SHLD1 is part of the Shieldin (SHLD) complex, which acts downstream of 53BP1 to counteract DNA double-strand break (DSB) end resection and promote DNA repair via non-homologous end-joining (NHEJ). While 53BP1 is essential for immunoglobulin heavy chain class switch recombination (CSR), long-range V(D)J recombination and repair of RAG-induced DSBs in XLF-deficient cells, the function of SHLD during these processes remains elusive. Here we report that SHLD1 is dispensable for lymphocyte development and RAG-mediated V(D)J recombination, even in the absence of XLF. By contrast, SHLD1 is essential for restricting resection at AID-induced DSB ends in both NHEJ-proficient and NHEJ-deficient B cells, providing an end-protection mechanism that permits productive CSR by NHEJ and alternative end-joining. Finally, we show that this SHLD1 function is required for orientation-specific joining of AID-initiated DSBs. Our data thus suggest that 53BP1 promotes V(D)J recombination and CSR through two distinct mechanisms: SHLD-independent synopsis of V(D)J segments and switch regions within chromatin, and SHLD-dependent protection of AID-DSB ends against resection.
Two programmed DNA double-strand break (DSB) repair processes participate in the generation of antigen receptor diversity in adaptive immune cells—V(D)J recombination that occurs in developing B and T lymphocytes and class switch recombination (CSR) that takes place in antigen-activated mature B cells. V(D)J recombination is a cut and paste mechanism that assembles variable region exons encoding the antigen-binding domains of the B and T cell receptors. It is initiated by recombination activating gene proteins RAG1 and RAG2 (forming the RAG endonuclease) which introduce DSBs between variable (V), diversity (D), and joining (J) segments and their flanking recombination signal sequences (RSSs). RAG-generated DSBs are subsequently repaired by proteins of the non-homologous end-joining (NHEJ) pathway within a so-called post-cleavage complex that is thought to participate in the stabilization and processing of DNA ends. MOD2A/XLF modifies antibody effector functions by replacing the isotype expressed from IgG to IgM, IgA, or IgE. At the DNA level, this is achieved by a deletional recombination event at the Igk locus between a donor (Sp) and an acceptor repetitive switch region (Sx) that brings into proximity the V region and the exons encoding a new constant region thus allowing the expression of an antibody with a different isotype but with the same specificity. During CSR, activation-induced cytidine deaminase (AID) deaminates cytosines into uracils in transcribed S-regions, which are converted to DSBs with the help of base excision and mismatch repair proteins. CSR is completed by fusing donor and acceptor S-region DSB ends by NHEJ and, in its absence, by alternative end-joining (alt-NHEJ) that is more biased to use longer junctional micro-homologies (MHs). In contrast to CSR, V(D)J recombination (alt-NHEJ) that is more biased to use longer junctional micro-homologies (MHs), is more reliant on NHEJ for the repair of RAG-DSBs and is comprised of REV7/MAD2L2, SHLD1, SHLD2, and SHLD3. Epistasis between SHLD1-RIF1 and the SHLD complex is remarkable in multiple NHEJ reactions, including CSR, the fusion of telomeres lacking TREC, and the repair of DSBs in BRCAl-deficient cells treated with PARP inhibitors. In AID-inducible B cell lines, loss of any of the SHLD complex members similarly impairs CSR that is associated with enhanced DSB end resection and the accumulation of chromosomal breaks at immunoglobulin switching sites. In mice, loss of REV7 or SHLD2 does not impact lymphocyte development while severely compromising CSR. The recruitment of SHLD to DSBs is mediated by the association of SHLD3 to chromatin-bound RIF1. SHLD3 also interacts directly with REV7 that bridges SHLD2 and SHLD1, with SHLD2 possessing a ssDNA-binding activity that is essential to inhibit resection. SHLD1 is a small (205 amino acid residues) low-abundance protein that lies at the very tip of the complex and for which clear functions have not yet been attributed.

Here, we generate mice and cells deficient for SHLD1, XLF, and XRCC4 to test the role of SHLD1 in antigen receptor diversification. We show that SHLD1- and SHLD1/XLF-deficient mice have normal lymphocyte development and perform robust RAG-DSB repair, demonstrating that SHLD1 is dispensable for V(D)J recombination. By contrast, we show that SHLD1 is essential to limit the extent of DNA end resection during CSR, providing an end-protection mechanism that enables both NHEJ and alternative end-joining pathways to functionally operate at AID-induced DSBs. Finally, we show that this end-protection function is required for the orientation-specific joining of AID-initiated DSBs. Thus, while SHLD1 is dispensable for V(D)J recombination, it is essential for productive CSR by controlling the processing of AID-DNA ends during CSR and alternative end-joining repair.

Results

Shld1−/− and Shld1−/− Xlf−/− mice are viable and show no overt developmental phenotype. To elucidate SHLD1 function in vivo, we analyzed Shld1 knockout mice (Shld1em1(IMPC)Wtsi) referred to hereafter as Shld1−/−) that we generated by CRISPR/Cas9-mediated deletion of the Shld1 coding sequence (Supplementary Fig. 1a–d). Shld1−/− mice were born at the expected Mendelian frequencies, were fertile and displayed no developmental abnormalities (Fig. 1a, b and Supplementary Fig. 1e, f).

Total cell numbers in the thymus and spleen of 6- to 8-week-old Shld1−/− mice were, on average, similar to those of wild-type (WT) (Fig. 1c, d). Flow cytometry revealed that the distribution of progenitor (pro-) B (CD19+CD43+B220+IgM−),...
**Fig. 1** *Shld1*−/− and *Xlf*−/− mice are viable and show no overt developmental phenotype. **a** Number of live-born mice obtained from crosses between *Shld1*−/− *Xlf*+/+ mice (left) or *Shld1*+/− *Xlf*−/− mice (right). Expected versus observed numbers were used to calculate one-sided Chi-square. *Shld1*−/− *Xlf*+/+ cross: n = 83 observed live-born mice; *Shld1*+/− *Xlf*−/− cross: n = 68 observed live-born mice. **b** Total body weight (in grams) of adult mice (males and females, 6–8-week-old) from the different genotypes as indicated. Bars represent mean ± s.e.m., n = 9 independent mice (*Shld1*−/−), n = 6 (53bp1−/−), n = 19 (*Shld1*+/− *Xlf*+/+), n = 5 (*Xlf*+/+), n = 6 (*Shld1*+/− *Xlf*−/−), n = 16 (*Shld1*−/− *Xlf*−/−) two-sided Wilcoxon–Mann–Whitney test (*p = 0.047 (Xlf+/+ vs Shld1−/−); p = 0.0268 (Xlf−/− vs Shld1−/− *Xlf*−/−)). **c** Total thymocytes numbers. Bars represent mean ± s.e.m., n = 10 (WT; *Shld1*−/−), n = 4 (53bp1−/−), n = 8 (*Xlf*+/+), n = 9 (*Shld1*+/− *Xlf*−/−), two-sided Wilcoxon–Mann–Whitney test (*p = 0.014; **p = 0.008; ***p = 0.0005). WT: dark blue, *Shld1*−/−: red, 53bp1−/−: light blue, *Xlf*−/−: green, *Shld1*+/− *Xlf*−/−: yellow. **d** Total splenocytes numbers. Bars represent mean ± s.e.m., n = 13 (WT), n = 10 (*Shld1*−/−), n = 4 (53bp1−/−), n = 11 (*Xlf*+/+), n = 11 (*Shld1*+/− *Xlf*−/−), two-sided Wilcoxon–Mann–Whitney test (*p = 0.0396; ***p = 0.002; **p = 0.0008; ***p < 0.0001). **e** Analysis of B cell development. Representative FACS analysis of bone marrow using B cell markers. **f** Ratio of CD43−*B220*−*CD19*−*IgM*+ pre-B cells versus CD43+*B220*+*CD19*+*IgM*+ pro-B cells. Bars represent mean ± s.e.m., n = 12 (WT), n = 10 (*Shld1*−/−), n = 4 (53bp1−/−), n = 11 (*Xlf*+/+), n = 11 (*Shld1*+/− *Xlf*−/−), two-sided Wilcoxon–Mann–Whitney test (**p = 0.0014 (Xlf+/+ vs Shld1−/− *Xlf*−/−); *p = 0.0011 (WT vs 53bp1−/−); **p = 0.0004 (Shld1−/− vs 53bp1−/−)). **g** Analysis of T cell development. Representative FACS analysis of thymus using T cell markers. **h** Ratio of CD4−*CD8*−*CD44*−*CD25*− (DN4) cells versus CD4−*CD8*+*CD44*−*CD25*− (DN3) cells. Bars represent mean ± s.e.m., n = 10 (WT; *Shld1*−/−), n = 4 (53bp1−/−), n = 8 (*Xlf*+/+), n = 9 (*Shld1*+/− *Xlf*−/−), two-sided Wilcoxon–Mann–Whitney test (**p = 0.0044 (Xlf+/+ vs 53bp1−/−); **p = 0.002 (WT vs 53bp1−/−); **p = 0.002 (Shld1−/− vs 53bp1−/−); ***p < 0.0001 (Shld1−/− vs Shld1−/− *Xlf*−/−)). n.s. non-significant (p ≥ 0.05), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Source data are provided as a Source Data file.
precursor (pre-) B (CD19+/CD43−/B220+/IgM−), newly-generated immature B (IgM+/B220low) and recirculating B (IgM+/B220high) cells in Shld1−/− bone marrow were similar to those of WT littermates (Fig. 1e and Supplementary Table 1). In addition, Shld1−/− mice contained splenic CD19+IgM− total B cells, Follicular (B220+/CD93−/CD23−/CD21+) and Marginal Zone (B220+/CD93−/CD23−/CD21high) B cells at distributions and numbers equivalent to WT (Supplementary Fig. 1g-i and Supplementary Table 2). Similarly, the distribution of CD4−CD8− (double-negative, DN), CD4+CD8+ (double-positive, DP), and CD4+CD8− or CD4−CD8+ (single-positive, SP) Shld1−/− thymocytes was comparable to those of WT littermates (Fig. 1g and Supplementary Table 3) and Shld1+/− mice contained normal populations of splenic CD3+TCR8+ T cells (Supplementary Fig. 1j) and T cell development in the absence of XLF.

H2AX, or 53BP1, SHLD1 is dispensable for overall mammalian recombination. Atm embryonic lethality and 53bp1−/− mice harbored impaired B and T cell development, with a moderate block at the CD43+ B220−/− IgM−/− pro-B cell (Fig. 1f) and CD4−CD8−CD44−CD25−/− DN3 thymocyte (Fig. 1h) stages during which antigen receptor gene assembly is initiated suggests that V(D)J recombination is normal in Shld1−/− developing lymphocytes. Consistent with normal V(D)J recombination, we generated Shld1−/− mice with Xlf−/− mice38,39 to generate Shld1−/− Xlf−/− animals (Supplementary Fig. 1b, d). Shld1−/− Xlf−/− mice were born at the expected Mendelian frequencies (Fig. 1a) and did not display growth defects (Fig. 1b and Supplementary Fig. 1f), indicating that in contrast to ATM, H2AX, or 53BP1, SHLD1 is dispensable for overall mammalian development in the absence of XLF.

To test for a potential functional redundancy between SHLD1 and XLF during lymphocyte development, we analyzed lymphocyte differentiation in Shld1−/− Xlf−/− mice. Strikingly, absolute numbers of thymocytes, total splenocytes as well as splenic B cells and T cells in Shld1−/− Xlf−/− mice were comparable to those of Xlf−/− mice (Fig. 1c, d, Supplementary Fig. 1g, j, and Supplementary Tables 2, 3). In addition, in Shld1−/− Xlf−/− mice, there was no exacerbated block in B cell development in the bone marrow and T cell development in the thymus, particularly at pro-B or DN3 populations in which V(D)J recombination occurs (Fig. 1e-h and Supplementary Tables 1, 3). Together, these results show that SHLD1 is dispensable for lymphocyte development in the sensitized XLF-deficient background.

53BP1 displays SHLD1-independent functions during V(D)J recombination. Consistent with the analysis of lymphocyte progenitor populations, semi-quantitative PCR analysis of Dβ1 to Jβ1 and DJβ2 to Jβ2 rearrangements in thymocytes from WT, Shld1−/−, Xlf−/−, and Shld1−/− Xlf−/− mice showed similar levels of rearrangements (Fig. 2a-c and Supplementary Fig. 2a, b). Notably, DJβ1 to Jβ1 and DJβ2 to Jβ2 rearrangements were also robustly detected in 53bp1−/− mice (Fig. 2a-c and Supplementary Fig. 2a, b). We next tested whether SHLD1, similar to 53BP116, might facilitate the joining of more distant V(D)J gene segments. We performed quantitative PCR assays of partial (Dβ2-Jβ1 and Dβ1-Dβ2) and complete (Vβ5-Dβ1 and Vβ2-Dβ8) rearrangements at the Tcrδ locus (Fig. 2d, e). Consistent with previous findings, we found that short-range (Dβ2-Jβ1 and Dβ1-Dβ2) rearrangements were similar to or even more abundant in 53bp1−/− than in WT thymocytes (Fig. 2d, e). In contrast, long-range Vβ6-to-Dβ6 recombination was significantly reduced in 53bp1−/− thymocytes (Fig. 2d, e). Strikingly, Shld1−/− thymocytes showed similar levels of rearrangements as WT thymocytes (Fig. 2d, e), demonstrating that SHLD1 is dispensable for long-range V(D)J recombination.

To more robustly quantify V(D)J recombination, we generated viral-Abl kinase (v-Abl) transformed pro-B cell lines from wildtype (WT) and Shld1−/− mice40. Treatment of v-Abl transformed pro-B cells with a v-Abl kinase inhibitor (hereafter named ABLki) leads to G1 cell cycle arrest, the rapid induction of RAG1/2 gene expression, and rearrangement of the Igk locus or introduced V(D)J recombination reporter substrates49. PCR analysis of IgkV6−23-J1 rearrangements revealed normal levels of recombination in Shld1−/− pro-B cells (Fig. 2f and Supplementary Fig. 2e). In addition, quantitative flow cytometry analysis of v-Abl pro-B cells carrying the pMX-RSS-GFP/ires-cD4 retroviral recombination substrate (pMX-INV) that allows for GFP expression upon successful chromosomal inversionsal RAG-mediated recombination revealed similar levels of recombination in WT and Shld1−/− v-Abl cells (Fig. 2h and Supplementary Fig. 2d, e). We next employed CRISPR/Cas9-mediated gene editing to delete Shld1 from WT and Xlf−/− v-Abl pro-B cells38,41, generating Shld1−/− and Xlf−/− Xlf−/− pro-B cell clones. In addition, we deleted Xlf from 53bp1−/− v-Abl pro-B cells21, generating Xlf−/− 53bp1−/− pro-B cell clones. Consistent with the previous work20,21,38,41, both PCR analysis of Igk rearrangements and flow cytometry analysis of pMX-INV recombination revealed robust recombination in XLF-deficient pro-B cells and a severe defect in V(D)J rearrangements in 53BP1/XLF-doubly deficient pro-B cells (Fig. 2f-i and Supplementary Fig. 2e). Strikingly, in contrast to 53BP1/XLF-deficient pro-B cells, SHLD1/XLF-deficient pro-B cells perform robust V(D)J recombination at the Igk locus as well as at the pMX-INV substrate (Fig. 2f-i and Supplementary Fig. 2e). Together, these results demonstrate that SHLD1 is dispensable for V(D)J recombination even in the absence of XLF and that 53BP1 has unique functions during V(D)J recombination that are independent of SHLD1.
Fig. 3a), suggesting that 53BP1 also fulfills SHLD1-independent functions during CSR.

To investigate further the function of SHLD1, we have tested a series of deletion and substitution mutations for their ability to rescue CSR in stimulated Shld1−/− primary B cells (Fig. 3f). SHLD1 is encoded by two exons, exon 2 that encodes for amino acids 1 to 60 and exon 3 that encodes for amino acids 61 to 205. The SHLD1 N-terminus (1–70) is predicted to be intrinsically disordered while its C-terminal part is more structured and may harbor one- or two-winged helix domains. To investigate the function of the N- and C-terminal regions of SHLD1, we tested WT SHLD1 as well as SHLD1Δ1–60, SHLD1Δ1–70, and SHLD1Δ71–205 deletion mutants (Fig. 3f). In addition, we generated a series of deletion and combined substitution mutations at strongly conserved residues (SHLD1Δ18–21) and SHLD1Δ195A, R96A, S98A, L99A, F102A, Y103, SHLD1L125A, I129A, L132A, SHLD1Y140A, M147A, V150A, I151A, D155A, F169A, and SHLD1P186A, G187A, L188A, S189A, D191A, I192A, F195A, L196A, L197A (referred to as mutants 1, 2, 3, and 4, respectively (Fig. 3f, and Supplementary Fig. 4a). We found that SHLD1Δ1–60 and SHLD1Δ1–70 partially restored CSR in stimulated Shld1−/− cells while SHLD1Δ71–205 and any of the tested SHLD1 proteins mutated for highly conserved residues in the C terminus failed to rescue CSR in stimulated SHLD1-deficient B cells (Fig. 3g). Of note, we could not detect SHLD1Δ71–205 by Western Blot suggesting that this mutant form of SHLD1 is unstable (Supplementary Fig. 4b). In contrast, SHLD1Δ18–21 restored CSR to levels equivalent to those measured after complementation with WT SHLD1 indicating that these four amino acid residues, recently implicated in CTC1-STN1-TEN1
Fig. 2 53BP1 displays SHLD1-independent functions during V(D)J recombination. a Schematic representation of the murine Tcrβ locus. Variable (V), diversity (D), joining (J), and constant (C) segments are shown. The star marks a pseudogene (Jβ2.6). b PCR analysis of DJ1 to Jβ1 rearrangements in Rag2−/−, WT, Shld1−/−, 53bp1−/−, Xlf−/−, and Shld1−/−Xlf−/− thymocytes. The arrows indicate the region in germline configuration. The bands marked by arrowheads represent rearrangements of DJ1 to one of the Jβ segments. IL2 gene PCR was used as a loading control. c PCR analysis of DJ2 to Jβ2 rearrangements. d Schematic representation of the mouse Tcrα locus with the four different TcR four rearrangements depicted individually below. e Relative frequency of TcR locus rearrangements in total thyocytes from WT, Shld1−/− and 53bp1−/− mice. Quantitative assessment of genomic DNA rearrangements of Dα1 to Dα2, Dα2 to Jα1, and Vβ2 and Vδ5 to (D)Jβ1 genes were performed by qPCR and normalized to the signal of the non-rearranging DNA 3′ of Jβ2. Histograms represent the average relative WT to mice for each rearrangement ± s.e.m., (n = 5 independent samples except 53bp1−/− for which n = 4), two-sided Wilcoxon–Mann–Whitney test (p-value = 0.0179). f Schematic of in situ FACS gating of Iκγα1−/−Jβ2 rearrangement (position of primers (arrows) used to assay coding joint are shown). g Semi-quantitative nested PCR analysis of Igκα1−/−Jβ2 coding joint (CJ) in αβ T cells from wildtype, and 53bp1−/− mice. Quantitative assessment of genomic DNA rearrangements of Dα1 to Dα2, Dα2 to Jα1, and Vβ2 and Vδ5 to (D)Jβ1 genes were performed by qPCR and normalized to the signal of the non-rearranging DNA 3′ of Jβ2. Histograms represent the average relative WT to mice for each rearrangement ± s.e.m., (n = 5 independent samples except 53bp1−/− for which n = 4), two-sided Wilcoxon–Mann–Whitney test (p-value = 0.0179). h Schematic of in situ FACS gating of Iκγα1−/−Jβ2 rearrangement (position of primers (arrows) used to assay coding joint are shown). i Bar plot showing V(D)J recombination efficiency of pmx-INV recombination substrate. j Pair-wise comparison of SHLD1 and 53BP1 mutant B cells (inserts). (WT: clone #O38 (n = 4 independent experiments), #G8B (n = 4), #12095 (n = 11), 53bp1−/−: clones #9999 (n = 7), #110 (n = 3), #BP95-2 (n = 3) and #BP95-5 (n = 3); Shld1−/−: clones #O32 (n = 3), #307 (n = 6) and #O44 (n = 3); 53bp1−/−: clones #1648B (n = 10) and #X95-3 (n = 2); Shld1−/−Xlf−/−: clones #XO2-8 (n = 4) and #XO2-24 (n = 6); 53bp1−/−: clones #9X1 (n = 6) and #9X2 (n = 6). n.s. non-significant (p ≥ 0.05), *p < 0.05, ***p < 0.0001. Source data are provided as a Source Data file.

(CST)-Polo-primase binding42, are dispensable for CSR (Fig. 3g). Together, these results show that the C-terminal (i.e., amino acids 71 to 205) portion of SHLD1 forms the core region of SHLD1 that is required for CSR. Finally, we generated substitution mutations at residues Y103 (SHLD1Y103F) and K203 (SHLD1K203A) that are invariably conserved in diverse species and represent potential phosphorylation and ubiquitylation sites, respectively43. We found that SHLD1K203A fully rescued CSR in SHLD1-deficient B cells. By contrast, SHLD1Y103F only partially restored IgG1 class switching suggesting that this residue might be implicated in SHLD1 function during CSR. SHLD1Y103F and SHLD1K203A were still able to interact with REV7/MAD2L2 (Supplementary Fig. 4b), suggesting that the Y103 residue and the SHLD1 N-terminus are dispensable for SHLD1 binding to upstream SHLD complex components.

53BP1-SHLD1 and NHEJ synergize during class switch recombination. We next investigated potential synergistic or epistatic functions between SHLD1 and XLF during CSR. Strikingly, Shld1−/− Xlf−/− mice displayed a significant increase of serum IgMs as compared to wildtype and single mutant mice that was associated with an almost complete lack of total IgG and IgG1 antibodies, indicating that CSR is highly defective in SHLD1/ XLF-deficient activated B cells (Fig. 3a). Consistent with this, class switching to IgG1, IgG2b and IgG3 was almost completely abrogated in SHLD1/XLF-deficient stimulated splenic B cells (Fig. 3b, c and Supplementary Fig. 3a, b) without noticeable defects in cell proliferation, Aid expression, and IgM germline transcripts levels (Fig. 3e and Supplementary Fig. 3d–f). In addition, defective CSR in Shld1−/−, Xlf−/−, and Shld1−/− Xlf−/− splenic B cells induced to switch to IgG1 was associated with a large population of IgMlow IgG1− B cells that inversely correlated with the levels of switched IgG1+ cells in the respective mutant cells (Fig. 3b, d). Together, these results demonstrate that SHLD1 and XLF act synergistically to promote productive CSR.

To probe the capacity of SHLD1 to promote CSR in the absence of XRCC4, another NHEJ factor, we generated SHLD1-proficient and SHLD1-deficient mice that harbored two copies of a loxP-flanked (floxed) Xrc4 allele (Xrc4f/f)44 plus a transgene that drives Cre recombinase expression in late stages of the B lineage from a CD21 promoter45, termed CD21-creTg Xrc4f/f mice and Shld1−/− CD21-creTg Xrc4f/f mice respectively. Consistent with previous work46, flow cytometry assays for surface IgG1 and IgG2b revealed substantial switching by appropriately stimulated CD21-creTgXrc4f/f B cells with average levels of IgG1 and IgG2b that were at about 25% of those of WT controls (Fig. 3h). Similar to SHLD1/XLF-deficient B cells, class switching to IgG1 or IgG2b was significantly diminished in SHLD1/XRCC4-deficient stimulated B cells and was associated with a large population of IgMlow IgG1− B cells (Fig. 3i, j). Thus, SHLD1 supports CSR in the absence of XRCC4.

We next investigated the capacity of 53BP1 to promote CSR in the absence of XRCC4. We used CRISPR/Cas9 gene editing to delete Xrc4 from wildtype, Shld1 knockout, and 53bp1 knockout CH12F3 (CH12) B cell clones, generating Xrc4−/−, Shld1−/− Xrc4−/−, and 53bp1−/− Xrc4−/− B cell clones, respectively. We established that combined loss of SHLD1 and XRCC4 or 53BP1 and XRCC4 almost completely abrogates the production of IgA-switched CH12 cells as measured by flow cytometry after stimulation with anti-CD40, IL-4, and TGF-β (Fig. 3i). Together, these results indicate that 53BP1-SHLD1 is essential for “productive” CSR—that is the ability of switched cells to express a new immunoglobulin isotype at the cell surface – in both NHEJ-proficient and NHEJ-deficient B cells.

SHLD1 promotes productive CSR in XLF-proficient and XLF-deficient cells by limiting DNA end resection. The most severe CSR defect of B cells deficient for SHLD1 and XLF could be due to a lack of repair of AID-induced DSBs or, as another non-mutually exclusive possibility, to hyper-resection leading to deletions of coding or regulatory regions essential for the expression of a functional switched immunoglobulin. To distinguish between these possibilities, we first measured the levels of IgG-DSBs by performing IgM locus-specific DNA–FISH on chromosome spreads prepared from wildtype, Shld1−/−, Xlf−/−, and Shld1−/− Xlf−/− splenic B cells stimulated to undergo IgM-to-IgG1 class switching (Fig. 4a). Analysis of metaphase spreads revealed low levels of IgM locus-associated chromosomal breaks in WT B cells (1.2%). Xlf−/− and Shld1−/− B cells contained a statistically significant increase in aberrant metaphases (4.1% and 9.4% respectively) that could reflect either a slower repair or a partial lack of repair of AID-induced DSBs in these cells (Fig. 4a, b and Supplementary Table 4). Interestingly, despite an almost complete absence of IgG1 switched cells 4 days after stimulation, Xlf−/− Shld1−/− B cells contained similar levels of Igm-associated chromosomal breaks (8.8%) as SHLD1 single-deficient cells (Fig. 4b). Similarly, metaphase spreads from Shld1−/− CD21-creTg Xrc4f/f stimulated B cells contained approximately the same level
of *Igh*-associated chromosomal breaks as metaphases from *Shld1*−/− B cells (Supplementary Fig. 5a, b and Supplementary Table 5). These results indicate that, in the absence of both NHEJ (i.e. XLF or XRCC4) and SHLD1, AID-induced DSBs might be aberrantly resolved, leading to the generation of unproductive switched-joins, rather than accumulating in the form of unrepaired DNA breaks.

To analyze AID-mediated recombining products, we extracted genomic DNA from stimulated splenic B cells and adapted a long-range PCR assay to amplify a region between the *Igh* intronic enhancer iEμ (primer EμF) and the *Igh* γ1 constant exon 6 (primer Cγ1R2) (Fig. 4c). The EμF and Cγ1R2 primers locate more than 100 kb apart and therefore do not allow amplification of the genomic sequence in the germline configuration or after AID-mediated inversional recombination between the Sμ region and the Sγ1 region. Amplification of genomic DNA from stimulated WT splenic B cells generated a clear band whose size corresponds to the expected deletional recombination products.
between the \(S_4\) region and the \(S_3\) region (i.e., from 7160 bp to 16,760 bp product sizes) (Fig. 4d). Amplification of genomic DNA from \(Xlf^{−/−}\) and \(Shld1^{−/−}\) B cells generated a weaker band of the expected recombination size that is consistent with the reduced percentage of IgG1+ cells in these cells and, instead, produced smaller PCR fragments that might correspond to resection of DNA ends prior to joining (Fig. 4d). The proportion of small PCR fragments was quite similar in SHLD1 knockout B cells (mean length 7035 bp) with 46.8% of the reads smaller than 7160 bp, which correspond to recombination products that suffered intense resection of DNA ends prior to joining (Fig. 4e, f and Supplementary Table 6). XLF-deficiency led to the generation of smaller recombination products (mean read length = 7035 bp) with 46.8% of the reads smaller than 7160 bp (Fig. 4e, f and Supplementary Table 6). Most strikingly, \(Shld1^{−/−}\) B cells harbored recombination products of various sizes that could be visualized as a smear on the agarose gel and that suggest intense resection of AID-induced DSBs in these cells (Fig. 4d and Supplementary Fig. 5c). To confirm these results, we sequenced PCR products obtained from wildtype and mutant B cells using long-read high-throughput sequencing to analyze hundreds of individual sequences from each genotype (Fig. 4e). This analysis revealed that in WT B cells the vast majority of the sequences corresponded to recombination products with 5′ and 3′ breakpoint sites mapping within the \(S_4\) core region and the \(S_3\) core region (Fig. 4e) with a mean read length of 8625 bp and with 15.6% of the reads being smaller than 7160 bp, which correspond to recombination products that suffered intense resection of DNA ends prior to joining (Fig. 4e, f and Supplementary Table 6). XLF-deficiency led to the generation of smaller recombination products (mean read length = 7035 bp) with 46.8% of the reads smaller than 7160 bp (Fig. 4e, f and Supplementary Table 6). Most strikingly, SHLD1-deficient B cells contained switched products with 66.4% of the reads smaller than 7160 bp in \(Shld1^{−/−}\) B cells (mean length = 5478 bp) and 85.3% of the reads smaller than 7160 bp in \(Shld1^{−/−}\) \(Xlf^{−/−}\) B cells (mean length = 4175 bp) (Fig. 4e, f and Supplementary Table 6). The SHLD1 promotes productive CSR in XLF-proficient and XLF-deficient B cells by antagonizing and controlling the extent of DNA end resection. ATM plays a dual role in CSR by promoting 53BP1-RIF1–XLF4–Shld1-deficient CSR and participating in CtIP-mediated nuclease activities that contribute to DNA end resection. To test the impact of ATM ablation in the context of SHLD1 deficiency, we bred our \(Shld1^{−/−}\) mice with \(Atm^{−/−}\) mice to generate doubly deficient animals. \(Shld1^{−/−}\) \(Atm^{−/−}\) mice were born at expected frequencies and displayed no additional deleterious developmental or immune phenotype compared with \(Atm^{−/−}\) mice (Supplementary Fig. 6a–d), indicating that ATM and Shld1 are epistatic during development and for immune functions such as V(D)J recombination. Analysis of class switching to IgG1 and IgG2b in stimulated naive B cells revealed similar levels of class switching in \(Shld1^{−/−}\) \(Atm^{−/−}\) cells as compared to \(Atm^{−/−}\) and \(Shld1^{−/−}\) cells (Supplementary Fig. 6e). Although not statistically significant, we noticed a small increase in the percentage of switched cells in \(Shld1^{−/−}\) \(Atm^{−/−}\) as compared to \(Shld1^{−/−}\) cells, suggesting that ATM deficiency might improve the production of functional joins in SHLD1-deficient B cells rather than aggravate it, possibly by dampening DNA end resection in these cells. Long-range PCR analysis of recombining products recovered from stimulated \(Shld1^{−/−}\) \(Atm^{−/−}\) splenic B cells showed that the loss of ATM in SHLD1-deficient cells partially restored high molecular weight PCR products in SHLD1-deficient B cells (Supplementary Fig. 6f), indicating that ATM might promote end resection in SHLD1-deficient B cells. Consistent with this, inhibition of the ATM kinase activity in stimulated SHLD1-deficient splenic B cells substantially restored CSR levels and high molecular weight recombination products (Supplementary Fig. 6g, h).

SHLD1 deficiency and long DNA end resection leads to unbiased directional joining during CSR. CSR is programmed to occur in a productive deletional orientation through a Cohesin-mediated loop extrusion mechanism that juxtaposes AID-initiated DNA ends within donor and acceptor S-regions for deletional CSR. Deletional repair of AID-induced DSBs at the \(IgH\) locus is dependent on 53BP1 as residual junctions from 53BP1-deficient cells display a more normalized ratio of deletional versus inversional end-joining. To test the potential role of SHLD1 in orientation-specific CSR, we used high-throughput genome-wide translocation sequencing (HTGTS), a linear amplification-based method that identifies “prey” DSB junctions...
to a fixed “bait” DSB with nucleotide resolution to simultaneously quantify end resection, microhomology (MH) usage and orientation of AID-generated DNA end-joining between the 5′ region of Sμ (the bait) and the downstream core Sγ1 region (the prey)13,14,48 (Fig. 5a). For this study, we analyzed >5,000 junctions from three independent mice of each wildtype, Shld1−/−, Xlf−/−, and Shld1−/−Xlf−/− genotype. Results from these experiments were highly reproducible (Fig. 5b, c and Supplementary Fig. 7).

Consistent with class switching levels in these cells, XLF-deficient B cells, SHLD1-deficient B cells, and SHLD1/XLF-deficient B cells had reduced Sγ1 junctions compared to wild-type

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**Fig. 4** SHLD1 promotes functional CSR in XLF-proficient and XLF-deficient cells by limiting DNA end resection. 

**a** Representative images of Igh breaks in aberrant metaphases, as quantified in b. Scale bars, 3 μm. **b** Quantification of Igh breaks (clear bar) and translocations (filled bar) in metaphases of WT, Shld1−/−, Xlf−/−, and Shld1−/−Xlf−/− cells. Bars represent means. n = 1005 (WT), n = 1075 (Shld1−/−), n = 1201 (Xlf−/−), n = 1000 (Shld1−/−Xlf−/−), two-sided Fisher’s exact test from four independent experiments (**p < 0.0001). See also Supplementary Table 4. **c** Schematic representation of the murine Igh locus. Arrows indicate primers used for the long-range PCR. **d** Long-range PCR analysis of Sμ to Sγ1 rearrangements in WT, Xlf−/−, Shld1−/−, and Shld1−/−Xlf−/− stimulated B cells. Il2 gene PCR was used as a loading control. **e** Normalized coverage of the mapped PacBio reads in WT, Shld1−/−, Xlf−/−, and Shld1−/−Xlf−/− stimulated B cells. Vertical dashed lines indicate the Sμ switch region and vertical lines indicate the Sγ1 region. **f** Distribution of read length (in bp) in WT, Shld1−/−, Xlf−/−, and Shld1−/−Xlf−/− cells. n = 3 independent samples. For all boxplots: minima is minimum value, maxima is maximum value, center is median and quartiles shown by box and whiskers, and cross is mean. n.s. non-significant (p ≥ 0.05), ****p < 0.0001. Source data are provided as a Source Data file.
B cells with Shld1−/− Xlf−/− cells having the most dramatic decrease in Sμ-Sγ1 joins (Fig. 5b–d). In WT cells, breakpoint junctions are almost exclusively located in S-regions with a very small fraction of the Sγ1 breakpoint sites being outside the core Sγ1 region (0.45%) and corresponding to long resection4 (Fig. 5c). Junctions were approximately 30% direct, with most of the others using 1 bp to 3 bp of MH and a small fraction (11%) using longer MHs (Fig. 5e–g). In agreement with previous studies in NHEJ-deficient cells13,49,50, XLF-deficient B cells had a marked increase in MH-mediated Sμ-Sγ1 junctions, with about 17%
Fig. 5 SHLD1 deficiency and long DNA end resection leads to unbiased directional joining during CSR. a Illustration of activation of CSR in normal B cells stimulated with anti-IgD dextran/LPS/IL-4 which induces AID and activates transcription of ly1. Deletional CSR events are indicated with a blue arrow and inversional recombination events are indicated with a red arrow. Intra-switch recombination events are indicated with a dotted line. b CSR-HTGTS-seq analysis of break joining between Sµ-Sα and downstream acceptor S-regions in WT, Shld1−/−, Xlf−/−, and Shld1−/−/Xlf−/− splenic B cells stimulated with anti-IgD dextran/LPS/IL-4. The blue line indicates deletional joining, and the red line indicates inversional joining. c Zoom-in view of CSR-HTGTS-Seq junctions located in the AID-targeted ectopic Sµl region from WT, Shld1−/−, Xlf−/−, and Shld1−/−/Xlf−/−. Cells. Junctions are plotted at 200 bp bin size. d Bar graph showing percentages of junctions located in Sµ and Sy1 regions from WT, Shld1−/−, Xlf−/−, and Shld1−/−/Xlf−/−. Cells. Junctions are plotted as a percentage of total junctions. n = 3 independent samples. e Percentages of Sµ-Sy1 direct joins in f. g Percentages of Sµ-Sy1 direct joins in f and Sµ-Sy1 joins with 4-bp or longer MH in g differ between pools. d Data represent mean ± s.e.m. from three independent repeats. f Microhomologies (MH) usage of Sµ-Sy1 junctions, plotted as a percentage of total junctions. n = 3 independent samples. g Percentages of Sµ-Sy1 direct joins in f and Sµ-Sy1 joins with 4-bp or longer MH in g differ between pools. h Inversional/deletional joining ratio at the Sy1 region in the different genetic backgrounds are compared. Bars represent mean ± s.e.m. n = 3 independent samples, unpaired two-tailed t-test, p values are indicated in Source Data file. i Percentage of resection at the Sy1 region in the different genetic backgrounds as indicated. Bars represent mean ± s.e.m., n = 3 independent samples, unpaired two-tailed t-test, p values are indicated in Source Data file. j Working model for the specific roles of 53BP1 and components of the SHLD complex in antigen receptor gene diversification. The 53BP1-dependent chromatin response acts independently of SHLD to promote chromosome structural changes that are essential for long-range V(DJ) recombination and CSR as well as for RAG-DSB repair in the context of an unstable post-cleavage complex (i.e., XLF-deficient cells) (left panel); while the 53BP1-SHLD axis limits resection at AID-induced DSB ends, providing an end-protection mechanism that permits productive CSR by NHEJ and alt-NHEJ (right panel).

being direct and a correspondingly increased fraction of MH-mediated junctions (MH >3 bp = 21%), indicating increased use of alternative end-joining in these cells. In addition, in XLF-deficient B cells, a small but significantly increased fraction of Sµ–Sy1 junctions harbored long resection (6%). Consistent with results from long-range PCR, SHLD1-deficient B cells had greater long resection increases (23%) and SHLD1/XLF-deficient B cells far greater increases (36%) that were also apparent as a long resection increases (23%) and SHLD1/XLF-deficient B cells far greater increases (36%) that were also apparent as a ‘flattening’ of Sy1 junction profile relative to other backgrounds (Fig. 5c, h). Notably, MHs were also significantly more present and longer at junctions recovered from SHLD1-deficient and, to a greater extent, SHLD1/XLF-deficient B cells (Shld1−/−: cells: direct joints = 15%, MH >3 bp = 29%; Shld1−/−/Xlf−/−: cells: direct joints = 6%, MH >3 bp = 37%) as compared with XLF-deficient and wild-type B cells (Fig. 5e–g). Thus, SHLD1 restricts end resection and controls microhomology-mediated alternative end-joining of AID-initiated DSBs in both NHEJ-proficient and NHEJ-deficient B cells.

Remarkably, SHLD1-deficient B cells displayed a profound defect in orientation-specific joining (Fig. 5i), as observed in 53BP1-deficient cells. Compared to wild-type B cells, where Sµ–Sy1 recombination occurs predominantly by deletion (>95%), in Shld1−/−/B cells deletions and inversions were present at 70 and 30%, respectively (Fig. 5i). In Shld1−/−/Xlf−/−/B cells, deletions and inversions were present at nearly equal frequencies (57 vs 43%) (Fig. 5i). This normalization of the orientation ratios correlated with the level of DNA end resection in these two B cell types (Fig. 5h), suggesting that, in the absence of SHLD1, exacerbated resection hinders orientation-specific CSR. In support of this and in agreement with previous work in NHEJ-deficient cells, XLF deficiency, which has a much less severe impact on CSR than SHLD1 deficiency and does not promote nearly the same degree of long S resections, also moderately impacted orientation-specific CSR (87 deletion vs 13% inversion). Altogether, these results suggest that the extent of AID-DSB end resection impacts orientation-specific CSR.

Discussion

The SHLD complex acts downstream of 53BP1-RIF1 to counteract DSB end resection and promote NHEJ, a DSB repair pathway essential for V(DJ) recombination and CSR. Here we find that SHLD1 is dispensable for lymphocyte development and V(DJ) recombination, but does however play an essential role in ensuring productive CSR in B cells. We propose a model in which 53BP1 promotes antigen receptor gene diversification through two distinct mechanisms. First, the 53BP1-dependent chromatin DSB response acts independently of SHLD to promote chromosome structural changes that are essential for long-range V(DJ) recombination, and RAG-DSB repair in the context of an unstable post-cleavage complex (i.e., XLF-deficient cells). Second, the 53BP1-SHLD axis limits resection at AID-induced DSB ends, providing an end-protection mechanism that permits productive CSR by NHEJ and alt-NHEJ (Fig. 5i).

Contrary to 53bp1−/− mice that display mild lymphopenia (16–18,30 and this study), we find that Shld1−/− mice harbor normal numbers of B and T cell populations in lymphoid organs. Shld2−/− mice27 and REV7-deficient animals30 also show normal lymphocyte differentiation, suggesting that all components of the SHLD complex are dispensable for lymphocyte differentiation and V(DJ) recombination. These results imply that RAG-generated DSB ends do not require SHLD protection against resection prior to joining. Consistent with this, we find that Shld1−/−/Xlf−/− mice support lymphocyte development and Shld1−/−/Xlf−/− lymphocytes perform RAG-induced DSB repair at levels similar to those observed in Xlf−/− lymphocytes. This is again in striking contrast to the severe combined immunodeficiency phenotype observed in 53bp1−/−/Xlf−/− animals and the accumulation of resected RAG-induced DSBs observed in 53bp1−/− and 53bp1−/−/Xlf−/− progenitor lymphocytes.18,20,21 Why is the SHLD complex, contrary to 53BP1, dispensable for RAG-induced DSB repair? Specific features of the V(DJ) recombination reaction might provide clues to this question.2,21 First, the RAG-cleavage and repair steps of the reaction are coupled, possibly limiting fortuitous end resection events. Second, RAG-generated DSB end structures are prone to repair by NHEJ, which means that hairpin-sealed coding ends are open, processed, and repaired by NHEJ and blunt signal ends require minimal end-processing prior to ligation by LIG4. Third, RAG-cleavage occurs in a very strict G1 environment that offers limited end resection activities. Additionally, during V(DJ) recombination, ATM and downstream chromatin DSB response factors such as H2AX and 53BP1 contribute to DSB end-tethering and synopsis, a function that is thought to be redundant with XLF.19 Functional compensation for DSB end-tethering/synapsis in the absence of XLF may also reside in additional post-cleavage complex proteins including RAG38, MR192, and ERC662.19 In addition, XLF-deficiency is largely compensated by PAXX during end-joining41,53,54. Thus, even in the absence of XLF, RAG-generated DNA ends benefit from robust end-joining as well as a chromatin environment that alleviates the need for SHLD to...
limit resection. Our finding that Shld1−/− Xlf−/− mice do not suffer developmental growth defects, as opposed to 53bp1−/− Xlf−/− mice, indicates that the ATM-H2AX-53BP1-mediated chromatin DSB response also supports important SHLD-independent functions during mammalian development.

Two distinct features of 53BP1 explain its essentiality for CSR; the ability to form stable oligomeric assemblies at DSB sites and the faculty to protect DNA ends against resection mediated by RIF1 and components of the SHLD complex. It has been proposed that tetramerization (and higher-order assembly) of 53BP1 might provide a tethering activity to bridge and stabilize distally located DNA ends such as in the case of AID-induced DSBs in donor and acceptor switch regions and RAG-induced DSBs in distant V(D)J segments. Notably, mutations that interfere with 53BP1 higher-order oligomer formation abrogate CSR without substantially affecting DSB end resection, suggesting that this function is independent of downstream RIF1–SHLD factors. In agreement with this, we show that 53BP1 knockout splenic B cells have a CSR defect approximately twice as severe as SHLD1 knockout splenic B cells, possibly illustrating the dual property of 53BP1. In addition, we find that recombination of distant V(D)J segments is independent of SHLD1. It was recently reported that CSR end protection might in fact play a limited role in the ability of 53BP1 to support CSR, based on the observation that a mutant form of 53BP1 defective for RIF1 recruitment still supports robust CSR. Yet, B cells deficient for any component of the SHLD complex as well as for RIF1 display defective CSR. The recent identification of distinct modes of RIF1 and SHLD recruitment at DSBs and action to promote DNA repair might partially explain this discrepancy.

We find that murine Shld1−/− B cells are defective for CSR due to unbiased orientation-joining of AID-DSBs and extensive DSB end resection in these cells. Inversional recombination leads to non-productive CSR as constant region coding sequences downstream of the donor S region would be inverted and incapable of being translated. Overactive resection also leads to non-productive CSR due to the loss of coding exons in the downstream acceptor constant region.

Orientation-specific CSR is known to be dependent on 53BP1 which could reflect the two aforementioned aspects of 53BP1: (1) a specialized structural role in promoting synopsis and stabilization of S-regions and/or (2) the ability to limit DNA end resection and promote rapid DNA repair, thus preventing the lingering of S-region broken ends within resection complexes and the loss of their orientation-specific joining properties. Interestingly, treatment of 53BP1 knockout activated B cells with an ATM kinase inhibitor substantially diminishes long S-region resection machinery(ies), the 53BP1–SHLD axis does not simply act during CSR, but its action is required for NHEJ or alt-NHEJ pathways to permit productive CSR in rapidly dividing activated B cells. This model is consistent with previous work suggesting that 53BP1 acts primarily to ensure the integrity of DSB repair end resection, as the complex forms at DSB ends through the interaction of SHLD2 with >50 nt-long ssDNA. AID-generated DSBs or at least a fraction of them are predicted to contain either 5′ or 3′ overhangs of variable lengths that offer an ideal substrate for SHLD. By protecting AID-DSB ends against resection, we propose that SHLD permits ssDNA processing and end-joining—by NHEJ or alt-NHEJ—to take place within switch regions, leading to productive CSR. The proliferative nature of switching B cells might also account for the need to protect AID-DSB ends, specifically in the S/G2-M phase where there is more extensive DNA end resection. In that regard, while NHEJ dominates in G1, alt-NHEJ acts predominantly in S/G2/M where it is associated with extensive resection and frequent microhomologies at junctions.
investigated. Regardless of the nature of ALT-NHEJ, our findings predict that the SHLD complex promotes its fidelity for the benefit of productive CSR. SHLD1 being the most recently evolved component of the DSB response apparatus that co-emerged with CSR in vertebrates\(^2\), it thus could have provided an ultimate evolutionary-selected brick to a complex protein network that led to the emergence of antibody class switching.

**Methods**

**Mice.** Shld1\(^{−/−}\), Xlf\(^{−/−}\) mice were bred with Shld1\(^{−/−}\) Xlf\(^{−/−}\) mice, and Shld1\(^{−/−}\) Xlf\(^{−/−}\) to generate double deficient mice. Shld1\(^{−/−}\) Xlf\(^{−/−}\) mice were crossed to CD21\(^{−/−}\), CD23\(^{−/−}\) mice in previous studies. Mice were bred under specific-pathogen-free (SPF) conditions and housed at ambient temperature and humidity with 12 h light/12 h dark cycles. In all experiments, 6–17-week-old sex- and age-matched littermates were used. Mice euthanasia was performed by carbon dioxide exposure. All experiments were performed after authorization was granted by the institutional animal care and ethical committee of Institut Pasteur/CETEA n°39 under protocol numbers 180006/14778.

**Lymphocyte development.** Lymphocyte development was analyzed in the thymus, bone marrow, and spleen from 6–17-week-old sex- and age-matched mice. Single-cell suspensions were treated with Fc-blocking antibody (CD16/32) and data were collected on a Fortessa analyzer (BD Biosciences). Data were analyzed by FlowJo v10.4.2 software. The gating strategy is provided in Supplementary Fig. 8.

**CRISPR/Cas9 editing of pro-B and CH12 cell lines.** v-ABL pro-B cell lines were generated as previously described\(^3,4\). Briefly, total bone marrow from WT and Shld1\(^{−/−}\) 7-week-old mice was cultured and infected with a retrovirus encoding v-ABL kinase to generate immortalized pro-B cell lines\(^4\). v-ABL transformed pro-B cell lines were then transduced with pMSCV-Bcl2-puro retrovirus\(^5\) to protect them from v-ABL kinase inhibitor-induced cell death. v-ABL pro-B cells were maintained in RPMI 1640 ( Gibco 18700044) supplemented with 10% fetal bovine serum (Sigma F6178), penicillin (100 U/ml) streptomycin (100 μg/ml) (Gibco) and 50 μM 2-mercaptoethanol (Gibco 11289262).

**CRISPR/Cas9 editing of v-ABL pro-B and CH12 cell lines.** v-ABL pro-B and CH12 knockout cell clones were generated as previously described\(^6,7\). Briefly, cells were nucleofected with two sgRNA-encoding plasmids and the pCas9-GFP plasmid (see Supplementary Table 7 for sgRNAs sequences) using an Amaxa Nucleofector strategy is provided in Supplementary Fig. 8.

**Lymphocytes from the spleen were identified and gated as previously described\(^8\), 500 ng of genomic DNA was amplified using pkl1a2 and pkl6c primers. The following parameters were used: 1x (95 °C 5 min); 17x (94 °C 30 s, 60 °C 30 s, and 72 °C 30 s); 1x (72 °C 5 min). Serial fourfold dilutions of these reactions were amplified using pkl2a and pkl6d primers and the following cycles: 1x (95 °C 5 min); 25x (94 °C 30 s, 60 °C 30 s, and 72 °C 30 s); 1x (72 °C 5 min). IL2 gene was amplified using IMR42 and IMR43 primers and was used as a loading control. The PCR gel images were acquired and analyzed using Image Lab v6.0 (Biorad). See Supplementary Table 7 for primer sequences.

**Quantitative PCR analysis of TCRβ rearrangements.** 100 ng of genomic DNA from thymocytes was amplified with a combination of gene-specific primers (see Supplementary Table 7 for primer sequences). PCR reactions were performed in triplicates and data were collected on a QuantStudio 3 Real-Time PCR System and analyzed using the QuantStudio Design & Analysis Software v2.6.0. For each assay, aliquots of DNA were analyzed for a control, non-rearranging DNA 3′ of Jκ2. The cycle threshold numbers for each primer combinations (ΔC\(_T\)) were used to calculate the absolute amount of PCR signal. The relative ratios of each rearrangement were averaged and plotted together with the standard error of the mean. See Supplementary Table 7 for primer sequences.

**Ex vivo CSR assay.** Splenic B cells were purified from 6–17-week-old sex- and age-matched mice using magnetic CD19 beads according to the manufacturer’s instructions (Miltenyi Biotec). B cells were cultured in 4 ml of complete medium consisting of RPMI 1640 supplemented with 12% FBS, penicillin (100 U/ml) streptomycin (100 μg/ml), 50 μM 2-mercaptoethanol, and incubated at 37 °C in a humidified atmosphere containing 5% CO\(_2\). To induce specific isotype switching, B cells were stimulated with either LPS (25 μg/ml, Sigma-Aldrich), IL-4 (10 ng/ml, Millteny) and anti-IgD dextran (3 ng/ml, Fina Biosolutions) or anti-CD40 antibody (1 μg/ml, Milteny) and IL-4 for IgG1, and LPS for IgG2b and IgG3. Cells incubated with either anti-IgD dextran or anti-CD40 antibodies were used as a negative control. After 4 to 5 days, cells were assayed for class switching by flow cytometry using CD19-450 (BD Biosciences 563705, clone 1D3, 1:200 dilution); B20-200 ACP (BD Biosciences 575699, clone RA3-6B2, 1:200 dilution); B20-200 ACP (BD Biosciences 533092, clone RA3-6B2, 1:200 dilution); IgG1-APC (BD Biosciences 550874, clone X56, 1:500 dilution), IgG2b-PE (Biotiful 4067087, clone RMG2b-1, 1:500 dilution), IgG3-FTC (BD Biosciences 533403, clone R40-82, 1:500 dilution), IgM-PE-Cy7 (BD Biosciences 553467, clone R60-202, 1:500 dilution), and IgM-APC (BD Bioseics 550874, clone X56, 1:500 dilution). Data were analyzed by FlowJo v10.4.2 software. The gating strategy is provided in Supplementary Fig. 8.

**Knockout clones were then validated by Western blot (Supplementary Fig. 9).**

**V(D)J recombination assay.** V(D)J recombination assays were performed as previously described\(^9,10\). Briefly, v-ABL infected pro-B cells were infected with MAV-INV retroviral vector and cells that had integrated the recombination substrate were enriched based on LD44 expression using LC4D4 Microbeins (Miltenyi 130-045-101). Purified v-ABL infected pro-B cells (10⁶ per ml) were treated with 3 μM of the v-ABL kinase inhibitor STI571 (Novartis) for 72 h and assayed for rearrangement levels. EAC's analysis of GFP+ PE (BD Biosciences 581210, clone CD138-113-254, clone M-T466, 1:100). V(D)J recombination efficiency was scored as the percentage of GFP-positive cells among hCD4-positive cells. FACS data was acquired by FACS Diva v8.0.1 (BD Biosciences) and analyzed and visualized by Flowjo v10.4.2 (TreeStar). The gating strategy is provided in Supplementary Fig. 8.

**PCR analysis of endogenous Igκ rearrangements.** Endogenous Vκ(2-4)κ, coding joints were amplified as previously described\(^11,12\). 300 ng of genomic DNA was amplified using pkla2 and pk6c primers. The following parameters were used: 1x (95 °C 5 min); 17x (94 °C 30 s, 60 °C 30 s, and 72 °C 30 s); 1x (72 °C 5 min). Serial fourfold dilutions of these reactions were amplified using pkla2 and pk6d primers and the following cycles: 1x (95 °C 5 min); 25x (94 °C 30 s, 60 °C 30 s, and 72 °C 30 s); 1x (72 °C 5 min). IL2 gene was amplified using IMR42 and IMR43 primers and was used as a loading control. The PCR gel images were acquired and analyzed using Image Lab v6.0 (Biorad). See Supplementary Table 7 for primer sequences.

**PCR analysis of TCRβ rearrangements.** 100, 50, and 10 ng of gDNA from thymocytes was amplified using either TCRB-D1US and TCRB-D1D-A (D1–Jβ1), or TCRB-D2US and TCRB-D2D-A (D2–Jβ2). PCR reactions (20 μl) contained genomic DNA template, 0.5 μM of each primer, and 1X Platinum SuperFi II PCR Master Mix (Invitrogen). The following parameters were used: (98 °C 30 s) 10 cycles; (58 °C 30 s) 10 cycles; (72 °C 30 s) 30 cycles; (72 °C 5 min). IL2 gene was amplified using IMR42 and IMR43 primers and was used as a loading control. The PCR gel images were acquired and analyzed using Image Lab v6.0 (Biorad). See Supplementary Table 7 for primer sequences.

**Purified genomic DNA template, 0.5 μM of each primer and 1X Power Sybr Green Master Mix (Applied Biosystems) and data were collected on a QuantStudio 3 Real-Time PCR System and analyzed using the QuantStudio Design & Analysis Software v2.6.0. For each assay, aliquots of DNA were analyzed for a control, non-rearranging DNA 3′ of Jκ2. The cycle threshold numbers for each primer combinations (ΔC\(_T\)) and for the control amplification (ΔC\(_T^{ΔC_{β2}}\)) were used to calculate the absolute amount of PCR signal. The relative ratios of each rearrangement were averaged and plotted together with the standard error of the mean. See Supplementary Table 7 for primer sequences.
CSR assay in CH12 cells. CH12 cells were plated at 50,000 cells per ml in complete RPMI supplemented with anti-CD40 antibody (1 mg/ml, Miltenyi), IL-4 (20 ng/ml, Miltenyi), and TGFI-β (1 ng/ml, R&D Biotech) to induce IgM to IgA switching. After 3 days, cells were assayed for class switching by flow cytometry using IgA-PE (biosciences 12-4204-82, clone mA-6E1, 1:200) and IgM-PE-Cy7 (BD Biosciences 552867, clone R6-60.2, 1:200 dilution) antibodies, and a Fortessa analyser (BD Biosciences). Data were analyzed by Flowjo v10.4.2 software. The gating strategy is provided in Supplementary Fig. 8.

Immunoprecipitation. Nuclear extracts were prepared by resuspending 40 million fresh cells in ice-cold 10 ml buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1% NP-40, protease inhibitors cocktail, phoSTOP) and rotating for 10 min at 4 °C. Nuclei were centrifuged at 800 x g for 10 min at 4 °C and resuspended in 1 ml IP buffer C150 (20 mM HEPES pH 7.9, 150 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.25% NP-40, 0.05% glycerol, protease inhibitors cocktail, phoSTOP). Lyssates were briefly sonicated followed by Benzonase (Sigma E8263) digestion for 30 min at 4 °C. Finally, lysates were cleared through centrifugation at 13,000 x g for 20 min before incubation with 50 μl of anti-Flag M2 magnetic beads (Sigma M8823) overnight at 4 °C. Beads were washed five times in an IP buffer. Washed beads were directly resuspected in Sample Buffer Laemmli 2x (Sigma S3401), and boiled at 95 °C for 10 min.

DNA-FISH on metaphase spreads. DNA-FISH was performed as previously described.13,14 Slides were treated with RNase A for 40 min, dehydrated in 70, 90, and 100% ethanol for 3 min each, denatured in 70% formamide/2x SSC for 3 min at 70 °C, and dehydrated in cold ethanol. Slides were hybridized with 30 μg of end-labeled biotin overnight at 37 °C in a humid chamber. The next day, slides were washed three times in 50% formamide/0.5x SSC for 5 min each at 37 °C and twice in 0.5x SSC for 10 min and 20 min at 37 °C. Finally, slides were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen P36931) to counterstain total DNA.

Metaphases were imaged using a Zeiss AxioImager.Z2 microscope and the Metafer automated capture system (MetaSystems) and counted manually. Probes used in this study: IgC C BAC probes (RP24-134G24, IgH BAC probe (RP24-386017), and XCyting Mouse Chromosome 12_paint (MetaSystems). Analysis of the percentage of aberrant metaphases was performed using Microsoft Excel 16.12.6.

Long-range PCR and sequencing. Long-range PCR of the Ighu/Ighy1 ampiclon was accomplished using Platinum SuperFi II polymerase (Invitrogen 164435839). A total of 100 ng of gDNA was amplified using Euf and Cy1R2 primers and the following cycles: 1× (98 °C 30 s); 30× (98 °C 10 s, 57 °C 10 s, and 72 °C 8 min); 1× (72 °C 5 min). See Supplementary Table 7 for primer sequences. Prior to PCR library preparation, all amplicons were purified using Ampure PB beads at 1:8X and controlled using a Fragment Analyzer with a High Sensitivity gDNA kit. Amplicon Sequencing library was prepared according to the Pacific Biosciences protocols (Fertigk 101-791-700 version 11 (January 2021)). We sequenced PCR products obtained from three replicates of wildtype and mutant splenic B cells using long-range PCR. Although for each of them, three replicates were included accounting for a total of 12 samples that were multiplexed in one sequencing run. The resulting sequences were demultiplexed and consensus circular read (CCS) were constructed using the SMRTLINK software from PacBio (v9) (smrtlink: https://www.pacb.com/support/software). CCS reads were quality filtered into Fastq and mapped onto the Mus musculus genome (reference GRCm38 v92) using the Sequana library (BAM reader) (https://www.pacb.com/support/software)). Then, CCS reads were converted into Fastq SMRTLINK software from PacBio (v9) (smrtlink: https://www.pacb.com/support/software). For each of them, three replicates were included accounting for a total of 25 μg DNA from the stimulated splenic B cells was sonicated (25 s ON and 60 s OFF, two cycles with low-energy input) on a Diagonode Bioruptor sonicator. The templates were amplified with a biotinylated 5′ mouse primer after sonication. The PCR products were purified over streptavidin C1 beads (#65001, Thermo Fisher Scientific) for 4 h at room temperature. The enriched biotin-labeled LAM-PCR products were ligated with an adapter with the following program (25°C 1 h, 22 °C 3 h, 16 °C overnight). The adapter-ligated products were amplified by nested PCR with barcode primers and tag PCR with P5-15 and P7-17 primers. The PCR products from 500 bp to 1000 bp were purified by separation on 1% TAE gel. HTGTS libraries were sequenced by paired-end 150 bp sequencing on a NextSeqlluma (Illumina). More details of the method have been described75. Libraries were processed via the published pipeline76. The libraries from Shld1/−/−, Xlf−/−, Xlf−/− and their WT C37BL/6 control mice were mapped against the mm9 genome as described previously47,48. Data were analyzed and plotted after removing duplicates47,48. Each experiment was repeated three times for statistical analyses. The junction numbers within the Sγ containing portion of the IgH were plotted in Fig. 5 and Supplementary Fig. 7. The numbers and percentages were indicated in the corresponding panels. The MH analysis were described in a previous study33.

Statistics and reproducibility. Statistical analyses were performed with GraphPad Prism V7.04 and BiostaTGV (https://biostatgv.sentiweb.fr/) and using the tests as indicated in the respective figure legends. Significance; ns, not significant (p > 0.05), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Unless indicated otherwise, all immunoblots are representative of at least two independent experiments, with uncropped blots shown in the Source data file.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The CSR-HTGTS data have been deposited in NCBI’s Gene Expression Omnibus under the accession number GSE202567. The Long-range PCR sequencing (Pacbio) data have been deposited in NCBI’s Sequence Read Archive under the accession number PRJNA361666. All other data can be found in the Supplementary Data of this paper or in Figs. 1--5 in the published paper. All raw sequencing data are available in the specified datasets, including the Supplementary Figures. All data are available from the authors upon reasonable request. Source data are provided with this paper.

Codability
All of the code used in the paper was published from studies and is cited in the manuscript.
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Author contributions
L.d. conceived the project and the studies. L.D. and C.L. supervised the work. L.D., C.L., and E.V. designed experiments, analyzed data, discussed the interpretation of the results, and wrote the manuscript. E.V. and C.L. performed all experiments with contributions from W.W. who performed V(DJ) recombination experiments in pro-B cells, X.Z. and F.W.A who conducted the HTGTS experiments and analysis of the data, C.P. and H.M. who conducted the serum Ig titration experiments and analysis of the data, W.Y. who generated CH12 knockout clones, H.L.-H. who generated pro-B cell knockout clones and, T.C. and J.P.d.F. performed long-range PCR sequencing and analyzed the data. D.J.A., S.P.J., and G.B. provided the SHLD1 knockout mouse line. S.P.J. and G.B. discussed the project and provided advice. All authors read and approved the manuscript.

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The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Chloé Lescale or Ludovic Deriano.

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Author contributions
L.D conceived the project and the studies. L.D. and C.L. supervised the work. L.D., C.L., and E.V. designed experiments, analyzed data, discussed the interpretation of the results, and wrote the manuscript. E.V. and C.L. performed all experiments with contributions from W.W. who performed V(DJ) recombination experiments in pro-B cells, X.Z. and F.W.A who conducted the HTGTS experiments and analysis of the data, C.P. and H.M. who conducted the serum Ig titration experiments and analysis of the data, W.Y. who generated CH12 knockout clones, H.L.-H. who generated pro-B cell knockout clones and, T.C. and J.P.d.F. performed long-range PCR sequencing and analyzed the data. D.J.A., S.P.J., and G.B. provided the SHLD1 knockout mouse line. S.P.J. and G.B. discussed the project and provided advice. All authors read and approved the manuscript.

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