Fixing DNA breaks during class switch recombination

Christopher J. Jolly, Adam J.L. Cook, and John P. Manis

Immunoglobulin (Ig) class switch recombination (CSR) involves the breakage and subsequent repair of two DNA sequences, known as switch (S) regions, which flank IgH constant region exons. The resolution of CSR-associated breaks is thought to require the nonhomologous end-joining (NHEJ) DNA repair pathway, but the role of the NHEJ factor DNA-dependent protein kinase catalytic subunit (DNA-PKcs) in this process has been unclear. A new study, in which broken IgH-containing chromosomes in switching B cells were visualized directly, clearly demonstrated that DNA-PKcs and, unexpectedly, the nuclease Artemis are involved in the resolution of switch breaks.

Unrepaired DNA double-strand breaks (DSBs) pose a tremendous threat to cells. Correspondingly, multiple pathways have evolved that allow cells to sense, respond to, and repair chromosomal breaks. DSBs can initiate programs that pause replication and repair DNA damage. DSBs can also initiate apoptosis, which may avoid the risk of improper repair that could lead to gene inactivation or oncogenic chromosomal translocations. DSB response and repair pathways are thus a major bulwark against cancer. The field of DNA repair has recently undergone a major transformation, due in part to the ability to better characterize the factors involved in the repair of cellular DNA breaks, and in part to an improved ability to induce and monitor chromosomal breaks. On page 557 in this issue, Franco et al. (1) test the role of end-joining factors, including DNA-PKcs, in B cells undergoing CSR by monitoring the appearance of broken IgH-containing chromosomes. In this commentary, we discuss how these findings help resolve the controversy regarding the role of DNA-PKcs in class switching. The utility of directly monitoring chromosomal events in addition to measuring levels of Ig produced when analyzing the function of repair factors in Ig class switching is also highlighted.

C.J. Jolly is at Centenary Institute, The University of Sydney, Sydney NSW 2006, Australia.

A.J.L. Cook is at UMR 218 Centre National de la Recherche Scientifique/Institut Curie, 75248 Paris, France.

J.P. Manis is at Joint Program in Transfusion Medicine and Department of Laboratory Medicine, Children's Hospital Boston, and Department of Pathology, Harvard Medical School, Boston, MA 02115.

CORRESPONDENCE
J.P.M.: manis@enders.tch.harvard.edu

DSB repair pathways

There are two principal pathways used to repair DSBs in mammalian cells: homologous recombination (HR) and NHEJ (2). HR uses an identical or homologous sequence as a template to extend DNA ends past a breakpoint, usually with high fidelity, whereas NHEJ simply joins DNA ends independent of sequence homology. Because DSB repair frequently requires the processing of the broken DNA ends before direct ligation, NHEJ is much more prone to sequence alterations at the junction than is HR. To date, there are seven factors known to function in the NHEJ pathway. Ku70 and Ku80 form a heterodimer that binds to broken DNA ends and recruits the DNA-PKcs, forming a triad complex termed DNA-PK. DNA-PKcs binds and phosphorylates Artemis, activating its DNA end-processing activities. Next, the DNA ends are ligated by a complex of DNA ligase IV (Lig4) and XRCC4, factors thought to function solely in the resolution phase of end joining. Cernunnos (also known as XLF), a recently identified NHEJ factor whose function is under intense investigation, also associates with XRCC4. Cells deficient for Ku, XRCC4, or Lig4 have growth defects, are more sensitive to ionizing radiation, and undergo premature senescence. In contrast, cells deficient for DNA-PKcs, Artemis, or Cernunnos are more sensitive to ionizing radiation but lack overt cellular growth defects.

Programmed DNA breaks and repair in B cells

B and T lymphocytes are developmentally programmed to undergo ordered DNA breaks that somatically alter their genome and generate a large and diverse range of antigen receptor variable regions. Thus, these cells offer an ideal system to examine physiological DNA repair mechanisms. In B cells, Ig variable region exons are assembled from component germline variable (V), diversity (D), and joining (J) gene segments in a highly regulated lymphocyte-specific process, called V(D)J recombination, which is catalyzed by the recombination-activating gene (RAG) protein complex (3). Joining of RAG-cleaved V(D)J exon–coding components involves all of the known NHEJ components, whereas direct joining of the excised DNA fragment between the recombination signal sequences requires the Ku heterodimer, XRCC4, Lig4, and Cernunnos, but is less reliant on DNA-PKcs and is independent of Artemis activity. Low levels of coding end joining occur in some mouse models of Ku70, DNA-PKcs, or Artemis deficiency, leading to "leaky" blocks in lymphocyte development, whereas Ku80, XRCC4, or Lig4 deficiency leads to a complete block. However, mice lacking any of the NHEJ factors are severely immunodeficient due to defective repair of the programmed

DSBs that initiate functional antigen receptor gene assembly early in lymphocyte development.

Once mature IgM$^+$ B cells are generated in the bone marrow, they exit to the spleen, lymph nodes, and Peyer's patches where they participate in immune responses. Two additional genomic alterations of Ig genes can take place in activated B cells (4). Somatic hypermutation (SHM) introduces point mutations into Ig V(D)J exons at a high rate, thereby allowing for selection of B cells that produce higher affinity antibodies. IgH CSR irreversibly replaces the constant region (CH) Cμ gene with one of the downstream CH genes, allowing the generation of a different antibody class (IgG, IgA, or IgE). CSR thus alters Ig effector function while leaving the specificity of the rearranged V(D)J exon unaltered. The mouse germline IgH locus contains eight sets of CH genes, with the Cμ gene directly proximal to the rearranged V(D)J exon, and the other CH genes positioned within a 200-kb region downstream of Cμ. Upstream of each CH gene, except IgD, is a repetitive DNA sequence referred to as a switch (S) region. CSR involves the breakage and subsequent synapsis and repair of two target S regions. Both CSR and SHM require the B cell–specific enzyme activation-induced cytidine deaminase (AID; Fig. 1) (5, 6), which acts on single-stranded DNA. AID initiates SHM or CSR by converting cytosine to uracil in Ig S regions or V regions, respectively. Deamination of cytidine residues within S regions generates DNA mismatches that are converted into strand lesions and, subsequently, DSBs that initiate CSR.

For CSR to occur, separate DSBs within two participating S regions (which can be separated by up to 200 kb) are joined rather than simply repaired individually (7, 8). NHEJ was long suspected to be the dominant pathway in CSR, as sequences derived from S region joins lack long stretches of homology (9). Direct testing of the role of NHEJ necessitated the generation of NHEJ-deficient B cells with prerearranged heavy and light chain transgenes "knocked-in" to bypass the early developmental block in V(D)J recombination. This strategy showed that CSR depends on Ku70 and Ku80, although Ku-deficient cells have general cellular defects, including impaired proliferation, which may impact CSR independent of end-joining defects (10–12). In contrast, relatively robust CSR (25–50% of wild-type levels to all Ig classes) was recently demonstrated in XRCC4- and Lig4-deficient B cells, factors that appear to function only in NHEJ (13). Thus, class switching is not entirely dependent on "classical" NHEJ. Careful sequence analysis revealed that S region junctions were joined predominantly by microhomology in XRCC4- or Lig4-deficient cells, uncovering a novel microhomology-driven end-joining pathway (13, 14).

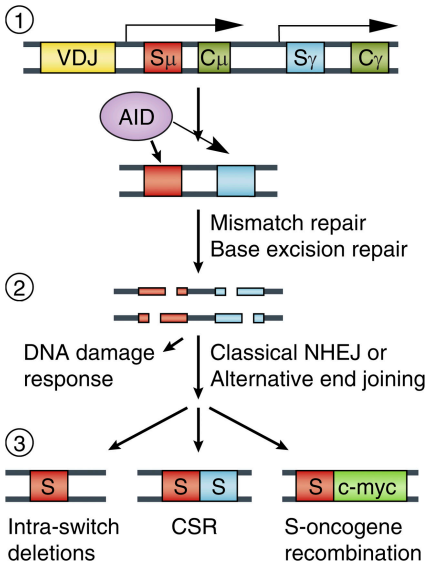

Figure 1. Mechanism of CSR. CSR involves three general steps. First (1), transcription is initiated upstream of the IgH CH regions undergoing CSR, proceeding through the S region and terminating after the CH exons. Transcription through the S region is thought to target AID to this region, resulting in deamination of cytidine residues to uridine. The U/G mismatches are then recognized and processed by various base excision repair factors and the mismatch repair pathway, leading to the generation of DNA strand breaks. Next (2), staggered DNA breaks on both strands result in DSBs, which in turn activate the DDR pathway and the DNA repair pathway. Finally (3), synapsis occurs between two S regions, and DSBs are repaired. S region DSBs can result in three different events: recombination within a single S region (intra-switch recombination), recombination between two S regions (or CSR), and recombination between an S region and another free DSB outside the IgH locus, which may result in an oncogenic translocation. All classical NHEJ factors tested to date are involved in CSR (Ku70, Ku80, XRCC4, DNA-Lig4, DNA-PKcs, and Artemis). An alternative end-joining repair pathway is uncovered in the absence of the NHEJ factors XRCC4 or DNA-Lig4.

Is DNA-PKcs required for switching?

The exact role for DNA-PKcs during CSR has been controversial. Severe combined immunodeficiency (*Scid*) mice harbor a spontaneous point mutation in the DNA-PKcs gene that results in the loss of the last 83 amino acids of DNA-PKcs and decreased protein expression (15). *Scid* mice are known to be leaky for V(D)J recombination and have small numbers of peripheral lymphocytes. In older *Scid* mice, the presence of switched Ig isotype classes indicates that CSR can occur, although it is difficult to quantify the efficiency of the CSR process given the ability of only a limited number of B cells to reconstitute peripheral compartments over time (16). Subsequent studies using transformed *Scid* pre–B cell lines found that switching to IgE was heavily impaired, suggesting a necessity for functional DNA-PKcs for class switching at least to that isotype (17). Three independent laboratories have generated IgH knock-in DNA-PKcs–deficient mice

using differing DNA-PKcs mutations, mouse strains, and IgH transgene specificity. One version of the DNA-PKcs–null mice had no detectable DNA-PKcs protein and a complete block in lymphocyte development in the absence of Ig transgenes. In these mice, CSR to all CH genes was completely blocked, with the striking exception of Cγ1, indicating an important role for DNA-PKcs in class switching (18). Another group reconstituted BALB/c *Scid* mice with Ig knock-in transgenes and found variably reduced levels of CSR to all CH genes. They concluded that DNA-PKcs activity played no role in CSR (19). More recently, the same group analyzed a mouse model in which the C-terminal kinase domain of DNA-PKcs was deleted (in theory generating a truncated protein similar to that seen in *Scid* mice, and accounting for the reported leaky lymphocyte development), and found evidence for near-normal switching to all isotypes (20). The third group used an IgH knock-in transgenic *Scid* mouse on a pure C57BL/6 background. They found that switching occurred to all isotypes, but the level of switching was variably reduced from 25 to 75% of controls (21). This group also uncovered evidence of aberrant processing of S region DSBs in *Scid* B cells; nontemplate strand mutations at cytosine residues in CSR junctions were lost, supporting a role for DNA-PKcs in CSR. Careful sequence analysis of S region junctions in DNA-PKcs–deficient mice revealed an essentially wild-type distribution of sequence homology length and no evidence for the microhomology-mediated end-joining pathway that was uncovered in the studies of XRCC4 or Lig4 deficiency (13, 14, 18, 21). In contrast to DNA-PKcs–deficient Ig knock-in B cells, Artemis-deficient B cells assayed in parallel revealed no readily detectable defects in IgH class switching, suggesting that most ends joined during CSR do not require processing by Artemis and that DNA-PKcs functions in CSR in an Artemis-independent fashion (22).

Revelations from monitoring chromosomal breaks

The reports of end joining during CSR collectively support a role for the classical NHEJ pathway in achieving normal CSR. Yet the variable degree of impairment in the different models of DNA-PKcs deficiency is perplexing. The variability may result in large part from the method used to detect CSR products. Measurement of serum Ig produced in vivo may not directly reflect the degree of CSR impairment, given the variables inherent to Ig accumulation and preferential expansion of certain B cell subsets. CSR is regulated by cell division number (23), yet even careful measurement of the number of cells that express switched Ig per cell division (by CFSE staining and FACS) after in vitro stimulation creates the potential of misjudging the degree of impairment of CSR, especially in cases when the defects are mild. The value of using more direct techniques to investigate CSR is clearly demonstrated in the new study by Franco et al. (1).

In their study, Franco et al. (1) use a highly sensitive technique to monitor DNA breakage and repair in cells undergoing CSR by fluorescently labeling the 5′ and 3′ ends of the IgH locus to track AID-generated breaks. This group and others have previously used this method (known as IgH fluorescence in situ hybridization [FISH]) to observe IgH loci breaks resulting from AID activity, which are frequently involved in translocations (24, 25). Using this same strategy, the group now finds that DNA-PKcs–deficient B cells, which switch efficiently to IgG1, nonetheless harbor a remarkably high degree of breaks in chromosome 12 (Chr 12), the IgH-associated chromosome, when activated to switch to multiple isotype classes. The increased breakage of Chr 12 during CSR in DNA-PKcs–null B cells suggests that DNA-PKcs plays a functional role in CSR and that it functions by catalyzing the end-joining reaction between two switch regions.

A more striking finding of the study is the high degree of unresolved Chr 12 breaks in Artemis-deficient B cells undergoing CSR. These cells do not display any overt CSR defects when using standard assay criteria, such as surface Ig expression. The IgH FISH assay for CSR is more sensitive, as the background level of breaks detected in wild-type B cells is extremely low. Thus, Artemis functions to resolve a subset of AID-initiated DSBs during CSR.

These new findings underscore the importance of using the right tools to test factors that are involved in the response to or repair of AID-induced breaks. It is now clear that the ability of a population of genetically disrupted cells to resolve AID-induced breaks into successful Ig class switching events at apparently normal frequencies does not preclude the possibility that potentially disastrous AID-induced chromosomal damage is occurring in the few cells that did not correctly switch. We may have incorrectly ignored this side of the coin until now.

Resolving the role of DNA-PKcs in CSR

Kiefer et al. (20) previously concluded that DNA-PKcs is not involved in CSR, based on their findings that CSR to all IgH isotypes appeared relatively normal in Ig knock-in transgene-complemented B cells carrying a targeted DNA-PKcs mutation that eliminated the catalytic domain of DNA-PKcs. At the time, they suggested that the apparent discrepancy between their findings and those generated using the complete DNA-PKcs mutant might reflect strain differences, HL transgene differences, or putative inadequacies in the detection of IgH isotypes. However, major defects in CSR to all IgH isotypes except IgG1 were confirmed in two different mouse strains, in assays done in parallel with those that found intact CSR to all IgH isotypes for Artemis-deficient or control B cells. Furthermore, the markedly altered mutation pattern observed in *Scid* cell switch junctions (21) cannot be dismissed by such arguments. Thus, differences in the sensitivity of the detection assays used cannot account for the discrepancy.

In any case, the current work by Franco et al. (1) unequivocally demonstrates that DNA-PKcs is required for end joining of a subset of AID-induced CSR breaks. The reason for the less severe defect in CSR to IgG1 in the complete

DNA-PKcs knockout, and the intriguing question of whether or not the catalytic domain mutant and the complete null mutant differ in their effects on CSR, are still open. Future studies using the IgH FISH CSR assay to compare the various mutant DNA-PKcs mouse models should answer the latter question.

Another major message to be taken from this study is that different CSR activation cocktails can result in different outcomes in the FISH IgH assay. Lipopolysaccharide (LPS)-stimulated DNA-PKcs knockout B cells failed to switch to IgG3 or IgG2b but, unexpectedly, did not exhibit excessive Chr 12 breaks. However, when the p53 cell cycle checkpoint pathway is inactivated in LPS-stimulated DNA-PKcs knockout B cells, abundant Chr 12 breaks are observed, indicating that LPS-activated B cells harboring unresolved DSBs are normally eliminated via a p53-dependent mechanism. Despite unmasking the Chr 12 breaks, p53 deficiency does not rescue CSR to IgG3 nor IgG2b in DNA-PKcs knockout B cells, indicating that impaired switching is due to the inability to end join and not merely the elimination of switching cells in culture. DNA-PKcs knockout B cells treated with anti-CD40 antibodies and interleukin 4 exhibit relatively high levels of AID-induced Chr 12 breaks that are not modulated by p53 deficiency, supporting the notion that this specific activation treatment suppresses the p53 response in a similar fashion as that seen in Bcl-6–expressing germinal center B cells. The interplay between S region joining and cell cycle checkpoint regulation during CSR is also clearly demonstrated in IgH FISH studies of DNA damage response (DDR) factors that affect both these processes. B cells lacking ATM, H2AX, or 53BP1 are impaired in resolving CSR-associated breaks, and high levels of Chr 12 breaks were observed after LPS treatment (24, 25).

Future questions for IgH FISH

Definitive proof of the role of DNA-PKcs and Artemis deficiency during CSR provides motivation to extend IgH FISH studies to other DDR or DNA repair factors. For example, studies using IgH FISH in switching Ku-deficient B cells will further delineate the role of the DNA-PK complex versus that of DNA-PKcs, as the latter harbors functions apart from end joining, including phosphorylation of DDR pathway members. It may even be worth revisiting the role of HR in CSR or SHM, especially in the DDR- or in repair-deficient backgrounds. Classical switch experiments have shown that core HR proteins, such as Rad54, are not involved in CSR, but it remains possible that HR deficiencies could nonetheless lead to the accumulation of AID-induced S region breaks in activated B cells, particularly in S/G2 phase cells.

In addition, the IgH FISH assay will facilitate careful examination of the IgH chromosome in human diseases, such as immunodeficiencies that affect class switching to a higher degree than V(D)J recombination. This is important, as it has become increasingly clear that not all types of DNA breaks are treated equally. For example, CSR appears to require certain DDR factors such as 53BP1, yet V(D)J recombination appears relatively unaffected in the absence of 53BP1 (26, 27). Additionally, extending the IgH FISH technique to monitor breaks in the human IgH locus and in proto-oncogenes, as has been reported for mouse B cells, will lead to novel insights in lymphomagenesis.

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