines, in either strand of Ig V(D)J-regions, which creates a U\textsuperscript{G} mismatch in the DNA (Fig. S1). If replication proceeds without uracil excision, this produces a C\textsuperscript{G} to T\textsuperscript{A} transition mutation in one daughter cell, because A is incorporated opposite U (reviewed in ref. 1).

Most deaminations are excised by the base excision repair (BER) enzyme UNG2, a uracil-specific enzyme that converts U into an apyrimidinic (AP) site (Fig. S1). The enzymes SMUG1 and TDG can substitute for UNG2, but in UNG2-proficient cells, their contribution to Ig hypermutation is probably minor (2–5). Mismatch repair (MMR) is also recruited (Fig. S1), presumably by the U\textsuperscript{G} mismatches deamination induces. Processing via BER or MMR predominantly restores C in place of U (6–9) but is also error-prone. Error-prone BER converts deaminated sites into transition or transversion mutations at C\textsuperscript{G} and occasionally produces mutations at bases flanking deamination sites, including A\textsuperscript{T} bases (1). Mutation does not require the canonical BER polymerase, Pol \beta (10); instead, C\textsuperscript{G} to G\textsuperscript{C} conversion mutation requires the translesion polymerases REV1 and Pol \eta (11, 12). Bypass of persistent AP sites using translesion polymerases is therefore thought to be the main mechanism of UNG2-dependent Ig hypermutation (12, 13).

Mutation at Ig A\textsuperscript{T} base pairs is substantially reduced by inactivation of the MMR genes Msh2, Msh6, or Exo1, inactivation of Polh, or by mutation of the K164 ubiquitination site in mouse PCNA (14–19). This has led to a model in which A\textsuperscript{T} mutations are introduced by translesion Pol \eta, predominantly recruited by noncanonical MMR (1, 20), with minor recruitment during UNG2-driven long patch (LP) BER (21). In this model (Fig. S1), activated MutS\alpha recruits Exo1 to create excision patches. Monoubiquitinated PCNA then recruits error-prone Pol \eta during excision patch in-fill, which introduces mutations biased toward A:T base pairs. Until recently, it was unknown how the DNA was nicked to allow Exo1 entry, but it is now clear that APE1, APE2, and MutL\alpha produce nicks semiredundantly (4, 5, 22) (Fig. S1).

A major question raised by this model is why PCNA becomes ubiquitinated to recruit Pol \eta during AID-induced MMR. Only G\textsubscript{1}-phase activities of AID or UNG2 are mutagenic in mouse B cells (5, 6, 23). Strong evidence for mutagenic interaction between BER and MMR implies that mutagenic MMR also occurs in G\textsubscript{1} phase (2, 3, 8, 9, 12, 21), dNTP levels were reported to be lowest in G\textsubscript{1} phase, especially in the nucleus (24). We proposed, therefore, that dNTP pools may be inadequate to support long-patch DNA synthesis by conventional DNA polymerases during G\textsubscript{1}-phase MMR; polymerase stalling could then induce PCNA ubiquitination (ref. 25; also see ref. 20). The rate-limiting enzyme for dNTP production is ribonucleotide reductase (RNR), which is downregulated in G\textsubscript{1}-phase cells via turnover of the R2 subunit (26). However, the major regulator of dNTP levels is not in fact RNR activity, but the deoxynucleotide triphosphohydrolase SAMHD1; dNTPs increase more than 10-fold in resting and proliferating Samhd1\textsuperscript{-/-} cells (27, 28). SAMHD1 is a potent restrictor of virus

Significance

Antibody affinity rises during immune responses to viruses via antibody gene somatic hypermutation and Darwinian selection of mutated B cells—in a time frame of days. The enzyme activation-induced deaminase (AID) initiates hypermutation by deaminating genomic cytosines. Mutation is exacerbated by noncanonical G\textsubscript{1}-phase DNA repair pathways that deploy error-prone polymerases, including Pol \eta (gene Polh). In G\textsubscript{1} phase, dNTP levels are restricted to inhibit viral replication. We derestricted G\textsubscript{1}-phase dNTP supply in hypermutating B cells, which increased virus susceptibility in vitro and caused changes in antibody hypermutation in vivo akin to Polh inactivation. We conclude that G\textsubscript{1}-phase dNTP paucity contributes to antibody hypermutation and that the evolution of antibody hypermutation included the repurposing of intracellular antivirus mechanisms based on dNTP starvation.


The authors declare no conflict of interest.

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1To whom correspondence should be addressed. Email: c.jolly@centenary.org.au.

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replication (27, 28), and its activity is highest in G1 phase (29). We quantified Ig hypermutation in mouse germinal center B cells lacking SAMHD1. We observed an increase in transitions at C/G and major decreases in transversions at A/T and C/G, which suggests that SAMHD1-induced dNTP paucity contributes to AID-induced mutagenesis via both MMR and uracil BER.

Results

RNR and SAMHD1 Expression in Mouse B Cells. Ribonucleotide reductase (RNR) consists of R1/R2 interfaces (reviewed in ref. 30). Consistent with reports for other cell types (30), R2 levels were substantially lower in G1-phase B cells [i.e., in mKO2+/mAGvvv (orange) Fucci-transgenic B cells; ref. 31] than in the rest of the cell cycle [i.e., in mKO2+/mAGvvv (green) Fucci-transgenic B cells; Fig. L4], implying that B cells carry out far less de novo deoxynucleotidylate synthesis in G1 phase than in S phase. SAMHD1 levels were not obviously cell cycle regulated in mouse B cells (Fig. L4), which was consistent with posttranslational modification being the principal regulator of SAMHD1 activity (29).

Samhd1 Inactivation Substantially Decreased Transversions in Ig Genes. We CRISPR-targeted Samhd1 exon 1 in C57BL/6 mouse embryos. Samhd1 alleles carrying 25- or 41-bp frame-shift deletions (Δ) in exon 1 (Fig. S2) appeared to be knockout alleles (Fig. L4). CRISPR mutation of Samhd1 enhanced retroviral transduction (P = 0.0042; Fig. 1B) and substantially increased the levels of dNTPs in resting and blasting B cells (P < 0.0001; P = 0.0003, respectively) and in G1-phase cells (Fig. L1C). Purines (dATP and dGTP) were increased more than pyrimidines (dTTP and dCTP; Fig. 1C). These transduction and dNTP data closely reproduced data generated using conventional Samhd1−/− mice (28, 32, 33).

We four times back-crossed Samhd1Δα alleles to Ig-transgenic “SWHEL” mice, which have a C57BL/6 background, then bred Samhd1Δα/ΔαSWHEL mice (and later, Samhd1Δα/ΔαSWHEL mice). To quantify Ig hypermutation, we ex vivo transduced SWHEL B cells, which are specific for hen egg lysozyme (HEL), to express the PSB2 uracil glycosylase inhibitor and/or GFP then adoptively transferred GFP-positive cells into congenic hosts and immunized with HEL conjugated to SRBC, as outlined in Fig. 24 and described previously (6, 9, 25). Adoptive cells are T-dependent and concentrate in germinal centers soon after transfer (25, 34). Ig hypermutations were collated in a 560-bp window from ≤47 single HEL-binding GFP** cells per host, sorted from spleens 6 d after adoptive transfer, using a minimum of three donors and hosts per treatment (Methods). This procedure results in reproducible hypermutation with minimal impact on the mutation spectrum by antigen selection, because the BCR analyzed starts with high affinity for HEL and the duration of hypermutation (i.e., 6 d) is short (9). Our model predicted that loss of SAMHD1 would decrease mutation at A/T base pairs, and we indeed found this to be the case (P < 0.0001 for mutation at A; P = 0.029 for mutation at T; Fig. S3.A). Specifically, Samhd1 inactivation reduced the frequency of transversion mutations at A/T base pairs (hereon called “A/T transversions”), relative to wild-type cells (P < 0.0001; Fig. 2 B, i), but barely changed the frequency of A/T transitions (Fig. 2 B, ii). Samhd1 inactivation also substantially reduced C/G transversions (P < 0.0001; Fig. 2 B, iii) and nearly doubled C/G transitions (P = 0.0041; Fig. 2 B, iv). This increase in C/G transitions partly compensated for the loss of A/T and C/G transversion mutations; total Ig mutation in Samhd1Δα B cells was lower than in wild-type cells (Table 1), but not significantly so (P = 0.07 by one-way ANOVA). See Table 1 and Figs. S3 and S4 for overviews of the mutation data. Samhd1 inactivation reduced all classes of C/G transversions. That is C/G to G/C transversions, which are introduced by REV1 via BER and by Pol η via MMR (11, 12, 35), and C/G to A/T transversions, which are introduced by a polymerase or polymerases currently unknown (Table 1 and Fig. S3.A).

To test whether the impact of Samhd1 inactivation on Ig hypermutation was an artifact of antigen selection, we quantified mutation in intron sequences immediately 3′ to the VDJ exon (Fig. S3B). Our 560-bp sequence window included the 5′ 101 bp of the J-C intron (Fig. S4); this region is analogous to that commonly analyzed in Peyer’s patch B cells (5, 36)—albeit, with the SWHEL intron carrying a deletion that tags gene-targeted cells (34). Samhd1 inactivation caused significant reductions in transversions at A/T (P = 0.0089) and at C/G (P = 0.0001) in the J-C intron and increased C/G transitions, albeit not significantly (P = 0.065; Fig. S3B), relative to wild-type cells. We conclude that the impact of Samhd1 inactivation on Ig hypermutation—in particular, reduced transversion mutation—was not an artifact of antigen selection.

![Western blot](Western blot showing mAG and mKO2 expression in R1 and R2 subunits of SAMHD1 and Memcode staining in SAMHD1 and SAMHD1Δα samples. (A) Western blot for RNR subunits (R1 = 90 kDa; R2 = 45 kDa) or SAMHD1 (76 kDa) extracted from splenic B cells after culture for 3 d in LPS. Fucci-red/ Fucci-green transgenic B cells were sorted into green (mAG/mKO2) and orange (mAG/mKO2) populations. Memcode staining (~10- to 20-kDa region) indicates protein loading and transfer. In Fucci cells, mKO2+ cells are in G1 phase and mAG+ cells are in S, G2, and M phases (6, 60). (B) Transducibility of Samhd1Δα/+ B cells. Splenocytes were cultured with LPS for 1 d to activate B cells, then transduced in triplicates with serially diluted pMIG retrovirus. Frequencies of GFP** cells were determined by cytometry 2 d later. **P = 0.0042, two-way ANOVA. (C) dNTPs measured in whole-cell extracts. (i) G0. (ii) Blasting ratios. (iii) G1. One day after LPS activation, cells were transduced to express mKO2-cdt fusion protein, which tagged G0, cells with orange fluorescence (6). Blasting mKO2** cells were purified by flow cytometry 2 d later.

![Transducibility](Transducibility of Samhd1Δα/+ B cells. Splenocytes were cultured with LPS for 1 d to activate B cells, then transduced in triplicates with serially diluted pMIG retrovirus. Frequencies of GFP** cells were determined by cytometry 2 d later. **P = 0.0042, two-way ANOVA. (B) dNTPs measured in whole-cell extracts. (i) G0. (ii) Blasting ratios. (iii) G1. One day after LPS activation, cells were transduced to express mKO2-cdt fusion protein, which tagged G0, cells with orange fluorescence (6). Blasting mKO2** cells were purified by flow cytometry 2 d later.)
Samhd1 Inactivation Does Not Phenocopy MMR Inhibition. Like Samhd1 inactivation, Msh2 knockout decreases A/T mutations and C/G transversions in SWHEL B cells (9) (Fig. 2, Table 1, and Fig. S3). This raised the possibility that Samhd1 inactivation acted by partially inhibiting AID-induced MMR. A hallmark feature of MMR knock-out is the focusing of AID-induced mutation on hotspot motifs, especially AGCW motifs (see ref. 9 and Table S1). We therefore examined our mutation data for hotspot focusing (Fig. 3) and measured hypermutation in adoptive Msh2Δ−/− Samhd1Δ−/Δ SWHEL B cells (Figs. 2B and 3A, Table 1, and Figs. S3 and S4). In summary, we found that Samhd1 inactivation did not focus AID-induced mutation on AGCW hotspots, an outcome quite different to loss of MMR (see overviews in Table S1).

The strand bias of mutation changes further distinguished Samhd1Δ−/Δ from Msh2Δ−/− cells. As described before (e.g., ref. 9), Msh2Δ−/− altered mutations at C nearly twice as much as at G. In contrast, Samhd1Δ−/Δ inactivation altered mutation at C and at G to comparable extents (Fig. S3A).

Samhd1 Inactivation Does Not Inhibit Uracil Repair via UNG2. Reduced C/G transversion in Samhd1Δ−/Δ B cells raised the obvious possibility that uracil excision by UNG2 might partially depend on SAMHD1, via some unexpected mechanism. To measure UNG2-mediated uracil repair, we blocked UNG2 activity in adoptive Samhd1Δ−/Δ cells using retroviral expression of the uracil glycosylase inhibitor (ugi) from bacteriophage PSB2 (37). Previous blockades of UNG activity in wild-type SWHEL B cells with ugi increased IgC/G transitions 2.3-fold, in a strand-unbiased manner, presumably by increasing “ignorant” replication opposite persistent uracils (6, 9). In Samhd1Δ−/Δ B cells, retroviral expression of ugi-UNG increased C/G transitions by 1.9-fold (P < 0.0001), relative to expression of GFP alone (Fig. 2B, iii), and this increase was strand-unbiased (Table 1 and Fig. S3A). This demonstrated that UNG2 mediates reversal of similar frequencies of U:G base pairs into C:G base pairs in hypermutating lgh genes regardless of SAMHD1 activity, so it is unlikely SAMHD1 alters uracil excision rates by UNG2.

Discussion
Samhd1 Inactivation Partially Phenocopies Genotypes That Block Pol η Recruitment. We, and later others, proposed polymerase stalling induced by dNTP imbalances as a G1-phase mechanism that could explain PCNA ubiquitination, Pol η recruitment, and nucleotide misincorporation during AID-induced MMR (20, 25). Our finding here of reduced A/T mutation in Samhd1Δ−/Δ cells (Fig. S3A) was predicted by this model but does not prove it. The unequal impact of Samhd1 inactivation on A/T transversions versus A/T transitions (Fig. 2B and Fig. S3B) was not expected but might be explained by the greater impact of Samhd1 inactivation on purine dNTPs over pyrimidine dNTPs (see below and Fig. 4). Samhd1-CRISPR also decreased C/G transversions

Table 1. Raw mutation data

| Cells            | Protein | Hosts | Sequences | Deletions* | Insertions* | A   | C   | T   | G   | A   | T   | G   | C   | A   | G   | Per sequence |
|------------------|---------|-------|-----------|------------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|---------------|
| WT or Samhd1Δ−/Δ | GFP     | 13    | 565       | 9,2,1      | 0           | 190 | 74  | 35  | 142 | 26  | 27  | 125 | 170 | 87  | 64  | 44  | 28           | 1.79          |
| Samhd1Δ−/Δ       | GFP     | 8     | 354       | 1,1        | 1           | 129 | 11  | 4   | 144 | 10  | 2   | 60  | 15  | 7   | 32  | 11  | 8  | 1.40         |
| Msh2−/− Samhd1Δ−/Δ| GFP     | 5     | 213       | 1,2,15,5,1 | 0           | 129 | 11  | 3   | 122 | 2   | 5   | 3   | 1   | 2   | 1   | 0  | 1.31         |
| Msh2−/−          | GFP     | 10    | 446       | 10,3       | 0           | 183 | 40  | 18  | 210 | 5   | 7   | 5   | 5   | 3   | 2   | 2   | 0   | 1.08         |
| Samhd1Δ−/Δ       | ugi-GFP | 5     | 218       | 0          | 0           | 235 | 0   | 1   | 163 | 0   | 1   | 61  | 20  | 12  | 34  | 6   | 8  | 2.48         |

*The size (bp) of all deletions or insertions observed is listed, separated by commas.
and increased C/G transitions in a strand unbiased manner that was not focused on AGCW hotspots (Figs. 2 and 3, Figs. S3 and S4, and Table S1). We largely ruled out reduced uracil excision via MMR or Ung2 as the cause of this. Reduced A/T mutations decoupled from an apoptosis on AGCW hotspots was observed previously in Polh<sup>−/−</sup> and Pcn<sup>−/−</sup> mice (33, 38), and contrasts with MMR deficiency, which couples these two mutation characteristics, regardless of Samhd1, Pcnα, or Polh genotype (Fig. 3; see also phenotypes summarized in Table S1). The contrast in hotspot focusing among Samhd1<sup>ΔΔ</sup>−/−, Polh<sup>−/−</sup>, and Pcn<sup>−/−</sup> on the one hand, and Msh2<sup>−/−</sup>, Msh6<sup>−/−</sup>, and ExoI<sup>−/−</sup> on the other, can be explained by the likelihood that the former genetic manipulations do not prevent mismatch excision and only impact excision patch in-fill, while the latter manipulations prevent both. We interpret the similarities in the hypermutation phenotypes of Polh<sup>−/−</sup>, Pcn<sup>−/−</sup> and Samhd1<sup>ΔΔ</sup>−/− in this time during UNG2-induced lesions bypass events, as illustrated in Fig. 4. It is possible that Samhd1’s exonuclease activity and/or its capacity to recruit the endonuclease Cpf1 (41, 42) induces Ig mutation, rather than its dNTPase activity. The production of double-strand breaks (DSB) downstream of AID is critical for Ig class switching (1), but DSB formation plays a minor role in V-region hypermutation (43–45). In our experiments, insertions and deletions, which likely arise from DSB, were not discernably affected by Samhd1 inactivation (Table 1) and clustered near AGCW motifs regardless of Samhd1 genotype (Fig. S4).

**An Entirely G1-Phase Model of Ig Hypermutation.** We suggested previously that MMR-independent lesion bypass might occur when G1-phase AP sites persist into S phase and are replicated (6; see Fig. S1). This model is not explicitly contradicted by the Samhd1<sup>ΔΔ</sup> phenotype we report here, because Samhd1 inactivation produced supraoptimal dNTPs in all cell cycle phases. However, we recently identified an ~20-bp footprint flanking AGCW sites within which C/G transversions occurred independently of MMR (9). In remarkable convergence with this footprint size, Woodruff et al. (46) showed that LP-BER produces excision patches centered on the excised AP site that are ~20 nucleotides long; they further showed that LP-BER predominates over single-nucleotide BER in living cells. AGCW motifs are the only hotspots where AID can deaminate either DNA strand with comparable efficiency in vivo (8, 9, 47). This leads us to update the Ig hypermutation model and propose that MMR-independent C/G transversions occur via simultaneous G1-phase LP-BER of deaminations that have accumulated in a by a combination of both polymerase encounter with AP sites and SAMHD1-induced dNTP paucity.

The hypermutation phenotypes of Ung<sup>−/−</sup>, Rev1<sup>−/−</sup>, and Polb<sup>−/−</sup> B cells suggest that most C/G transversions are created by translesion bypass of AP sites, with a minority (~15%) created by simple misincorporation at C/G during MMR, long-patch BER, or single-nucleotide BER (10, 13, 35). We speculate that the substantially reduced C/G transversion and increased C/G transition mutation in Samhd1<sup>ΔΔ</sup> cells is again most simply explained by bias toward purine misincorporation over pyrimidine misincorporation—this time, due to UNG2-deleted lesion bypass events, as illustrated in Fig. 4. It is possible that SAMHD1’s exonuclease activity and/or its capacity to recruit the endonuclease Cpf1 (41, 42) induces Ig mutation, rather than its dNTPase activity. The production of double-strand breaks (DSB) downstream of AID is critical for Ig class switching (1), but DSB formation plays a minor role in V-region hypermutation (43–45). In our experiments, insertions and deletions, which likely arise from DSB, were not discernably affected by Samhd1 inactivation (Table 1) and clustered near AGCW motifs regardless of Samhd1 genotype (Fig. S4).

**Fig. 3.** Occurrence of C/G transitions (x axes) or transversions (y axes) outside or within AGCW motifs. The mean (±SEM) number of C/G transitions or transversions per 560-bp sequence were calculated on a per host basis, as in Fig. 2, for PCG located outside AGCW motifs (A) or AG located inside AGCW motifs (B). Significant differences to the vector-transduced Samhd1<sup>+</sup> means are indicated with asterisks (one-way ANOVA, Holm-Sidak test); vertical and black for C/G transversions; horizontal and gray for C/G transversions. All means lying to the right of the vertical dotted line represent significantly elevated AGCW transition frequencies, and all means lying below the gray dotted line represent significantly reduced AGCW transition frequencies, relative to the Samhd1<sup>+</sup> Msh<sup>−/−</sup> data.

**Fig. 4.** A completely G1-phase model of Ig hypermutation that incorporates SAMHD1 and a major role for LP-BER. If both strands of an AGCW motif are deaminated, then LP-BER in one strand can expose an AP site in the other, whereas single-nucleotide BER cannot. At deamination sites >10 bases away from AGCW motifs, the chance that excision during LP-BER will expose another AP site in the opposite strand is much lower. Recruitment of ExoI via MutSα produces much longer excision patches (>90 bases long; ref. 61) than LP-BER’s 20-base excision patch (46). In Ung<sup>−/−</sup> cells, these long excision patches have a roughly equal chance of exposing an AP site in the opposite strand regardless of where the deamination site that recruited MutSα lies. SAMHD1 activity determines the dNTP milieu, thus dictating the misincorporation spectrum for both MMR and LP-BER. This model explains (i) the hyperfocusing of Ig mutation on AGCW motifs that occurs only in Ung<sup>−/−</sup> MMR-deficient B cells and not in Ung2/MMR-deficient cells (9, 21), (ii) the near complete MMR dependence of C/G transition mutation at sites >10 bases from AGCW motifs (9), (iii) increased OG transitions, and (iv) substantially decreased A/T and C/G transversions in Samhd1<sup>ΔΔ</sup>−/− B cells (this work).
opposite strands of AGCW sites, as illustrated in Fig. 4, without any requirement for AP sites to persist into S phase. A key strength of this model is its ability to explain why Ung2 is at its most error-prone in regions where AGCW motifs are concentrated.

**Similarities Between Ig Hypermutation and Intracellular Virus Restriction Mechanisms.** Cell cycle turnover of RNR and the dNTPase activity of Samhd1 are intracellular antiviral mechanisms that evolved long before the appearance of adaptive immunity (48). The APOBEC3 family of AID paralogs also restrict viral infection by deaminating viral genomes; Ung2 and APE activities downstream of APOBEC3 can contribute to viral restriction (49, 50). AID/APOBEC-like deaminases occur across metazoan, dicyostelidila, and even algae, and may have evolved in an arms race with viruses and retro elements (51). Here, we’ve shown that Samhd1 is another virus restriction factor that contributes to the mutagenicity of AID—by promoting transversion mutation downstream of AID. Transition mutation is the most common form of point mutation in the genome. Codon degeneracy has evolved to accommodate this, which means that transition mutations are generally more mutagenic than transversion mutations (52), implying that Ig point mutations likely to cause the greatest changes in antibody binding characteristics are promoted by Samhd1. For example, the generation of high-affinity anti-phenylalanine antibodies in BALB/c mice predominantly involves H34N and Y36F mutations of V_{	ext{H}}\_\text{C}1/V_{	ext{H}}\_\text{J}5 rearrangements: a C to T transversion and an A to T transversion, respectively (53). It’s conceivable that G1-phase MMR can also act as an antiviral mechanism, because repeated recruitment of Exol to viral genomes that misincorporated G1-phase dNTPs would inhibit viral replication. Given that G1-phase nicking by MutLα in B cells does not discriminate between mutated and nonmutated DNA strands (5), it is further conceivable that G1-phase MMR evolved to attack viral genomes and trigger apoptosis, rather than to repair errors, and was then coopted by Ig hypermutation, along with Ung2 and Samhd1, as part of a preexisting suite of ancient intracellular antivirus mechanisms.

**Materials and Methods.**

**Mice.** Male C57BL/6 host mice were initially purchased from Animal Resources Centre (Canning Vale, Western Australia) and later from Australian BioResources (Moss Vale, New South Wales), and were used in experiments when 8–16 wk old. Msh2/++, Rag1−/−, Igh−/−, Igl−/−, Igh−/−Igl−/−, and C57BL/6 interspecific Fucci-mouse (29, 31) were interbred and maintained on a C57BL/6 background under SPF conditions in the Centenary Animal Facility.

**Samhd1Δ25/Δ25 mice were produced by the Mouse Engineering Garvan/ABR (Mega) Facility (Moss Vale and Sydney, Australia) by CRISPR/Cas9-mediated gene targeting in C57BL/6 mouse embryos following established molecular and animal husbandry techniques (55). To minimize off-target genome modifications, the double-nicking approach employing the single-strand cleaving mutant of the Cas9 endonuclease (Asp10Ala) was employed (56). For this purpose, paired single-guide RNAs (sgRNAs) were designed to target within the first coding exon (Exon 1) of Samhd1. The sites used were CCTCAAGGGTGGCCTGCTAGG and GCTAAGGGACCGCGTCGAG (protospacer-associated motifs = PAMs underlined).

To target Samhd1, a solution consisting of the two sgRNAs (15 ng/μL each) and full-length, polyadenylated Cas9 mRNA (30 ng/μL) was prepared and microinjected into the nucleus and cytoplasm of SW40 × C57BL/6 myocytes. Microinjected embryos were cultured overnight and introduced into pseudogestant foster mothers. Pups were screened by PCR across the two target sites and Sanger sequencing to detect those with modifications to Samhd1. Independent founders carrying 25- and 41-bp frame-shift deletions within Exon 1 were selected, backcrossed to wild-type SW40 (C57BL/6) mice four times, and the progeny was intercrossed to derive homozygous Samhd1Δ25/Δ25 and Samhd1Δ41/Δ41 lines.

All work was carried out with approval from and oversight by the Royal Prince Alfred Hospital Animal Welfare and Biosafety Committees, in accordance with NSW and Federal Australian legislation and the Australian Code for the Care and Use of Animals for Scientific Purposes (57).

Plasmids and Retroviruses. pMiG-based retroviruses expressing mKO2-cdt fusion protein (whose accumulation is restricted to G2/M-phases by the degron from human Cdt1), EGFP, or ugi-EGFP fusion protein have been described (6). All CDNs were cloned into the pMiG vector (58) using conventional techniques and verified by Sanger sequencing (Macrogen Inc.). Ecotropic viral supernatants were produced by calcium phosphate co-transfection of the Platinum-E retroviral packaging cell line (Cell Biolabs, Inc.) as described (9). Culture medium (DMEM, 10% bovine calf serum, 1 mM sodium pyruvate, 2 mM L-alanyl-L-glutamine dipeptide, penicillin/streptomycin) was then used for 12 h, at which point supernatants were harvested at 60 h, 0.45-μm filtered, snap frozen in liquid nitrogen, and stored at −70 °C.

**Transduction and Adoptive Transfer of SW40.**

**Splenocytes and Mutation Analysis.** Activation of mouse splenocytes with recombinant CD40L for 24 h, followed by transduction in culture for a further 48 h has been described (9). GFP+ cells were sorted using a BD FACS Aria II or BD influx sorter, mixed with sheep red blood cells (SRBC; Applied Biological Products Management) conjugated to hen egg lysozyme (HEL; Sigma-Aldrich) in B cell medium (54) and injected into host tail veins as a bolus of ±105 HEL-binding B cells plus ±107 HEL-SRBC into 8- to 12-wk-old male hosts that had been primed i.p. 7 d prior with ±105 SRBC in PBS, as described (34). Six days after adoptive transfer, host spleens were harvested, depleted of RBC using Histopaque 1083 fraction (Sigma-Aldrich), and GFP+ HEL-binding cells were sorted using a BD FACSAria II or BD influx, one cell per well into 96-well PCR plates (9). dNTPs were then quantified using 15 μL per well of ice-cold Mg-free 1× Taq DNA polymerase buffer (Promega “Go-Taq” buffer) supplemented to include 0.1 mM EDTA, 0.25 mg/mL proteinase K (Roche Diagnostic). Single-cell nested PCR of the singular gene-targeted SW40 VDJ, V\_\text{D}1 rearrangement present in each cell was performed as described (25). Single allele mutation data were collected from up to 47 single cells using direct sequencing of single-cell PCR products, as described (25), except that the sequencing primer (jol27: 5′-S'ACTC AACCA CCAT CACA C3-3′) was positioned further 3′ to the start codon than previously, enlarging the sequence window from 523 bases to 560 bases. Each treatment involved at least three individual donors and hosts. Mutation data were analyzed by one-way ANOVA and Holm-Sidak’s post hoc multiple comparisons tests with Prism 7 for MacOS (GraphPad Software, Inc), using the mean mutation of each host’s sampled cells as a single data point (9).

**Western Blot Analysis.** Cells from Fucci-red/Fucci-green double-transgenic mice (31) were cultured in 20 μg/mL S. typhosa LPS (Sigma-Aldrich) plus 20 ng/mL mouse IL-4 (BD Biosciences) for 3 d to induce robust B cell proliferation. B cells from Fucci mice express an orange fluorescent fusion protein (“mKO2-cdt”) in G1 phase of the cell cycle or a green fluorescent fusion protein (“mAG-mgm”) in G2, S, and M phases (31). Transduced cells were sorted into mKO2-mAG (G1 phase) and mKO2-mAG (S/G/M phase) fractions using a BD Influx sorter. Proteins were extracted from cells by vortexing cell pellets at 2 × 106 cells per mL in Laemmli sample buffer (0.1% SDS, 5% mercaptoethanol, 2% SDS, 5% glycerol, 0.0005% bromophenol blue, 63 mM Tris Cl, pH 6.8), then incubating for 30 min at 20 °C with 250 units/mL Benzonase nuclease (Millipore). Extracts were electrophoresed using 4–12% Bolt Bis-Tris Plus polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes using a Novex semi-dry blower (Invitrogen) according to manufacturer protocols. Membranes were blocked for 1 h with 5% (wt/vol) skim milk powder in TBST [0.5% (wt/vol) NaCl, 0.1% (vol/vol) Tween-20 and 0.1 M Tris HCl, pH 7.4] at room temperature, then incubated overnight at 4 °C with mouse anti-Samhd1 IgG2b monoclonal OTI1A1 (Abcam), goat anti-R2 IgG polyclonal E-16 (Santa Cruz Biotechnology), or rabbit anti-R1 IgG monoclonal EPR4883 (Abcam) in skim milk/TBST. Washed membranes were incubated with species-appropriate secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 1 h at 20 °C in skim milk/TBST, then washed again and incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher). Luminescence was recorded using a ChemiDoc MP (Bio-Rad).

**dNTP Quantitation.** Samhd1Δ41/Δ41 or Samhd1Δ25/Δ25 placentomes were cultured with LPS plus IL-4, for 24 h as above. Activated cells were then transduced with fresh retroviral supernatants of pMiG-derived retroviruses that expressed mKO2-cdt; cells expressing mKO2-cdt fluoresce red when they are in G2/M phase (6, 31). Two days later, orange-fluorescent (i.e., G1-phase) cells were sorted using a BD influx sorter into ice-cold bovine serum. Total dNTPs were extracted from PBS-washed sorted cells using ice-cold 60% aqueous methanol, as described (59). Resting or nonfractionated proliferating Samhd1Δ41/Δ41 or Samhd1Δ25/Δ25 spleenocytes depleted of RBC and dead cells using density gradient centrifugation over Histopaque 1083 (Sigma-Aldrich) were also extracted. dNTPs were then quantified using an HIV reverse transcriptase primer extension assay, as described (59).
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