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Immunology

Opinion

DNA repair and antibody diversification: the 53BP1 paradigm

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The DNA double-strand break (DSB) repair factor 53BP1 has long been implicated in V(D)J and class switch recombination (CSR) of mammalian lymphocyte receptors. However, the dissection of the underlying molecular activities is hampered by a paucity of studies [V(D)J] and plurality of phenotypes (CSR) associated with 53BP1 deficiency. Here, we revisit the currently accepted roles of 53BP1 in antibody diversification in view of the recent identification of its downstream effectors in DSB protection and latest advances in genome architecture. We propose that, in addition to end protection, 53BP1-mediated end-tethering stabilization is essential for CSR. Furthermore, we support a pre-DSB role during V(D)J recombination. Our perspective underscores the importance of evaluating repair of DSBs in relation to their dynamic architectural contexts.

53BP1 in antibody diversification: placing DNA repair in context

V(D)J recombination and CSR are antigen receptor diversification reactions that are crucial for adaptive immune responses. V(D)J recombination occurs in developing T and B lymphocytes and generates highly diverse **T and B cell receptor (TCR/BCR)** (see Glossary) repertoires, respectively [1–4]. CSR occurs in mature B cells and produces various **antibody [also known as immunoglobulin (Ig)] classes** or isotypes, thus diversifying the effector component of antibody responses [3,4]. At the molecular level, V(D)J recombination and CSR both depend on the formation and repair of programmed **DNA DSBs** [1–4] (Boxes 1 and 2). Among all DNA repair factors that have been implicated in these reactions, few have stimulated so much debate and discussion as 53BP1. Since the first reports of its involvement in CSR [5,6], 53BP1 has both frustrated and fascinated molecular immunologists for the severity of the CSR defect and the plurality of immune-related phenotypes described over the years [5–12]. These reports have collectively depicted a complex, and sometimes contradictory, picture of the molecular bases of 53BP1's contribution to antigen receptor gene diversification.

Here, we revisit the different B and T cell phenotypes of 53BP1 in view of the recent dissection of its downstream effectors in DNA repair and the latest advances in antigen receptor loci configuration dynamics. We provide evidence in support of pre- and post-DSB roles for 53BP1 in both CSR and V(D)J and argue in favor of a DNA end-tethering stabilization function that is essential for CSR. In general, we highlight the key role played by the relative kinetics of the DNA metabolic activities at play during the two reactions, thus providing an integrated perspective for the mechanistic dissection of antigen receptor diversification.

53BP1: the master regulator of S region break resection

One of the most interesting enigmas in the CSR field arose with the observation, nearly two decades ago, that *ex vivo* stimulated B cells from mice deficient for the DSB repair protein 53BP1 (*Trp53bp1*$^{-/-}$) displayed near-complete abrogation of CSR and a consequent decrease in the generation of Ig classes other than IgM [5,6]. The defect severity was at odds with the

Highlights

Mammalian 53BP1 and replication timing regulatory factor 1 (RIF1) protect DNA double-strand breaks (DSBs) against nucleolytic degradation through the recruitment of the REV7-SHLD1-SHLD2-SHLD3 (shieldin) and CTC1–STN1–TEN1 (CST) complexes.

Mouse models deficient in downstream effectors of DSB protection have enabled the comparative assessment of defects in V(D)J recombination and class switch recombination (CSR).

Regulation of DSB resection is crucial for CSR but dispensable for V(D)J recombination.

The DSB protection function of mammalian 53BP1 does not explain the CSR defect severity associated with its deficiency.

V(D)J recombination and CSR rely on the dynamic reconfiguration of *Tcr/Ig* loci that is contributed by cohesin-mediated loop extrusion.

We propose that mammalian 53BP1 mediates pre- and post-break functions related to *Tcr/Ig* dynamics and DSB end-tethering, respectively, during the two recombination reactions.

Significance

The dissection of the key molecular activities contributed by 53BP1 to antibody gene diversification exemplifies the importance of considering the repair of V(D)J and class switch recombination DNA double-strand breaks in their architectural genomic contexts. The investigation of the close interplay between *Tcr/Ig* loci dynamics and the repair of these programmed breaks can provide a deeper understanding of the molecular bases of humoral immune responses.

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Box 1. The molecular basis of isotype switching

Naive B cells express IgM and IgD antibodies on their surface since the rearranged VDJ exon of the *Igh* locus is juxtaposed to the constant (C) μ-δ regions [3,4]. Coexpression of the two isotypes is mediated by alternative splicing of the VDJ-Cμ-Cδ pre-mRNA transcript [4]. Class switching to IgG, IgE, or IgA occurs after B cell activation via replacement of the donor Cμ with one of the downstream acceptor C regions (Cγ/Cε/Cα) [3,4]. The C gene, except Cδ, is preceded by a constitutively expressed (Cμ) or cytokine-inducible (Cx) promoter, an intervening (I) exon, and a 1–12-kb intronic sequence, known as the switch (S) region [3,4]. S regions are highly repetitive in nature, although they exhibit relatively different core repeat units of 10–80 bp in length [3,4].

CSR is initiated by the activation-induced priming of an acceptor S region via the process of GLT and the expression of the B cell-specific enzyme AID [82]. AID targets the recombining Sμ-Sx regions in a manner dependent on GLT, and its activity results in the formation of multiple DSBs per S region [83]. Productive CSR events rely on the inter-Sμ-Sx ligation of AID-induced breaks and deletion of the intervening sequence, which places the new Cx region close to the rearranged VDJ exon. CSR exhibits an orientation-specific recombination preference known as deletional bias (90:10 deletions over inversions), which sets CSR apart from the repair of ubiquitous DSBs (50:50 deletions to inversions) [7]. Ligation of CSR DSBs occurs predominantly by NHEJ and yields mainly blunt or 1–4-bp MH-bearing CSR junctions [4,21,84–86]. In the absence of a functional NHEJ pathway, and to a lesser extent in NHEJ-proficient cells, CSR is mediated by the A-EJ pathway [4,85–87]. A-EJ skews repair toward increased use and length of MHs and operates with reduced efficiency and slower kinetics [4]. Finally, breaks within the same S region can also be internally rejoined in a process known as intra-S-recombination, which leads to internal S region deletions but does not result in productive class-switching events [4].

much milder phenotype exhibited by the known, at the time, upstream components of the DSB signaling cascade, including the DSB repair kinase ATM [13–16]. Even more puzzling was the observation of a concomitant increase in the frequency of S region (Box 1) intra-recombination events [17]; the postulated DNA end-joining activity of 53BP1 could not explain the CSR versus intra-S-recombination antithetic phenotypes. The field was left wondering about these conflicting observations for a few years, until the turning point discovery of 53BP1's ability to inhibit the **resection** of breaks in the S regions [18–20]. This DSB end protection function places 53BP1 upstream of the resolution step of DSB repair, thus providing an *ad hoc* explanation for the apparently conflicting end-joining phenotypes associated with its deficiency. Since the repeated core sequences differ between the donor and various acceptor S regions [3,4], resection of breaks in the absence of 53BP1 would interfere with inter-S repair (CSR) while promoting **microhomology (MH)**-mediated end joining of proximal DSBs (intra-S recombination) (Box 1). The increased resection also explained the MH-skewed profile of residual CSR junctions [21] and suggested the intervention of the **alternative end-joining (A-EJ)** pathway (Box 1).

Box 2. The molecular basis of V(D)J recombination

V(D)J recombination is a lineage- and developmental stage-specific reaction that occurs in the bone marrow for B lymphocytes and in the thymus for T cells. The process assembles the variable portion of the BCR and TCR from the different variable (*V*), diversity (*D*), and joining (*J*) gene segments in the corresponding antigen receptor loci [81,88,89] (see Figure 1 in main text). The expression of a functional, non-autoreactive BCR or TCR is essential for the completion of lymphocyte maturation [1,2]. Thus, defects during V(D)J recombination result in a developmental block of B and T cells, which in human patients manifests in the form of severe combined immunodeficiency (SCID) syndrome [90]. SCID patients lack mature B and T lymphocytes in the periphery because of mutations in factors required for V(D)J recombination [90].

V(D)J recombination requires the expression of RAG 1 and 2, which form the RAG protein complex [81,88,89]. RAG introduces DSBs between the recombination signal sequences (RSSs) and the flanking V/D/J gene segment [81,88,89]. RSSs are non-coding DNA sequences that comprise conserved heptamer (7 bp) and nonamer (9 bp) sequences separated by a less conserved spacer of either 12 or 23 bp (12-RSS or 23-RSS) [81,88,89]. RAG synapses and introduces breaks only in RSS pairs comprising a 12-RSS and a 23-RSS, known as the 12/23 rule [81,88,89]. The presence of the RSS sites together with chromatin marked by active transcription or activating histone modifications recruits RAG to antigen receptor loci (encoding Igh, Igκ, and Igλ in B cells and TCRα, TCRβ, TCRγ, and TCRδ in T cells), thus leading to the formation of the RC [91,92]. RAG-mediated cleavage generates two DSB ends flanking the RSSs (signal ends) and two ends flanking the coding sequences (coding ends) [81,88,89]. RAG's presence *per se* generates a stable synaptic environment (post-cleavage complex), which channels the broken ends to the NHEJ pathway [93,94]. Whereas signal ends are blunt and can be directly joined, coding ends are covalently sealed hairpins that must be opened and processed to allow ligation. Finally, convergent orientation of the aligned RSS sites inside the RC will lead to a deletional recombination event, whereas divergent orientation generates an inversional outcome [68,69,81,88,89].

In the past few years, the molecular composition, and to some extent regulation, of the 53BP1 end protection apparatus has been defined. 53BP1 associates with DSB-containing chromatin, and following ATM-mediated phosphorylation recruits **replication timing regulatory factor 1 (RIF1)** [22–26] and the REV7-SHLD1-SHLD2-SHLD3 (**shieldin**) [27–35] and **CTC1–STN1–TEN1 (CST)** [36,37] protein complexes to the break sites, where they counteract DNA end resection. The analysis of **CH12** cell lines and mouse models deficient in these downstream DNA end protection factors (*Rev7*$^{f/f}$*Mb1*$^{Cre/+}$, *Shld1*$^{-/-}$, and *Shld2*$^{-/-}$) recently enabled further refinement of the original **nonhomologous end joining (NHEJ)** (Box 1)-versus-A-EJ hypothesis behind the CSR phenotypes of 53BP1-deficient B cells. SHLD1 deletion in NHEJ-deficient (*Xlf*$^{-/-}$*Shld1*$^{-/-}$ or *Shld1*$^{-/-}$*CD21-cre*^{Tg}*Xrcc4*$^{f/f}$) mouse splenic B cells further reduces the CSR levels compared with the single mutant counterparts [38]. These data imply that hyper-resection renders the broken ends incompatible with repair by either NHEJ or A-EJ, thus providing a plausible explanation for the severity of CSR loss in the double-deficient mutants. Overall, these studies consolidated 53BP1-dependent regulation of S region processing as a key determinant of successful CSR (Figure 1, Key figure).

53BP1 deficiency in CSR: a plethora of phenotypes

Despite the considerable class switching defect, B cells lacking RIF1 and SHLD1/2 do not exhibit the near-complete abrogation of CSR seen in 53BP1 knockout cells [23,35,38]. Accordingly, a 53BP1 mutant bearing alanine substitutions at 28 phosphorylation motifs (28A), which is unable to recruit the downstream end protection machinery (Box 3), increases CSR twofold over the empty vector levels in *Trp53bp1*$^{-/-}$ splenocytes reconstituted with 53BP1-expressing retroviral constructs [19,39,40]. Furthermore, inhibition of DSB end resection by either downregulation of the key end-processing factor CtIP or inhibition of ATM kinase activity only minimally rescues the CSR defect in *Trp53bp1*$^{-/-}$ B cells [18,20]. These observations led to the conclusion that the involvement of 53BP1 in CSR extends beyond its ability to regulate DNA end processing.

One of the first reports hinting at additional functions showed that residual CSR/*Igh* junctions from *Trp53bp1*$^{-/-}$ B lymphocytes display a 50:50 instead of a 90:10 ratio of deletional versus inversional repair events, which suggests that 53BP1 is required to enforce the CSR deletional bias (Box 1) [7]. Several pieces of evidence indicate a lack of correlation between deletional joining and DSB end processing. Specifically, inhibition of ATM kinase activity in *Trp53bp1*$^{-/-}$ B cells rescues the enhanced resection phenotype of S region breaks to a considerable extent without exerting any effect on the loss of the deletional bias [7]. Conversely, ablation of DSB protection factors differentially affects deletional joining, with *Rif1*$^{f/f}$*Cd19*$^{Cre/+}$ and *Shld1*$^{-/-}$ B cells exhibiting a considerably milder and a severe phenotype, respectively, compared with *Trp53bp1*$^{-/-}$ [7,38,41]. Finally, B cells lacking the recently described DSB repair factor **excision repair cross-complementation group 6 like 2 (ERCC6L2)** display loss of orientation-specific joining but proficient DSB end protection [41]. Collectively, these observations uncouple orientational joining from the regulation of DSB end resection, suggesting the existence of an independent molecular mechanism regulating the deletional bias during CSR.

The loss of 53BP1 has been suggested to impact the *Igh* locus chromatin structure. Initial **chromosome conformation capture (3C)** experiments showed that, in **naive B cells**, the 3′ locus superenhancer [3′ regulatory region (3′RR)] establishes contacts with regions at the 5′ of the Eμ enhancer [42,43] (Figure 1). On activation, specific acceptor S regions are recruited into the 5′Eμ-3′RR loop base in a cytokine-dependent manner [43]. This locus configuration has been proposed to facilitate both **germline transcription (GLT)** and **synapsis** of the recombining S regions, and as a consequence activation-induced deaminase (AID) targeting and DSB formation [8,9], all of which are indispensable steps for CSR (Box 1). Regarding

Glossary

3D DNA fluorescence *in situ* hybridization (3D FISH): fluorescence microscopy-based technique that uses reconstructed 3D images to determine the spatial arrangements of specific loci of interest in the nucleus.
Abelson-transformed pro-B cell lines: cell lines generated via immortalization of primary pro-B cells with the Abelson murine leukemia virus (A-MuLV).
Alternative end joining (A-EJ): DSB repair pathway mediated by a different set of factors than NHEJ, ligating DNA ends after short-range resection that uncovers up to 20 bp of nucleotides.
Antibody/Ig classes: also known as isotypes; the different types of Igs (IgM, IgD, IgG, IgE, and IgA), which are defined by the constant region of the heavy chain that they contain. The isotypes possess different effector functions.
CH12: mouse B cell lymphoma line that undergoes CSR to IgA after stimulation with a cytokine cocktail.
Chromosome conformation capture (3C/4C/Hi-C): a set of techniques that analyze the spatial organization of the chromatin in the nucleus by quantifying the interactions between loci that are distal in the linear genome but close in 3D genome folding.
Cohesin: chromosome-associated multisubunit protein originally characterized for its role in sister chromatid cohesion and later found to mediate DNA looping.
CTC1–STN1–TEN1 (CST): protein complex recently found to act as a downstream effector of DSB end protection.
DN3-cell stage: T cell developmental stage characterized by V(D)J recombination-mediated rearrangement of the TCRβ chain.
DNA double-strand breaks (DSBs): DNA lesions generated when the sugar-phosphate backbone is severed on both strands of the DNA double helix.
Excision repair cross-complementation group 6 like 2 (ERCC6L2): recently identified as a pro-NHEJ protein factor.
Germline transcription (GLT): non-coding transcription of I-Sx-Cx regions in the *Igh* locus resulting in the exposure of single-stranded stretches of DNA that are targeted by AID for DSB generation.
Immature B cells: B cell developmental stage that precedes exit from the bone

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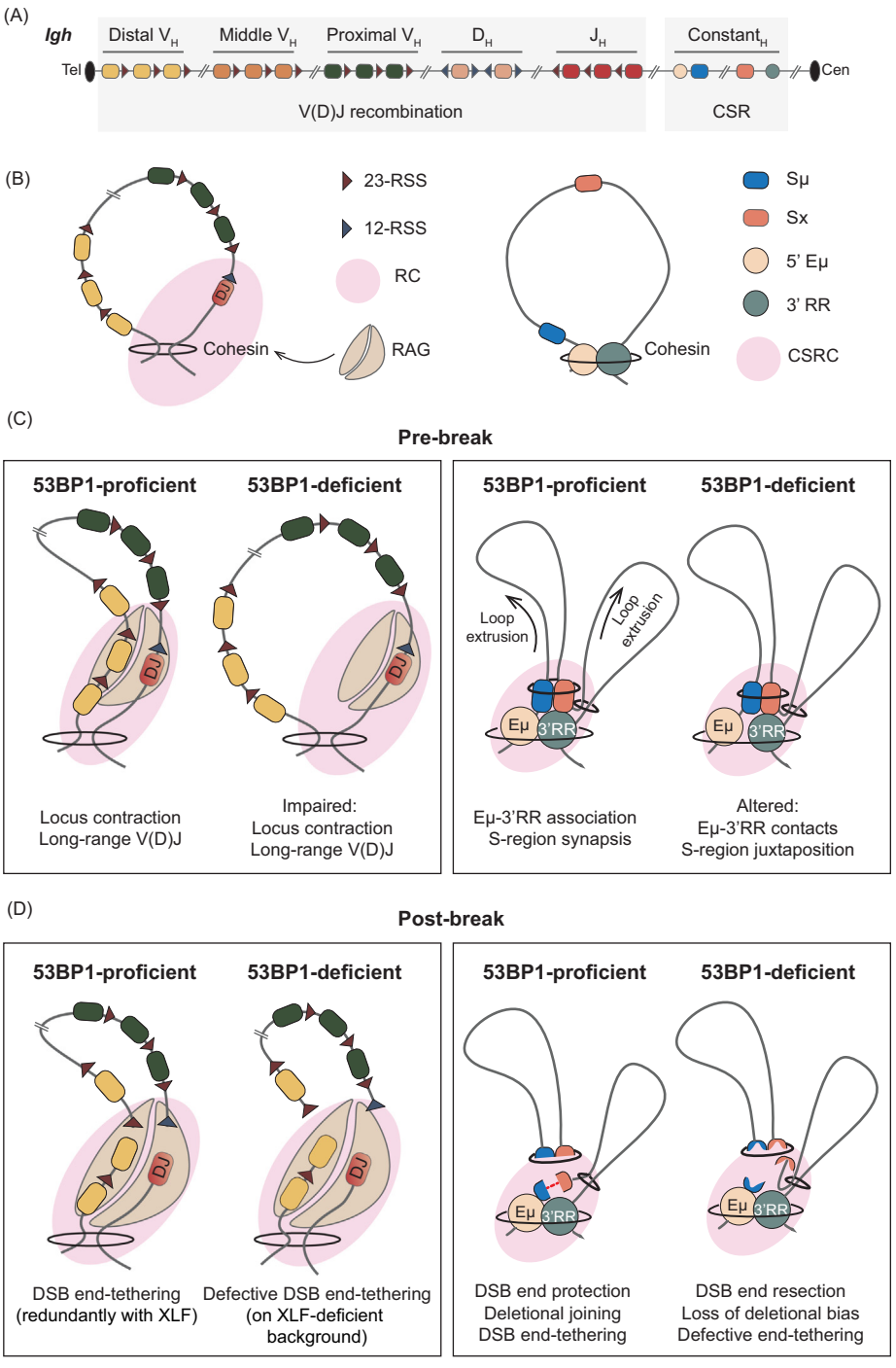
Key figure

53BP1's potential roles during antigen receptor locus diversification



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marrow, when cells have completed the V(D)J recombination of both heavy and light Ig chains and express a functional BCR.

Microhomology (MH): sequence in a junction that shares the longest stretch of uninterrupted homology with both of the DNA ends being ligated.

Naive B cells: also known as resting B cells; quiescent mature B cells that have not been exposed to an antigen.

Nonhomologous end joining (NHEJ): DSB repair pathway that ligates DNA ends after minimal processing, thus resulting in either direct (blunt) joins or junctions carrying up to 4 bp of MHs.

Replication timing regulatory factor 1 (RIF1): phospho-53BP1 interactor that acts as the upstream component of the 53BP1-mediated DSB end protection pathway.

Resection: nucleolytic degradation of the 5′ strands of DSBs to generate 3′ single-stranded DNA stretches.

Shieldin: protein complex that acts as the downstream effector in DSB end protection.

Synapsis: juxtaposition of donor and acceptor S regions brought forward by transcription-dependent *Igh* locus dynamics after B cell activation.

T and B cell receptor (TCR/BCR): receptor complex expressed on the surface of T (TCR) and B (BCR) cells to mediate antigen recognition.

V(D)J sequencing (VDJ-seq): next-generation sequencing-based technique to analyze B and T cell V(D)J repertoires.

Box 3. The molecular determinants of 53BP1's activities in antibody diversification

53BP1 is a large protein (1972 and 1969 amino acids in human and mouse, respectively) comprising various domains and motifs that support its activities in DSB repair [95–97]. The C-terminal portion is crucial for 53BP1 binding to chromatin. Specifically, 53BP1 displays constitutive chromatin association, which is mediated by the interaction between the Tudor domain and dimethylated Lys20 of histone H4 (H4K20me2) [95,96]. Following DSB formation, 53BP1 is actively recruited to the chromatin surrounding the break site via the additional binding of its ubiquitylation-dependent recruitment (UDR) motif to ubiquitylated Lys15 of histone H2A (H2AK15ub) [95,96]. The latter histone modification is the result of the DSB-induced ATM-γH2AX-MDC1-RNF8-RNF168 cascade that activates the cellular response to DNA damage [95,96]. 53BP1's N-terminal half comprises a long, intrinsically disordered region containing several SQ/TQ motifs [95,96]. ATM-mediated phosphorylation of a subset of these motifs is essential for 53BP1 DSB end protection, as it initiates the recruitment of RIF1-shieldin-CST to the DSBs [95,96]. Several studies have shown that all of the above domains and motifs are crucial for 53BP1's role in CSR [95,96]. By contrast, besides the requirement for the Tudor domain, little information is available regarding the molecular determinants of 53BP1's contribution to V(D)J recombination [12].

An additional 53BP1 motif has recently drawn attention in reference to a potential separation-of-function activity. 53BP1 forms dimers and oligomers via an oligomerization domain (OD) (amino acids 1231–1270) and the dynein light chain domain (LC8) (amino acids 1142–1181) [95,96]. Deletion of the OD has long been shown to abrogate CSR, and the defect has been attributed to the postulated dependency of DSB end protection on 53BP1 oligomerization [19]. However, a recent study from our group showed that *Trp53bp1*$^{-/-}$ B cells reconstituted with a 53BP1 OD core-deletion mutant protected S region DSBs to a considerable extent, although they still displayed near-complete abrogation of CSR [39]. This observation raised the attractive hypothesis that 53BP1 oligomerization might mediate an additional, yet-to-be-defined function in CSR other than DSB end protection [39]. The same activity was discussed as being potentially responsible for a 53BP1 short-versus-long-range recombination phenotype of the *Tcrd* locus because of the cooperative nature of protein oligomerization (see discussion in [10]). Whether 53BP1 oligomerization contributes DNA end protection-independent functions to CSR and V(D)J recombination represents an interesting area of investigation, which will require a comprehensive set of analyses under conditions of physiological expression in B and T cells.

53BP1, a study reported that its absence impairs the 5′Eμ-3′RR enhancer interaction but does not affect the post-activation recruitment of the acceptor S region to the loop base [9]. A different study using circular chromosome conformation capture (**4C**) described that 53BP1 deficiency in mouse B cells increased the chromatin interaction of Sμ with the primed acceptor S region [8]. This last study also showed that, while wild-type (wt) B cells preferentially introduced DSBs first in the donor Sμ and only subsequently in the acceptor S region, *Trp53bp1*$^{-/-}$ cells lost this break-order bias [8]. Collectively, these reports led to the hypothesis that 53BP1 exerts a pre-DSB role in CSR that is intimately linked to the *Igh* architecture and dynamics. However, defects in locus reorganization should impact GLT and DSB formation, both of which appear to be unaffected in 53BP1-deficient cells [5,6,9]. In this regard, the different experimental conditions and sometimes conflicting interpretations complicated the inference of a clear and unified mechanistic explanation. Furthermore, the studies did not provide a temporally resolved picture of the described phenotypes, leaving the contribution of 53BP1 to *Igh* locus dynamics an open question.

Figure 1. (A) Schematic representation of the germline mouse *Igh* locus indicating the genomic regions affected by V(D)J recombination- and class switch recombination (CSR)-mediated rearrangements (not to scale). (B) 3D configuration of the *Igh* variable (left) and constant (right) genomic regions. For the variable regions (left), the scheme depicts the locus configuration after successful D_H-J_H rearrangement. The recombination-activating gene (RAG) complex is recruited to the recombination center (RC) and starts to scan the chromatin for the respective V_H genes. The scheme shows one chromatin loop for simplicity. For the constant regions (right), the association of the 5′Eμ and 3′ regulatory region (RR) enhancers marks the base of the chromatin loop as seen in resting B cells. (C,D) Graphical representation of the pre-break (C) and post-break (D) involvement of 53BP1 in V(D)J recombination (left) and CSR (right). (C) In 53BP1-deficient progenitor B cells (left), locus contraction and access of the distal V_H genes to the RC and RAG are disrupted. In activated mature B cells (right), the mechanism ensuring 5′Eμ–3′RR enhancer interaction and/or Sμ–Sx juxtaposition is altered by 53BP1 ablation. (D) 53BP1's redundant contribution to the tethering of RAG-generated ends in the RC is unmasked by XLF deficiency (left). In the absence of 53BP1, S region breaks are subject to extensive resection and their tethering is not stabilized long enough for productive repair to occur. Unrepaired double-strand breaks (DSBs) diffuse away and might engage in both deletional and inversional joining events (right). Abbreviations: Cen, centromere; CSRC, CSR center; Tel, telomere.

Interplay between *Igh* locus dynamics and DSB repair during CSR

The high-resolution assessment of the interaction profiles of *Igh* elements in relation to their transcriptional status and recruitment of the **cohesin** complex has recently provided a mechanistic explanation for the previously reported *Igh* locus dynamics [42,43] and laid the basis for the cohesin-dependent loop extrusion model [44]. Specifically, the model proposes that both the 5′Eμ-3′RR loop in resting B cells and locus reorganization after activation are the result of dynamic interplay between opposing forces influencing the extrusion of *Igh* chromatin: cohesin activity and transcription-dependent impediments [44]. These forces drive the juxtaposition of enhancers and recombining regions to create the CSR center (CSRC) (Figure 1). The model implies that the same transcription-linked mechanisms that are important to establish the locus architecture are also responsible for key structural features promoting productive CSR events, namely: (i) synapsis of the donor Sμ and activated acceptor Sx region in the CSRC; (ii) the formation of DSBs in the recombining Sμ–Sx pair; and (iii) alignment of the broken DNA ends for deletional joining [44].

The cohesin-dependent loop extrusion model provides a framework for the formulation of non-mutually exclusive hypotheses to explain the mechanisms underlying the severe CSR defect and plurality of phenotypes associated with 53BP1 deficiency. In one scenario (the pre-break role hypothesis), 53BP1 contributes to the dynamics of the *Igh* locus architecture, although to an extent that would impact the S region synapsis for deletional joining without dramatically affecting locus reorganization (Figure 1), since GLT is not impaired in *Trp53bp1*$^{-/-}$ cells [5,6,9]. An alternative scenario, which we consider more attractive, takes into account the interplay between *Igh* locus dynamics and S region DSB formation, processing, and repair: the post-break role hypothesis. According to this hypothesis, although synapsis of recombining S regions is likely to favor productive repair of CSR breaks by promoting their local proximity, it is not sufficient for the actual repair to occur; presumably, an additional tethering activity is required, which is likely to stabilize DSB end proximity long enough to complete end joining. Based on this framework, we propose that 53BP1 has a major impact on stabilizing Sμ-Sx DSB end-tethering. Accordingly, 53BP1 might control the delicate interplay of CSRC's competing activities from a dual perspective, since, in its absence, not only are DSB ends susceptible to resection but their juxtaposition would not be stabilized for NHEJ-mediated Sμ-Sx repair to occur. MH-mediated pairing of minimally resected ends is likely to be responsible for the few productive CSR events but when resection extends over the capability to engage repair by A-EJ, DSBs diffuse away from the CSRC, although this scenario remains conjectural (Figure 1). Nevertheless, this hypothesis implies that stabilization of end tethering is a prerequisite for successful CSR and it justifies the more severe CSR defect of 53BP1-deficient cells. In addition, it is also tempting to consider the possibility that end-tethering stabilization might impart deletional joining bias, thus providing a mechanistic basis for the latter phenomenon and confirming its independence from DNA end protection. Mechanistically, the inability to stabilize DSB end tethering long enough to complete Sμ-Sx deletional repair might eventually result in diffusion of DSBs within the CSRC, thus also enabling inversional joining events. Whether defects in deletional bias reflect an active mechanism in end-tethering stabilization or an indirect consequence of impaired *Igh* locus dynamics and/or DSB repair remains to be addressed, but certainly represents a fruitful area for future investigation.

53BP1 in V(D)J recombination: another unresolved riddle

Many of the studies on the factors contributing to the repair of recombination-activating gene (RAG) 1 and 2 DSBs (Box 2) have been performed in V(D)J recombination assays using extrachromosomal plasmids or integrated retroviral constructs as substrates, initially in non-lymphoid cells, and later in **Abelson-transformed pro-B cell lines** (hereafter called v-abl pro-B cells). In addition to the analysis of V(D)J recombination at the endogenous loci, these assays highlighted the crucial

role played by core NHEJ factors (KU70, KU80, LIG4, XRCC4) in ligating broken DNA ends [45–54]. At the same time, this approach revealed the contribution, often with overlapping functions, of multiple DSB repair factors (XLF, PAXX, DNA-PKcs, ATM) to a key event occurring after RAG cleavage and before ligation; namely, the stabilization of DNA end tethering in the recombination center (RC) [55–59].

53BP1 was initially considered dispensable for V(D)J recombination, since germline deletion of 53BP1 in mice does not block B and T lymphocyte development [6,60]. In addition, V(D)J recombination assays in v-abl pro-B cells show normal recombination efficiencies in 53BP1-deficient cells [11,12,38]. However, a thorough assessment of the lymphoid compartments of *Trp53bp1*$^{-/-}$ mice showed a decreased proportion of **immature B cells** in the bone marrow and an increased percentage of **DN3-cell-stage** T cells in the thymus, relative to wt mice [10–12,31,38]. Collectively, these results suggest defects in V(D)J recombination. In addition, analysis of gene usage during *Tcrd* gene (encoding TCRδ) assembly revealed a reduced ability of *Trp53bp1*$^{-/-}$ progenitor T cells compared with wt cells to recombine *Vd* genes that are distant in the linear genome from the *Dd* and *Jd* segments, thus limiting TCRδ receptor repertoire diversity [10,38]. Finally, despite the fact that mice deficient for the NHEJ factor XLF (*Xlf*$^{-/-}$) display mild lymphocytopenia [61,62], combinatorial deficiency of both proteins results in a severely immunocompromised phenotype with complete loss of mature splenic B and T lymphocytes [11,12]. Accordingly, *Trp53bp1*$^{-/-}$*Xlf*$^{-/-}$ v-abl pro-B cells show a dramatic decrease in V(D)J joining events compared with their wt counterparts, as evidenced from V(D)J assays using integrated retroviral constructs [11,12,38]. These results suggested that 53BP1 acts redundantly with XLF to support DNA end tethering during V(D)J recombination [11,12,38], which is likely to provide the optimal time window for successful repair (Figure 1); this scenario, however, remains speculative. We posit that the redundancy of factors supporting the stabilization of DSB ends in the RC is likely to safeguard the development of B and T cell lineages in the case of loss-of-function mutations in a single contributing component. Furthermore, in agreement with the dependency of lymphocyte development on efficient V(D)J recombination, knock-in of prearranged *Tcra*/*Tcrb* (encoding TCRα/TCRβ, T cells) and *Igh*/*Igk* (Igh/Igκ, B cells) rescues the lymphocyte developmental defects of 53BP1- and 53BP1-XLF-double-deficient mice, respectively [10,11]. These results prove that the loss of mature B and T lymphocytes in the mutant mice is due to the inability of developing lymphocytes to efficiently perform V(D)J recombination.

53BP1 in V(D)J recombination: DSB end protection takes a step back

Under conditions of ATM kinase inhibition, which is thought to destabilize the RC [63], *Xlf*$^{-/-}$ v-abl pro B cells accumulate unrepaired signal and coding ends as seen in V(D)J assays using integrated retroviral constructs [56]. Ablation of 53BP1 in this context results in resection of these DSB ends, which indicates that 53BP1 can also protect V(D)J DSBs [11,12]. However, the fact that 53BP1-deficient mice develop mature B and T cells argues against a fundamental role for 53BP1's DSB protection function in V(D)J recombination. In support of this conclusion, two recent reports showed that *Shld1*$^{-/-}$ or *Rev7*$^{f/f}$*Mb1*$^{Cre/+}$ mice do not exhibit any of the B and T cell developmental defects seen in *Trp53bp1*$^{-/-}$ mice [31,38]. Furthermore, combined ablation of SHLD1 and XLF does not further reduce the recombination frequency of the single knockout v-abl pro B-cells in V(D)J assays [38]. Finally, SHLD1 deficiency does not recapitulate the *Tcrd* recombination defects observed in 53BP1-null cells [38]. Collectively, these findings uncouple the DSB end protection function of 53BP1 from its role in DSB end-tethering stabilization and *Tcrd* long-range recombination.

Revisiting 53BP1's role in V(D)J recombination: a pre-break role?

Recent advances in the genome architecture field have recently proposed that, similar to CSR, V(D)J recombination is highly dependent on the dynamic reconfiguration of the respective loci

[64–72]. In their linear configuration, the antigen receptor loci extend from 1 Mb (*Tcrb*, encoding TCRβ) to over 3 Mb (*Igk*, encoding Igκ) [73]. **3D DNA fluorescence *in situ* hybridization (3D FISH)** and **Hi-C** assays revealed that, in recombining cells, these loci transition from an extended to a reversibly contracted state [64–72,74–80]. It is now well established that cohesin-dependent loop extrusion represents the dominant mechanism controlling contraction of the *Igh* locus [68,69,81].

The short-versus-long-range V(D)J defect observed in the *Tcrd* locus in the absence of 53BP1 cannot be solely explained by its potential end-tethering activity and suggests a pre-break role for the protein in this setting. Whereas defects in end tethering can probably explain the reduced frequency of long-range recombination events, they cannot explain why short-range joining reactions are unaffected, or even increased [10,38]. In support of a pre-break hypothesis, 53BP1 has been implicated in the efficient contraction of the *Tcra* locus (encoding TCRα), as assessed by 3D DNA FISH experiments in thymocytes [10] (Figure 1). However, the relationship between the long-range recombination and locus contraction phenotypes as well as the underlying molecular mechanism remains an unexplored research area. In addition, a potential involvement of 53BP1 in long-range V(D)J recombination of *Ig* loci in B cells, to our knowledge, remains to be assessed. Thus, unbiased **V(D)J sequencing (VDJ-seq)** approaches in *ex vivo* progenitor B and T cells might pave the way to the unambiguous dissection of 53BP1's pre-DSB contribution to V(D)J recombination.

Concluding remarks

Resolution of programmed DSBs is a prerequisite for protective immunity. Here, we focused on 53BP1, the master regulator of DSB end protection, and discussed its multiple roles during antigen receptor diversification. Although, 53BP1's ability to inhibit DSB resection is a key determinant of CSR, this function *per se* does not explain the full extent of the defect caused by its deficiency. In reference to V(D)J, it is now clear that activities other than DSB protection underlie 53BP1's roles, although the limited number of studies to date has prevented the mechanistic dissection of its contribution to the reaction. The precise nature and molecular determinants of these additional activities remain to be elucidated (see Outstanding questions; Box 3).

In general, the plurality of phenotypes associated with 53BP1 deficiency in CSR and V(D)J recombination exemplifies how the architecture and dynamics of antigen receptor loci are intimately connected with the DSB repair machinery. Productive repair of both V(D)J and CSR breaks requires the timely integration of structural and DNA repair components. Accordingly, we emphasize that there are pitfalls and limitations when considering the repair of these programmed DSBs independent of their dynamic architectural context since this may lead to oversimplified data interpretation and conclusions. We envision that future experiments addressing the molecular interplay between *Tcr/Ig* loci dynamics and DSB repair kinetics will provide a more in-depth understanding of antigen receptor diversification reactions (see Outstanding questions) and ultimately of the molecular bases of humoral immunity.

Acknowledgments

We thank A. Rahjouei (Di Virgilio laboratory, MDC, Berlin) and L. Deriano (Institute Pasteur, Paris) for valuable discussions. The project was supported by the Initiative and Networking Fund of the Helmholtz Association (to M.D.V.).

Declaration of interests

The authors declare no competing interests.

Outstanding questions

To what extent do *Igh* configuration-related phenotypes of 53BP1 deficiency contribute to the severity of the CSR defect? How does 53BP1 impact *Igh* locus dynamics? 53BP1's postulated pre-break role might be explained by its ability to influence the loop extrusion process that directs 5′Eμ–3′RR interaction and S region synapsis.

Is stabilization of S region DSB end tethering a prerequisite for successful CSR? Is this activity mechanistically equivalent to that contributed by 53BP1 to the repair of V(D)J DSBs in an XLF-redundant manner? While the mechanism underpinning 53BP1's DSB end tethering might be the same in V(D)J recombination and CSR, the differential repair requirements of RAG- versus AID-mediated breaks could justify the distinct functional relationship between 53BP1 and XLF in the two reactions.

What are the molecular determinants (domains and post-translational modifications) responsible for the postulated new activities of 53BP1 in antibody diversification? 53BP1 is extensively modified at the post-translational level and the different modifications and/or combinations thereof are likely to underpin its distinct functions.

Do *Tcra* locus contraction and *Tcrd* long-range recombination phenotypes reflect a general pre-DSB role of 53BP1 in the dynamic reconfiguration of *Tcr/Ig* loci during V(D)J recombination in T and B cells? The dissection of 53BP1's pre-DSB roles in V(D)J recombination requires a comprehensive assessment of *Tcr/Ig* loci configuration as well as antigen receptor repertoire diversity in *Trp53bp1*$^{-/-}$ primary lymphocytes.

Does orientation-specific joining during CSR reflect an active mechanism contributed by 53BP1 (and other factors) or is it an indirect consequence of impaired *Igh* locus dynamics and/or DSB repair? Even when assuming that deletional joining of S region breaks is enforced by an active mechanism, the close interplay between DNA metabolic reactions and *Igh* locus dynamics in the CSRC may still impact joining bias.

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