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Sha Luo, Ruolin Qiao, Hailiang Zha, Leyi Yu, Xiaoling Shan, Shuchan Li, Zifan Yang, Aoyu Ma, Yaxin Cheng, Yao-Feng Zhao & Xuefei Zhang

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**Chromatin-intrinsic mechanisms determine orientation-specific class switch recombination**

Sha Luo<sup>1,2,3#</sup>, Ruolin Qiao<sup>1,2,3#</sup>, Hailiang Zha<sup>1,2#</sup>, Leyi Yu<sup>1,2#</sup>, Xiaoling Shan<sup>1,2</sup>, Shuchan Li<sup>1,2</sup>, Zifan Yang<sup>1,2</sup>, Aoyu Ma<sup>1,2</sup>, Yaxin Cheng<sup>4</sup>, Yao-Feng Zhao<sup>4</sup>, Xuefei Zhang<sup>1,2,3\*</sup>

<sup>1</sup>Biomedical Pioneering Innovation Center (BIOPIC), School of Life Science, Peking University, 100871 Beijing, China

<sup>2</sup>Beijing Advanced Innovation Center for Genomics (ICG), Peking University, 100871 Beijing, China

<sup>3</sup>Joint Graduate Program of Peking-Tsinghua-NIBS, Academy for Advanced Interdisciplinary Studies, Peking University, 100871 Beijing, China

<sup>4</sup>State Key Laboratory of Animal Biotech Breeding, College of Biological Sciences, National Engineering Laboratory for Animal Breeding, Frontiers Science Center for Molecular Design Breeding, China Agricultural University, 100193 Beijing, China

<sup>#</sup>These authors contributed equally to this work.

\*Correspondence to Xuefei Zhang: [Xuefei\\_zhang10@pku.edu.cn](mailto:Xuefei_zhang10@pku.edu.cn)

## Abstract

**Activation-induced cytidine deaminase (AID)-initiated immunoglobulin heavy chain (*Igh*) class switch recombination (CSR) replaces donor  $C_{\mu}$  constant region exons ( $C_{HS}$ ) with a downstream acceptor  $C_H$  to generate different functional antibody isotypes. However, mechanisms governing orientation-specific productive CSR remain incompletely understood. Through analysing the characteristics of evolved constant regions and constructing diversified constant regions to recapitulate productive CSR in jawed vertebrates to systematically dissect productive CSR determinants, we find that switch topological configuration (STC), including transcriptional orientation, chromatin distance, and chromatin domain of *Igh*, determines orientation-specific joining of AID-initiated breaks for productive CSR. Long-distance  $C_{HS}$  under co-oriented transcription within *Igh* domain foster predominantly deletional joining-mediated productive CSR. In contrast, oppositely transcribed and short-distance  $C_{HS}$  affect end-joining bias via promoting diffusion-mediated inversional joining for CSR with lower efficiency. Moreover, AID-initiated breaks in different domains, facilitate more diffusion-mediated orientation-unbiased end-joining for non-productive CSR. Our findings uncover chromatin-intrinsic mechanisms safeguarding orientation-specific productive CSR throughout evolution.**

## Introduction

The functional efficacy of adaptive immunity is a defining feature of jawed vertebrates that equips them to counteract rapidly evolving pathogens (e.g., viruses, bacteria), which is crucially hinge on two synergistic processes: V(D)J recombination and class switch recombination (CSR)<sup>1</sup>. During B lymphocyte development, RAG endonuclease cleaves immunoglobulin heavy chain (*Igh*) V, D, and J gene segments to assemble a V(D)J exon adjacent to the  $C_{\mu}$  constant region exons ( $C_{HS}$ ), encoding the default IgM antibody<sup>2,3</sup>. Upon B lymphocyte activation, *Igh* CSR replaces  $C_{\mu}$  with one of several downstream acceptor  $C_{HS}$  to change antibody isotypes and functions<sup>3,4</sup>. Each  $C_H$  unit contains an I-promoter, a long repetitive switch (S) region intron, and several  $C_H$  coding exons<sup>5</sup>. Chromatin loop extrusion<sup>6,7</sup>-mediated *Igh* 3' regulatory region (3'RR) enhancer scanning activates transcription of acceptor  $C_{HS}$  and promotes S-S synapsis for recombination<sup>5,8-10</sup>. Activation-induced cytidine deaminase (AID)<sup>11</sup> targets the transcribed donor  $S_{\mu}$  region and an acceptor S region to initiated deamination lesions<sup>12</sup>, which are converted to double-strand breaks (DSBs) and joined by DNA damage repair factors to accomplish CSR<sup>4,13-15</sup>. The *Igh* domain boundary termed 3'CBEs downstream of 3'RR, consisted of ten consecutive CTCF-binding elements (CBEs)<sup>16</sup>, safeguards loop extrusion-mediated transcriptional and CSR activities within *Igh* domain<sup>17,18</sup>.

Unlike the orientation-unbiased random joining of most chromosomal DSB ends genome-wide<sup>19-21</sup>, AID-initiated CSR within *Igh* domain employs an orientation-specific joining mechanism to generate productive antibody isotypes<sup>22</sup>. The mouse *Igh* domain contains six acceptor  $C_{HS}$  in the same transcriptional orientation as the upstream donor  $C_{\mu}$ , indicating that only deletional joining between donor  $S_{\mu}$  breaks and acceptor S breaks results in productive CSR<sup>10</sup>. DNA damage repair factors, such as ATM<sup>22-25</sup>, H2AX<sup>22,25,26</sup>, 53BP1<sup>22,25,27,28</sup>, RIF1<sup>22,25,29,30</sup>, Rev7<sup>31,32</sup>, Shieldin<sup>33-37</sup>, and Ligase 4<sup>25,38</sup>, have been found to promote deletional joining-mediated productive CSR by facilitating DSB tethering, suppressing DSB processing and decreasing microhomology (MH) of CSR junctions in mice. Previous studies implicate the DNA damage repair factors in enforcing the deletional joining mechanism, but do not implicate them in providing a basis for the orientation-specific CSR<sup>22</sup>.

Chromatin loop extrusion has been proposed to be the underlying mechanism for CSR in *Mus musculus* and *Homo sapiens*, which promotes the formation of dynamic CSR centers where acceptor S regions get activated and synapsed with donor S $\mu$  for CSR<sup>9,10,39</sup>. Moreover, chromatin loop extrusion has also been proposed to explain the predominantly deletional CSR in *Mus musculus*, supported by the increase of inversional joining from 3% to 19% during S $\alpha$  CSR via inserting CBE-mediated chromatin loop extrusion blocks within *Igh* constant region in murine CH12F3 cells<sup>9,10</sup>. However, in some birds and reptiles, CSR must proceed by inversional joining to generate productive antibody isotypes due to the opposite transcriptional orientation of donor C $\mu$  and some acceptor C $_{HS}$ <sup>40,41</sup>. The chromatin loop extrusion-mediated predominantly deletional CSR in mice and the structural diversity of constant regions across jawed vertebrates suggest that regulation of orientation-specific joining for productive CSR is more than previously documented<sup>9,10,22,41</sup>. Thus, detailed mechanisms need to be investigated to comprehensively explain the joining features of productive CSR with different *Igh* architectures throughout evolution.

In this study, by comprehensively analysing the constant region across a series of species, we revealed that the distinct chromatin features play critical roles in regulating productive CSR. We constructed series of murine CH12F3 cell mutants with distinct *Igh* configurations and found that switch topological configuration (STC) including transcription orientation, chromatin distance, and chromatin domain, determines orientation-specific joining of AID-initiated breaks for productive CSR.

## Results

### Intrinsic chromatin features affect end-joining of CSR

To investigate the potential influencing factors promoting productive CSR with different *Igh* architectures throughout evolution, we conducted a detailed comparative analysis of constant region across multiple species. The sequence feature analysis of S regions indicated the GC content within S regions was increased in evolution (Supplementary Fig. 1a). In addition, RNA-seq analysis showed an adaptive fixation of unidirectional transcription polarity and a progressive elongation of *Igh* constant region along the evolutionary lineage from birds to mammals (Supplementary Fig. 1b). Moreover, Hi-C analysis indicated an increasing conservation of constant region within a domain across the Osteichthyes-to-Mammalia transition (Supplementary Fig. 1c). We further manipulated the *Igh* constant region of murine CH12F3 cells to investigate the roles of chromatin configurations in orientation-specific CSR.

CH12F3 cells undergo robust CSR between S $\mu$  and S $\alpha$  to generate predominantly deletional joining-mediated productive IgA antibodies upon  $\alpha$ CD40/IL4/TGF $\beta$  activation<sup>22,42</sup>. To eliminate the potentially confounding effects of non-productive *Igh* allele on the assessment of sequencing results, we employed Cas9/gRNA targeting to delete the entire non-productive *Igh* locus from upstream of the first V $_H$  to downstream of 3'CBEs in CH12F3N $\Delta$  cells<sup>9</sup>, thereby generating CH12F3PO cells (Supplementary Fig. 2a, b). We placed the C $\alpha$  unit next to the donor C $\mu$  with opposite transcriptional orientation by inverting the cluster of six acceptor C $_{HS}$  from upstream of C $\gamma$ 3 to downstream of C $\alpha$  in CH12F3PO cells to generate  $\gamma$ 3- $\alpha$ Inv cells (Fig. 1a, Supplementary Fig. 2c, d). Then we employed high-sensitive CSR-HTGTS-seq to assay the joining features of CSR junctions (Fig. 1b) and found that  $\gamma$ 3- $\alpha$ Inv cells exhibited predominantly deletional S $\gamma$ 3 CSR and a small but nonnegligible number of S $\alpha$  CSR (Fig. 1c). Remarkably, in contrast to the predominantly deletional S $\alpha$  CSR in CH12F3PO cells, S $\alpha$  CSR underwent

orientation-unbiased joining with 46% inversional joining of S $\mu$ -S $\alpha$  junctions in  $\gamma$ 3- $\alpha$ Inv cells without perturbing DNA damage repair factors (Fig. 1d, e).

We further used Pro-seq and 3C-HTGTS analyses to check the transcription status and chromatin interaction of *Igh* locus for revealing the underlying mechanisms. In contrast to the predominantly transcription of downstream C $\alpha$  unit in CH12F3PO cells,  $\gamma$ 3- $\alpha$  cluster inversion significantly activated transcription of downstream C $\gamma$ 3 unit and repressed upstream C $\alpha$  transcription in  $\gamma$ 3- $\alpha$ Inv cells (Fig. 1f-h, Supplementary Fig. 2e, f), suggesting that chromatin loop extrusion-mediated 3'RR scanning predominantly activates C $\gamma$ 3 unit instead of upstream C $\alpha$  unit in  $\gamma$ 3- $\alpha$ Inv cells. And the low transcription of C $\alpha$  unit is corresponding to the low level of AID-initiated S $\alpha$  CSR in  $\gamma$ 3- $\alpha$ Inv cells (Fig. 1c, g). In addition, unlike the dominant interaction between C $\alpha$  unit with iE $\mu$  and 3'RR in CH12F3PO cells, C $\gamma$ 3 unit interacted with iE $\mu$  and 3'RR within CSR center in  $\gamma$ 3- $\alpha$ Inv cells, facilitating S $\mu$ -S $\gamma$ 3 synapsis-mediated predominant deletional joining of S $\mu$ -S $\gamma$ 3 junctions (Fig. 1c, f, g, i, Supplementary Fig. 2e, f). Although the rarity of interactions between donor C $\mu$  and acceptor C $\alpha$  unit limited S $\mu$ -S $\alpha$  synapsis formation in  $\gamma$ 3- $\alpha$ Inv cells, low-frequent short-range diffusion could promote orientation-unbiased random joining of S $\mu$ -S $\alpha$  junctions, accounting for 46% inversional S $\alpha$  CSR for generating productive IgA antibodies (Fig. 1d-i, Supplementary Fig. 2e, f). These data suggest that chromatin configuration including transcriptional orientation and chromatin distance of C<sub>HS</sub> affects orientation-specific joining of CSR within *Igh* domain.

### Transcription orientation of C<sub>HS</sub> affects end-joining of CSR

Given that both transcriptional orientation and chromatin distance of C $\mu$  and C $\alpha$  units were changed in  $\gamma$ 3- $\alpha$ Inv cells by inverting the entire acceptor C<sub>H</sub> region (Fig. 1a), we further inverted different acceptor C<sub>H</sub> units individually to change the relative transcription orientation between donor C $\mu$  and acceptor C<sub>HS</sub>. We firstly inverted C $\alpha$  unit, the farthest downstream acceptor C<sub>H</sub> from donor C $\mu$  in CH12F3PO cells, to generate  $\alpha$ Inv cells (Fig. 2a, Supplementary Fig. 3a, b). The results showed that  $\alpha$ Inv cells slightly increased inversional joining of S $\mu$ -S $\alpha$  junctions from 4% to 8% (Fig. 2b-d). And this increase of inversional joining in  $\alpha$ Inv cells had little effect on the MH of S $\mu$ -S $\alpha$  junctions (Supplementary Fig. 3c), suggesting a distinct regulation from DNA damage repair factor-deficiency-mediated increase of inversional joining during CSR. Then we took advantages of the activated CSR from upstream acceptor C<sub>HS</sub> upon I $\alpha$  deletion in I $\alpha$  $\Delta$  CH12F3 cells<sup>9</sup>. We inverted C $\gamma$ 2a unit in I $\alpha$  $\Delta$  cells to generate  $\gamma$ 2aInv cells (Fig. 2e, Supplementary Fig. 3d, e) and found that  $\gamma$ 2aInv cells significantly increased inversional joining of S $\mu$ -S $\gamma$ 2a junctions from 12% to 25% without affecting MHs (Fig. 2f-h, Supplementary Fig. 3f). We further inverted C $\gamma$ 1 unit in I $\alpha$  $\Delta$  cells to generate  $\gamma$ 1Inv cells (Fig. 2i, Supplementary Fig. 3g, h) and found that  $\gamma$ 1Inv cells significantly increased inversional joining of S $\mu$ -S $\gamma$ 1 junctions from 5% to 43% without affecting MHs (Fig. 2j-2l, Supplementary Fig. 3i). We further inverted C $\alpha$  unit in  $\gamma$ 3- $\alpha$ Inv cells to generate  $\gamma$ 3- $\alpha$ Inv- $\alpha$ Inv cells (Fig. 2m, Supplementary Fig. 3j, k). Notably,  $\gamma$ 3- $\alpha$ Inv- $\alpha$ Inv cells significantly decreased inversional joining of S $\mu$ -S $\alpha$  junctions from 43% to 18% without affecting MHs (Fig. 2n-p, Supplementary Fig. 3l). All these data indicate that transcription orientation play distinct roles from DNA damage repair factors in regulating orientation-specific joining of CSR with more inversional joining from two opposite transcribed C<sub>HS</sub>.

### Chromatin distance of C<sub>HS</sub> affects end-joining of CSR

To further assess the effects of chromatin distance between donor  $C_{\mu}$  and acceptor  $C_H$  on orientation-specific joining of CSR, we changed the chromatin distance between  $C_{\mu}$  and  $C_{\gamma 3}$  unit. We firstly inverted  $C_{\gamma 3}$  unit in  $I\alpha\Delta$  cells to generate  $\gamma 3Inv$  cells, which have shorter distance between  $C_{\mu}$  and  $C_{\gamma 3}$  (Fig. 3a, Supplementary Fig. 4a, b), and  $\gamma 3Inv$  cells significantly increased inversional joining of  $S_{\mu}$ - $S_{\gamma 3}$  junctions from 8% to 20% (Fig. 3b-d). Given  $\gamma 3Inv$  cells changed both chromatin distance and transcription orientation of  $C_{\mu}$  and  $C_{\gamma 3}$  compared to  $I\alpha\Delta$  cells, we further inverted  $C_{\gamma 3}$  unit in  $\gamma 3Inv$  cells to generate  $\gamma 3Inv$ - $\gamma 3Inv$  cells (Fig. 3a, Supplementary Fig. 4a, c). We found that  $\gamma 3Inv$ - $\gamma 3Inv$  cells decreased inversional  $S_{\mu}$ - $S_{\gamma 3}$  junctions from 20% to 14% compared to  $\gamma 3Inv$  cells (Fig. 3c-e), consistent with the conclusion that opposite transcriptional orientation of  $C_{HS}$  increases inversional joining of CSR (Fig. 2). Notably, compared to  $I\alpha\Delta$  cells,  $\gamma 3Inv$ - $\gamma 3Inv$  cells increased inversional  $S_{\mu}$ - $S_{\gamma 3}$  junctions from 8% to 14% (Fig. 3b, d, e), indicating that chromatin distance contributes to orientation-specific joining of CSR. We also deleted the intergenic region between  $C_{\mu}$  and  $C_{\gamma 3}$  unit directly to generate  $\gamma 3IRD$  cells (Fig. 3a, Supplementary Fig. 4d, e), and  $\gamma 3IRD$  cells significantly increased inversional joining of  $S_{\mu}$ - $S_{\gamma 3}$  junctions from 8% to 15% (Fig. 3b, d, f), exhibiting similar trend as  $\gamma 3Inv$ - $\gamma 3Inv$  cells. And this short-distance-mediated increase of inversional joining was not dependent on changing MH-related DNA damage repair pathways (Supplementary Fig. 4f). Altogether, these data indicate that chromatin distance of  $C_{HS}$  play roles in regulating orientation-specific joining of CSR with more diffusion-mediated inversional joining from two short-distance  $C_{HS}$ .

### **Inverted I promoter-mediated block activates inversional CSR**

To reveal the regulatory mechanism of transcriptional orientation in orientation-specific joining of CSR, we further fused the full  $S_{\gamma 3}$  and  $S_{\alpha}$  in CH12F3PO cells and  $\alpha Inv$  cells to generate  $I\gamma 3$ -FS- $C_{\alpha}$  and  $I\gamma 3$ -FS- $I\alpha$  cells respectively (Supplementary Fig. 5a-c). In  $I\gamma 3$ -FS- $C_{\alpha}$  cells, both  $S_{\gamma 3}$  and  $S_{\alpha}$  were transcribed under  $I\gamma 3$  promoter with the same transcription orientation as donor  $C_{\mu}$ , synapsed with donor  $C_{\mu}$ , and underwent robust deletional  $S_{\gamma 3}$  CSR and  $S_{\alpha}$  CSR at similar levels (Fig. 4a, Supplementary Fig. 5d). However, in  $I\gamma 3$ -FS- $I\alpha$  cells, both  $S_{\gamma 3}$  and  $S_{\alpha}$  were transcribed under inverted  $I\alpha$  promoter instead of the upstream  $I\gamma 3$  promoter, suggesting that 3'RR enhancer scans to activate the closer promoter for transcriptional activation (Fig. 4b, Supplementary Fig. 5e). Compared to the robust synapsis between donor  $S_{\mu}$  and both  $S_{\gamma 3}$  and  $S_{\alpha}$  in  $I\gamma 3$ -FS- $C_{\alpha}$  cells (Fig. 4a, Supplementary Fig. 5d),  $I\gamma 3$ -FS- $I\alpha$  cells exhibited major interaction between donor  $S_{\mu}$  with inverted  $I\alpha$  promoter and adjacent  $S_{\alpha}$ , which blocked transcriptional activation of  $I\gamma 3$  promoter, leading to predominantly  $S_{\alpha}$  CSR and less frequent  $S_{\gamma 3}$  CSR (Fig. 4b, Supplementary Fig. 5e). Meanwhile, cohesin significantly accumulated at the inverted  $I\alpha$  promoter and this inverted  $I\alpha$  promoter with cohesin accumulation-mediated block effects increased inversional joining of  $S_{\mu}$ - $S_{\alpha}$  junctions from 8% to 19% and of less frequent  $S_{\mu}$ - $S_{\gamma 3}$  junctions from 10% to 36% (Fig. 4a, b, Supplementary Fig. 5f).

We also fused the partial  $S_{\gamma 3}$  and  $S_{\alpha}$  in CH12F3PO cells and  $\alpha Inv$  cells to generate  $I\gamma 3$ -PS- $C_{\alpha}$  and  $I\gamma 3$ -PS- $I\alpha$  cells respectively (Supplementary Fig. 5a, g, and h). In  $I\gamma 3$ -PS- $C_{\alpha}$  cells, both  $S_{\gamma 3}$  and  $S_{\alpha}$  were transcribed under  $I\gamma 3$  promoter with the same transcription orientation as donor  $C_{\mu}$ , synapsed with donor  $C_{\mu}$ , and underwent robust  $S_{\gamma 3}$  CSR and less frequent  $S_{\alpha}$  CSR (Fig. 4c, Supplementary Fig. 5i). Compared to the robust  $S_{\alpha}$  CSR in  $I\gamma 3$ -FS- $C_{\alpha}$  and  $S_{\gamma 3}$  CSR in  $I\gamma 3$ -PS- $C_{\alpha}$ , the less frequent  $S_{\alpha}$  CSR in  $I\gamma 3$ -PS- $C_{\alpha}$  cells also suggest that AID targets more 5'S

region for CSR (Fig. 4a, c). I $\gamma$ 3-PS-I $\alpha$  cells exhibited a similar trend of transcription status, chromatin interaction, cohesin accumulation, and CSR as I $\gamma$ 3-FS-I $\alpha$  cells (Fig. 4b, d, Supplementary Fig. 5e, j). The inverted I $\alpha$  promoter in I $\gamma$ 3-PS-I $\alpha$  cells dominated the transcription orientation, chromatin interaction, cohesin accumulation, and CSR with more diffusion-mediated inversional S $\mu$ -S $\alpha$  and S $\mu$ -S $\gamma$ 3 junctions (Fig. 4d, Supplementary Fig. 5j). These results indicate that unidirectional transcription polarity within *Igh* constant region orchestrates 3'RR-mediated transcriptional activation, cohesin accumulation, and chromatin interaction to regulate loop extrusion- and diffusion-mediated end-joining of CSR.

### Chromatin domains of *Igh* affect end-joining of CSR

To investigate the joining features of AID-initiated breaks located in different chromatin domains, we placed 3'RR and its associated C $\alpha$  unit outside *Igh* domain by inverting the C $\alpha$ -3'CBEs region in  $\alpha$ Inv cells to generate  $\alpha$ -3CBEInv cells, in which 3'RR and C $\alpha$  are located in a different domain from donor C $\mu$  unit (Fig. 5a, Supplementary Fig. 6a, b). Remarkably,  $\alpha$ -3CBEInv cells significantly increased inversional S $\mu$ -S $\alpha$  junctions from 8% to 53% without affecting MHs (Fig. 5b-d, Supplementary Fig. 6c). In contrast to the predominantly S $\mu$ -S $\alpha$  synapsis for robust deletional S $\alpha$  CSR in  $\alpha$ Inv cells (Fig. 5e, Supplementary Fig. 6d), C $\alpha$  unit in  $\alpha$ -3CBEInv cells was isolated by the new topologically-associated domain (TAD) formed by the inverted 3'CBEs and downstream CBE, which prevented S $\mu$ -S $\alpha$  synapsis even C $\alpha$  unit was highly transcribed (Fig. 5f-j, Supplementary Fig. 6e). These data indicate that the donor C $\mu$  and acceptor C $H$  located in different TADs undergo diffusion-mediated orientation-unbiased end-joining of CSR.

Given that the above CSR occurs between classical donor S $\mu$  and acceptor S regions, we also wanted to investigate the general joining mechanism between AID-targeted classical S region and off-targets. Within *Igh* domain, chromatin loop extrusion-mediated 3'RR scanning activates C $\alpha$  for CSR in CH12F3PO cells and scans upstream C $H$ S to activate their CSR activity upon the elimination of C $\alpha$  competition in I $\alpha$  $\Delta$  cells<sup>9</sup>. In  $\alpha$ -3CBEInv cells, 3'RR activates C $\alpha$  for CSR in a new TAD and we further deleted C $\alpha$  unit in  $\alpha$ -3CBEInv cells to generate  $\alpha$ -3CBEInv- $\alpha$  $\Delta$  cells (Fig. 6a, Supplementary Fig. 7a, b). The data indicated that 3'RR activated an ectopic S (eS) region immediately downstream of the deleted C $\alpha$  unit and donor S $\mu$  recombined, without orientation bias, to this eS region in  $\alpha$ -3CBEInv- $\alpha$  $\Delta$  cells (Fig. 6b-g). This eS region indicated a significant lower GC content than classical S $\mu$  and S $\alpha$  regions (Supplementary Fig. 7c), corresponding to the less AID targeting frequency (Fig. 6b, c). These data indicate that AID-initiated on- and off-targets located in different domains promotes diffusion-mediated orientation-unbiased recombination.

### Discussion

In this study, through analysing chromatin features of constant region across different species (Fig. 7a) and constructing a series of *Igh* constant regions to recapitulating productive CSR in jawed vertebrates, we have revealed some evolutionary adaptations of the constant region facilitating CSR in evolutionary species, including that transcription polarity, chromatin distance, and TAD of *Igh*, termed as switch topological configuration (STC), plays critical roles in regulating orientation-specific joining of AID-initiated CSR for generating productive antibodies (Fig. 7b, c). Some species, such as *Mus musculus* and *Homo sapiens*, have same transcription orientation between acceptor C $H$ S with donor C $\mu$  within *Igh* locus and undergo orientation-biased

deletional joining of CSR, leading to efficient generation of productive antibodies<sup>22,39</sup>. Chromatin loop extrusion can promote long-range S-S synapsis for predominantly orientation-biased deletional joining-mediated productive CSR in some species such as mice (Fig. 7b). In contrast, some species having opposite transcribed C<sub>H</sub>s, short-distance C<sub>H</sub>s, as well as C<sub>H</sub>s located in different TADs, strongly activate short-range diffusion-mediated random joining of AID-initiated breaks, leading to more inversional joining-mediated productive CSR (Fig. 7c).

Cohesin-mediated chromatin loop extrusion has been proposed to explain the packaging of large amounts of chromatin DNA into TADs within the nucleus<sup>6,7</sup>. Cohesin can be loaded onto chromatin and promote the formation of chromatin loop domains when cohesin reaches a pair of convergently oriented CBEs bound by CTCF<sup>6,7,43</sup>. In addition to CTCF, some factors (such as transcription factors, MCM and dCas9)<sup>44-46</sup> and chromatin structures (such as R-loops and replication forks)<sup>47,48</sup> have also been suggested as potential dynamic impediments during chromatin loop extrusion-mediated physiological processes. Our work indicates that the promoter polarity not only determines the transcriptional orientation, but also affects the chromatin loop extrusion and related DNA recombination process. Interestingly, elimination of chromatin loop extrusion only modestly affects gene expression and chromatin loop extrusion has been found to be required for long-range enhancer action<sup>49-51</sup>. And previous studies also suggest that chromatin loop extrusion plays more dominant roles in long-distance D to J<sub>H</sub> recombination and short-range diffusion dominates the DQ52 to J<sub>H</sub> recombination within V(D)J recombination center<sup>46</sup>. Moreover, a recent study suggests that chromatin loop extrusion brings V<sub>κ</sub> and J<sub>κ</sub> into close proximity around Cer and Cis elements, and local diffusion promotes the final V<sub>κ</sub> to J<sub>κ</sub> recombination within *Igκ* recombination center<sup>52</sup>. Our work suggests that chromatin loop extrusion plays more dominant roles in promoting orientation-specific end-joining of long-range DNA recombination. Although inversion of individual C<sub>H</sub> unit, which has a long distance from donor C<sub>μ</sub> unit, increases the inversion end-joining for various degree, deletional end-joining is still the dominant outcome due to the long-distance. Thus, the intrinsic chromatin features such as transcriptional orientation, chromatin distance, and chromatin domains orchestrate chromatin loop extrusion- and diffusion-mediated DNA damage repair to balance repair efficiency and outcomes.

Early studies exhibited numerous DNA repair factors participate in promoting deletional joining-mediated productive CSR by regulating DSB processing and repair pathways<sup>22-38</sup>, but the roles of these factors still fail to fully account for the regulation of orientation-specific joining mechanisms. Chromatin loop extrusion-mediated CSR model greatly improves the understanding of CSR including the formation of dynamic CSR centers, transcription activation of acceptor C<sub>H</sub> units, synapsis of acceptor C<sub>H</sub> and donor C<sub>μ</sub> units, and orientation-biased deletional joining of CSR in mice<sup>9,10</sup>. The chromatin loop extrusion-mediated predominantly deletional joining of CSR junction in mice extends the end-joining regulation from DNA damage repair-related trans-factors to dynamic chromatin regulation<sup>9,10</sup>. By further revealing the chromatin features of *Igh* constant region cross different species, we reveal that STC-related intrinsic chromatin features of *Igh* locus fulfil another layer of regulation in orientation-specific joining of AID-initiated CSR for generating productive antibodies. These detailed STC-mediated mechanisms of orientation-specific joining of DSBs not only deepen the understanding of productive CSR in different species throughout evolution and repair of pathogenic DSB genome-wide, but also provide new insights for antibody directed evolution.

## METHODS

### Experimental procedures

No statistical methods were used to predetermine sample size and experiments were not randomized in this study. Investigators were not blinded to allocation during experiments and outcome assessment.

### Cell culture

CH12F3 cells were cultured in medium R15 and stimulated with  $\alpha$ CD40 (1  $\mu$ g/ml, eBioscience), IL4 (20 ng/ml, PeproTech), and TGF $\beta$  (0.5 ng/ml, R&D systems) for 72hrs. CSR-HTGTS-seq was performed in AID-proficient CH12F3 cells. Pro-seq, 3C-HTGTS, and ChIP-seq were performed in AID-deficient CH12F3 cells to obviate confounding effects of CSR-related genomic rearrangements.

### Generation of mutant CH12F3 cell lines

gRNA design and vector construction: a Cas9/gRNA approach was employed to generate all the various mutant strains used in this study as described previously<sup>9</sup>. gRNAs were designed using the E-CRISP web tool (v5.4) with default parameters for SpCas9, targeting the *Mus musculus* genome. Candidate gRNAs were selected based on high predicted on-target efficiency scores (>80) and the absence of predicted off-target sites (with  $\leq 3$  mismatches) within protein-coding exons. gRNAs were individually cloned into the *BbsI* and *BsaI* site of the p332 vector following the standard protocol. The specific sequences for all gRNAs used are listed in Supplementary data.

Nucleofection and clonal selection: to generate mutant clones,  $2 \times 10^6$  CH12F3 cells were nucleofected with 2  $\mu$ g of p332-based Cas9/gRNA plasmids containing two gRNA sequences using the Lonza 4D-Nucleofector™ system (Solution SF, program CA-137). Twenty-four hours post-nucleofection, single cell subcloning was performed by fluorescence-activated cell sorting (FACS) on a BD FACS Aria™ SORP cell sorter to isolate one cell into one well of 96-well plates. Genomic DNA from individual clones was subsequently extracted for PCR-based genotyping and sequencing to confirm the desired genetic modification. Two pairs of genotyping primers were designed on either side of the two break sites for each mutant. Different combinations of these four primers can be used for PCR genotyping to detect WT bands, deletion bands, and inversion bands. The detailed primer pairs for genotyping were described in the corresponding figure legends. Based on the PCR genotyping and sequencing results, we can only detect WT bands for the control cells and positive bands (deletion or inversion) for the mutant clones. Detailed primers for selecting various mutant CH12F3 cell lines are listed in Supplementary data.

CH12F3PO cells were generated by deleting the entire non-productive *Igh* locus from upstream of the first V<sub>H</sub> to downstream of 3'CBEs in CH12F3N $\Delta$  cells<sup>9</sup> and confirmed by PCR genotyping and sequencing.  $\gamma 3$ - $\alpha$ Inv cells were generated by inverting the six acceptor C<sub>HS</sub> region from upstream of C $\gamma 3$  to downstream of C $\alpha$  in CH12F3PO cells and confirmed by PCR genotyping and sequencing.  $\alpha$ Inv cells were generated by inverting the C $\alpha$  unit in CH12F3PO cells and confirmed by PCR genotyping and sequencing. We generated the I $\alpha$  $\Delta$  cells by deleting I $\alpha$  promoter in CH12F3 cells in the previous study<sup>9</sup>.  $\gamma 2a$ Inv cells were generated by inverting the C $\gamma 2a$  unit in I $\alpha$  $\Delta$  cells and confirmed by PCR genotyping and sequencing.  $\gamma 1$ Inv cells were generated by inverting the C $\gamma 1$  unit in I $\alpha$  $\Delta$  cells and confirmed by PCR genotyping and

sequencing.  $\gamma 3$ - $\alpha$ Inv- $\alpha$ Inv cells were generated by inverting the C $\alpha$  unit in  $\gamma 3$ - $\alpha$ Inv cells and confirmed by PCR genotyping and sequencing.  $\gamma 3$ Inv cells were generated by inverting the C $\gamma 3$  unit in I $\alpha$  $\Delta$  cells and confirmed by PCR genotyping and sequencing.  $\gamma 3$ - $\alpha$ Inv- $\gamma 3$ Inv cells were generated by inverting the C $\gamma 3$  unit in  $\gamma 3$ - $\alpha$ Inv cells and confirmed by PCR genotyping and sequencing.  $\gamma 3$ IRD cells were generated by deleting the intergenic region between C $\gamma 3$  and C $\mu$  to position C $\gamma 3$  ~17kb away from C $\mu$  in I $\alpha$  $\Delta$  cells and confirmed by PCR genotyping and sequencing. I $\gamma 3$ -FS-C $\alpha$  cells were generated by fusing the entire S $\gamma 3$  with S $\alpha$  in CH12F3PO cells and confirmed by PCR genotyping and sequencing. I $\gamma 3$ -FS-I $\alpha$  cells were generated by fusing the entire S $\gamma 3$  with S $\alpha$  in  $\alpha$ Inv cells and confirmed by PCR genotyping and sequencing. I $\gamma 3$ -PS-C $\alpha$  cells were generated by fusing the partial S $\gamma 3$  with S $\alpha$  in CH12F3PO cells and confirmed by PCR genotyping and sequencing. I $\gamma 3$ -PS-I $\alpha$  cells were generated by fusing the partial S $\gamma 3$  with S $\alpha$  in  $\alpha$ Inv cells and confirmed by PCR genotyping and sequencing.  $\alpha$ -3CBEInv cells were generated by inverting the  $\alpha$ -3'CBEs region in  $\alpha$ Inv cells and confirmed by PCR genotyping and sequencing.  $\alpha$ -3CBEInv- $\alpha$  $\Delta$  cells were generated by deleting the C $\alpha$  unit in  $\alpha$ -3CBEInv cells and confirmed by PCR genotyping and sequencing. The corresponding AID-deficient cells were generated by deleting the *Aicda* gene from the above cells and confirmed by PCR genotyping and Western blotting. At least two independent clones were obtained for each derivative mutant genotype.

### Chromatin feature analyses of *Igh* locus across species

Reference genome assemblies used in this paper included *Danio rerio* (GRCz10), *Xenopus tropicalis* (UCB\_Xtro\_10.0), *Gallus gallus* (GRCg7w), and *Mus musculus* (mm9). Alligator *sinensis* was derived from Pan, T et al<sup>53</sup>. Splicing regions in *Mus musculus* have been well-annotated. For other species, we aligned the constant region sequences obtained from IMGT<sup>54</sup>. The splicing regions were defined as 4kb upstream of the first exon of the constant regions. Splicing region sequences were extracted by bedtools (v2.31.1) and the GC contents were calculated with seqkit (v2.8.2). Background GC content was derived genome-wide by sliding 4-kb windows.

Reads of RNA-seq from all species<sup>53,55-58</sup> were aligned to their respective reference genomes using STAR (v2.7.11a). Strand-specific bigwig files were generated with deeptools (v3.5.3) and strand orientation was defined from the alignment described above.

All Hi-C data<sup>53,59-61</sup> were processed with HiC-Pro (v3.1.0) under default settings, except the LIGATION\_SITE was species-specific, to generate valid interaction pair files. The output pair files were then converted to .hic files using the hicpro2juicebox utility. Hi-C files were also converted to .cool files using Hicexplorer (v3.7.2). Except for *Gallus gallus*, all species were analyzed at 25 kb resolution. *Gallus gallus* was analyzed at 5 kb resolution because the *Igh* locus is located on the microchromosome. Insulation scores and TADs were calculated with cooltools (v0.7.1) with a window size of 7 bins. All visualizations were generated by pyGenomeTracks (v3.8).

### CSR-HTGTS-seq library preparation and data analysis

CSR-HTGTS-seq libraries with 5'S $\mu$  bait were prepared from different CH12F3 mutants stimulated with  $\alpha$ CD40/IL4/TGF $\beta$  for 72hrs as described previously<sup>22</sup>. Briefly, 25  $\mu$ g gDNA from  $\alpha$ CD40/IL4/TGF $\beta$ -stimulated CH12F3 cells was sonicated on Covaris M220 sonicator. The sonicated DNA segments were amplified by LAM-PCR with biotinylated 5'S $\mu$  primer. The

amplified biotin-labeled LAM-PCR products were enriched with streptavidin C1 beads (Thermo Fisher Scientific, #65001) for 4hrs at room temperature, followed by adaptor ligation with the following PCR program: 25 °C 1hr, 22 °C 3hrs, 16 °C overnight. The adaptor-ligated products were subjected to nested-PCR with barcode primers and followed by tag-PCR with Illumina platform-matched P5-I5 and P7-I7 primers. 500-1000bp tag-PCR products were selected by separation on 1% TAE gel. CSR-HTGTS-seq libraries were sequenced by paired-end 150bp on a NovaSeq 6000 (Illumina) sequencer.

Libraries were processed via the published pipeline and mapped against the mm9 genome or corresponding modified genome<sup>22</sup>. Data were analyzed and plotted after removing the duplicates as described<sup>22</sup>. Each experiment was repeated at least three times from at least two independent clones. To plot the MH pattern, direct joins (MH=0) and junctions with MH were pooled and sorted by length, and the number of junctions with indicated length of MH were counted and calculated as percentage of the total number of junctions mapped to the region of interest. Primers used for CSR-HTGTS-seq are listed in Supplementary Table 1.

### 3C-HTGTS library preparation and data analysis

3C-HTGTS analyses were performed as previously described on AID<sup>-/-</sup> CH12F3 cells<sup>9</sup> stimulated with  $\alpha$ CD40/IL4/TGF $\beta$  for 72hrs. Briefly, 10 million cells were crosslinked with 2% formaldehyde for 10 minutes at room temperature and quenched with glycine at a final concentration of 125 mM. Then, the crosslinked cells were lysed in the 3C lysis buffer and nuclei were digested with NlaIII enzyme (NEB, R0125) at 37 °C overnight. The digested samples were ligated by T4 DNA ligase (NEB, M1801) at room temperature for at least 6hrs. The ligated products were de-crosslinked with Proteinase K (Roche, #03115852001) at 56 °C overnight and the 3C templates were purified by phenol/chloroform. The 3C templates were used for making 3C-HTGTS with different baits including the iE $\mu$ -I $\mu$ , 3'RR(HS4) or 3'CBEs locale. Besides the DNA templates and baits, the 3C-HTGTS library preparation procedures were similar as CSR-HTGTS-seq described above.

The 3C-HTGTS libraries were then sequenced by paired end 150 bp sequencing on a NovaSeq 6000 (Illumina) sequencer and data were processed as previously described<sup>9</sup>. Each experiment was repeated from at least two independent clones. Before plotting the data for comparison, libraries were size-normalized to total junctions of the smallest library in the set of libraries for comparison. For statistical analyses, we counted the number of junctions within the indicated bait-interacting locals for each sample. For bar graph presentations in Fig. 1 and Fig. 5, the junction number from control samples was normalized to represent 100% and relative experimental values listed as a percentage of the control values. Primers used for 3C-HTGTS are listed in Supplementary Table 1.

### Pro-seq library preparation and data analysis

Pro-seq libraries were prepared as described previously<sup>62</sup> from AID<sup>-/-</sup> CH12F3 cells stimulated with  $\alpha$ CD40/IL4/TGF $\beta$  for 72hrs. Briefly, 10 million cells were collected and permeabilized with permeabilization buffer. The permeabilized cells were resuspended in 100  $\mu$ l of storage buffer for nuclear run-on with 2X run-on mix at 37°C for 5 mins. RNA was extracted by Trizol and followed by hydrolyzation. The RNA was incubated with streptavidin C1 beads (Thermo Fisher Scientific, #65001) and the enriched run-on samples were incubated with hydroxyl repair with T4 PNK (NEB, M0201S) and RppH (NEB, M0356S), followed by ligating the 5' and 3' RNA adaptor. RT-PCR was performed from the adaptor-ligated RNA to obtain cDNA. The

cDNA was subjected to making Pro-seq libraries by two rounds of PCR with barcode primers. 200-500 bp products from the first round of PCR were subjected to the second round of PCR with the number of PCR cycles determined by test PCR amplification. The second round of PCR products were size-selected by SPRIselect beads (Beckman Coulter, B23318). Pro-seq libraries were sequenced via single end 50 bp sequencing on a HiSeq2500 (Illumina) sequencer or paired end 150bp sequencing on a NovaSeq 6000 (Illumina) sequencer. The processed reads were mapped to the mm9 genome or corresponding modified genome using Bowtie2 software. The read coverage for each sample was normalized to a coverage of 10 million 100nt reads for display. Each experiment was repeated three times from at least two independent clones.

### **ChIP-seq library preparation and data analysis**

ChIP-seq was carried out in AID-deficient cells stimulated with  $\alpha$ CD40/IL4/TGF $\beta$  for 72 hrs based on a prior protocol<sup>63</sup> with some modifications. Briefly, 20 million cells were collected and fixed with 1% formaldehyde for 10 mins at room temperature, and quenched with glycine at a final concentration of 125 mM. Cells were lysed and sonicated with a Covaris M220 sonicator for 5-8 min until the size of most fragments was in the range of 200–700 bp. The fragmented chromatin was incubated with Rad21 antibody (ab992, abcam) overnight, followed by incubating with magnetic protein A (Invitrogen, 10002D) for 4hrs at 4 °C. The enriched chromatin was used for tagmentation, followed by decrosslinking and elution. Purified DNA was subjected to make ChIP-seq libraries. The amplified libraries were purified by SPRIselect beads (Beckman Coulter, B23318) and were sequenced via paired end 150bp sequencing on a NovaSeq 6000 (Illumina) sequencer. The processed reads were mapped to the modified genome using Bowtie2 software. The uniquely aligned reads were retained, while duplicate reads and those located in blacklist regions were excluded prior to further analyses. The read coverage for each sample was normalized by RPKM.

### **Quantification and statistical analysis**

Statistical analyses for CSR-HTGTS-seq, 3C-HTGTS, and Pro-seq between two samples were performed via two-tailed, unpaired or paired Student's t-test. At least three biological repeats were done for each statistical analysis.  $p < 0.05$  is considered significant. P value shown in the bar graphs in the main and Supplementary Figures. The statistical analyses were also described in the corresponding Figure legends and Supplementary Figure Legends. Unless indicated otherwise, all agarose gel electrophoresis images are representative of at least two independent experiments, with uncropped gels shown in the Source Data file.

### **Data availability**

CSR-HTGTS-seq, 3C-HTGTS, Pro-seq, and ChIP-seq sequencing data analyzed here has been deposited in the GEO database. The GEO accession number for CSR-HTGTS-seq is GSE299392 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE299392>]. The GEO accession number for 3C-HTGTS is GSE299499 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE299499>]. The GEO accession number for Pro-seq is GSE299975 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE299975>]. The GEO accession number for ChIP-seq is GSE299391 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE299391>]. All other data can be found in the Supplementary Data of this paper or in the Source Data. This includes all uncropped gel electrophoresis images, and data shown in graphs throughout the manuscript, including the

Supplementary Figures. All data are available from the authors upon reasonable request. Source data are provided with this paper.

### **Code availability**

V(D)J-HTGTS-seq, CSR-HTGTS-seq, and 3C-HTGTS data was processed through published pipelines ([http://robinmeyers.github.io/transloc\\_pipeline/](http://robinmeyers.github.io/transloc_pipeline/)). Pro-Seq were aligned to either mm9 genome or modified genomes with bowtie2 (v.2.4.4) (<http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>), processed by samtools (v.1.15.1) (<https://sourceforge.net/projects/samtools/files/samtools/1.15.1/>), and generated graph files via RseqQ (v.5.0.1) (<https://rseqc.sourceforge.net/>) package. ChIP-Seq were aligned to the mm9-derived modified genomes with bowtie2 (v.2.4.4), processed by samtools (v.1.15.1), generated bigwig files via Deeptools (v.3.5.1) (<https://github.com/deeptools/deepTools>), and generated peak files via MACS2 (v.2.2.7.1) (<https://github.com/taoliu/MACS/>).

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## AUTHOR CONTRIBUTIONS

S.L. (Sha Luo), R.Q., and X.Z. designed the study; S.L. (Sha Luo), R.Q., X.S., S.L. (Shuchan Li), Z.Y., and A.M performed the experiments; H.Z., and L.Y. designed some of the bioinformatics pipelines; S.L. (Sha Luo), R.Q., H.Z., and X.Z. analysed the data; S.L. (Sha Luo), R.Q., H.Z., L.Y., and X.Z. prepared the figures with some input from Y.C. and Y-F.Z.; S.L. (Sha Luo), R.Q., H.Z., L.Y., and X.Z. wrote the manuscript. X.Z. managed and supervised the project.

## COMPETING INTERESTS STATEMENT

The Authors declare no competing interests.

## Figure Legends

**Figure 1.  $\gamma 3\text{-}\alpha$  inversion activates predominantly deletional  $S\gamma 3$  CSR and orientation-unbiased  $S\alpha$  CSR within *Igh* domain.**

**a** Schematic of *Igh* locus from V(D)J exon to 3' CBEs in CH12F3PO cells and illustration of the generation of  $\gamma 3\text{-}\alpha$ Inv cells. **b** Illustration of deletional and inversional joining between the donor  $S\mu$  and an acceptor  $Sx$  in CSR. The CSR joining features were measured by CSR-HTGTS-seq with 5' $S\mu$  bait. **c** CSR-HTGTS-seq analysis of break joining between 5' $S\mu$  and acceptor  $S$  regions in CH12F3PO and  $\gamma 3\text{-}\alpha$ Inv cells stimulated with  $\alpha$ CD40/IL4/TGF $\beta$  for 72 hrs. Junctions within the *Igh* constant region are plotted at 2.25kb bin size. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. **d** Joining features of  $S\mu$ - $S\alpha$  junctions in CH12F3PO and  $\gamma 3\text{-}\alpha$ Inv cells. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. **e** Bar graph shows the inversional joining of  $S\mu$ - $S\alpha$  junctions in CH12F3PO and  $\gamma 3\text{-}\alpha$ Inv cells. Data are presented as mean  $\pm$  s.e.m. from six independent experiments. *P* values were calculated via unpaired two-tailed Student's t-test. **f** Pro-seq and 3C-HTGTS with iE $\mu$ /I $\mu$  or 3'RR bait (blue asterisks) show the transcription status and chromatin interaction of *Igh* locus from CH12F3PO cells. **g** Pro-seq and 3C-HTGTS with iE $\mu$ /I $\mu$  or 3'RR bait (blue asterisks) show the transcription status and chromatin interaction of *Igh* locus from  $\gamma 3\text{-}\alpha$ Inv cells. **h** Bar graph shows the relative transcription activity of  $C\gamma 3$  unit and  $C\alpha$  unit in CH12F3PO and  $\gamma 3\text{-}\alpha$ Inv cells. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. *P* values were calculated via paired two-tailed Student's t-test. **i** Bar graph shows the relative interaction frequency between iE $\mu$ /I $\mu$  bait with  $C\gamma 3$  unit and  $C\alpha$  unit in CH12F3PO and  $\gamma 3\text{-}\alpha$ Inv cells. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. *P* values were calculated via paired two-tailed Student's t-test. Source data are provided as a Source Data file.

**Figure 2. Transcription orientation affects orientation-specific CSR.**

**a** Schematic of *Igh* locus from V(D)J exon to 3' CBEs in CH12F3PO cells and illustration of the generation of  $\alpha$ Inv cells. **b, c** Joining features of S $\mu$ -S $\alpha$  junctions in CH12F3PO (b) and  $\alpha$ Inv (c) cells. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. **d** Bar graph shows the inversional joining of S $\mu$ -S $\alpha$  junctions in CH12F3PO and  $\alpha$ Inv cells. Data are presented as mean  $\pm$  s.e.m. from five independent experiments. **e** Schematic of *Igh* locus from V(D)J exon to 3' CBEs in I $\alpha$  $\Delta$  cells and illustration of the generation of  $\gamma$ 2aInv cells. **f, g** Joining features of S $\mu$ -S $\gamma$ 2a junctions in I $\alpha$  $\Delta$  (f) and  $\gamma$ 2aInv (g) cells. **h** Bar graph shows the inversional joining of S $\mu$ -S $\gamma$ 2a junctions in I $\alpha$  $\Delta$  and  $\gamma$ 2aInv cells. **i** Schematic of *Igh* locus from V(D)J exon to 3' CBEs in I $\alpha$  $\Delta$  cells and illustration of the generation of  $\gamma$ 1Inv cells. **j, k** Joining features of S $\mu$ -S $\gamma$ 1 junctions in I $\alpha$  $\Delta$  (j) and  $\gamma$ 1Inv (k) cells. **l** Bar graph shows the inversional joining of S $\mu$ -S $\gamma$ 1 junctions in I $\alpha$  $\Delta$  and  $\gamma$ 1Inv cells. **m** Schematic of *Igh* locus from V(D)J exon to 3' CBEs in  $\gamma$ 3- $\alpha$ Inv cells and illustration of the generation of  $\gamma$ 3- $\alpha$ Inv- $\alpha$ Inv cells. **n, o** Joining features of S $\mu$ -S $\alpha$  junctions in  $\gamma$ 3- $\alpha$ Inv (n) and  $\gamma$ 3- $\alpha$ Inv- $\alpha$ Inv (o) cells. **p** Bar graph shows the inversional joining of S $\mu$ -S $\alpha$  junctions in  $\gamma$ 3- $\alpha$ Inv and  $\gamma$ 3- $\alpha$ Inv- $\alpha$ Inv cells. For (f-h), (j-l), and (n-p), data are presented as mean  $\pm$  s.e.m. from three independent experiments. *P* values were calculated via unpaired two-tailed Student's *t*-test. Source data are provided as a Source Data file.

**Figure 3. Chromatin distance affects orientation-specific CSR.**

**A** Schematic of *Igh* locus from V(D)J exon to 3' CBEs in I $\alpha$  $\Delta$  cells and illustration of the generation of  $\gamma$ 3Inv,  $\gamma$ 3Inv- $\gamma$ 3Inv, and  $\gamma$ 3IRD cells. **b, c, e, and f** Joining features of S $\mu$ -S $\gamma$ 3 junctions in I $\alpha$  $\Delta$  (b),  $\gamma$ 3Inv (c),  $\gamma$ 3Inv- $\gamma$ 3Inv (e), and  $\gamma$ 3IRD (f) cells. **d** Bar graph shows the inversional joining of S $\mu$ -S $\gamma$ 3 junctions in I $\alpha$  $\Delta$ ,  $\gamma$ 3Inv,  $\gamma$ 3Inv- $\gamma$ 3Inv, and  $\gamma$ 3IRD cells. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. *P* values were calculated via unpaired two-tailed Student's *t*-test. Source data are provided as a Source Data file.

**Figure 4. Inverted promoter of CHS promotes inversional CSR.**

**a** CSR-HTGTS-seq, Pro-seq, 3C-HTGTS with iE $\mu$ /I $\mu$  (blue asterisks), and Rad21 ChIP-seq to show the CSR junctions, transcription status, chromatin interaction, and cohesin binding of *Igh* locus from I $\gamma$ 3-FS-C $\alpha$  cells. **b** CSR-HTGTS-seq, Pro-seq, 3C-HTGTS with iE $\mu$ /I $\mu$  bait (blue asterisks), and Rad21 ChIP-seq to show the CSR junctions, transcription status, chromatin interaction, and cohesin binding of *Igh* locus from I $\gamma$ 3-FS-I $\alpha$  cells. **c** CSR-HTGTS-seq, Pro-seq, 3C-HTGTS with iE $\mu$ /I $\mu$  (blue asterisks), and Rad21 ChIP-seq to show the CSR junctions, transcription status, chromatin interaction, and cohesin binding of *Igh* locus from I $\gamma$ 3-PS-C $\alpha$  cells. **d** CSR-HTGTS-seq, Pro-seq, 3C-HTGTS with iE $\mu$ /I $\mu$  bait (blue asterisks), and Rad21 ChIP-seq to show the CSR junctions, transcription status, chromatin interaction, and cohesin binding of *Igh* locus from I $\gamma$ 3-PS-I $\alpha$  cells. For (a-d), data are presented as mean  $\pm$  s.e.m. from three independent experiments. Source data are provided as a Source Data file.

**Figure 5. AID-initiated on-targets located in different chromatin domains promote diffusion-mediated inversional CSR.**

**a** Schematic of *Igh* locus from V(D)J exon to downstream CBE outside *Igh* domain in  $\alpha$ Inv cells and illustration of the generation of  $\alpha$ -3CBEInv cells. **b** CSR-HTGTS-seq analysis of break

joining between 5'S $\mu$  and acceptor S $\alpha$  in  $\alpha$ Inv and  $\alpha$ -3CBEInv cells stimulated with  $\alpha$ CD40/IL4/TGF $\beta$  for 72 hrs. Junctions within the *Igh* constant region are plotted at 2.25kb bin size. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. **c** Joining features of S $\mu$ -S $\alpha$  junctions in  $\alpha$ Inv and  $\alpha$ -3CBEInv cells. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. **d** Bar graph shows the inversional joining of S $\mu$ -S $\alpha$  junctions in  $\alpha$ Inv and  $\alpha$ -3CBEInv cells. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. *P* values were calculated via unpaired two-tailed Student's t-test. **e** 3C-HTGTS with 3'CBEs, iE $\mu$ /I $\mu$  or 3'RR bait (blue asterisks), and Pro-seq to show the chromatin interaction and transcription status of the indicated region from  $\alpha$ Inv cells. **f** 3C-HTGTS with 3'CBEs, iE $\mu$ /I $\mu$  or 3'RR bait (blue asterisks), and Pro-seq to show the chromatin interaction and transcription status of the indicated region from  $\alpha$ -3CBEInv cells. **g-i** Relative interaction frequency between 3'CBEs (g), iE $\mu$ /I $\mu$  (h), or 3'RR (i) bait with the indicated regions in  $\alpha$ Inv and  $\alpha$ -3CBEInv cells. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. *P* values were calculated via paired two-tailed Student's t-test. **j** Relative transcription activity of C $\mu$  and C $\alpha$  unit in  $\alpha$ Inv and  $\alpha$ -3CBEInv cells. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. *P* values were calculated via paired two-tailed Student's t-test. Source data are provided as a Source Data file.

**Figure 6. AID-initiated off-targets located in different chromatin domains promote diffusion-mediated inversional CSR.**

**a** Schematic of *Igh* locus from V(D)J exon to downstream CBE outside *Igh* domain in  $\alpha$ -3CBEInv cells and illustration of the generation of  $\alpha$ -3CBEInv- $\alpha\Delta$  cells. **b** CSR-HTGTS-seq and Pro-seq analyses to show the CSR junctions and transcription status of the indicated region from  $\alpha$ -3CBEInv cells. CSR junctions are plotted at 3.55kb bin size. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. **c** CSR-HTGTS-seq and Pro-seq analyses to show the CSR junctions and transcription status of the indicated region from  $\alpha$ -3CBEInv- $\alpha\Delta$  cells. CSR junctions are plotted at 3.55kb bin size. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. **d, e** CSR-HTGTS-seq and Pro-seq to show the CSR junctions and transcription status around the eS region in  $\alpha$ -3CBEInv (d) and  $\alpha$ -3CBEInv- $\alpha\Delta$  (e) cells. **f** Bar graph shows the deletional and inversional joining of eS region junctions in  $\alpha$ -3CBEInv- $\alpha\Delta$  cells. Data are presented as mean  $\pm$  s.e.m. from six independent experiments. *P* values were calculated via unpaired two-tailed Student's t-test. **g** Bar graph shows the transcription activity of eS region in  $\alpha$ -3CBEInv and  $\alpha$ -3CBEInv- $\alpha\Delta$  cells. Data are presented as mean  $\pm$  s.e.m. from three independent experiments. *P* values were calculated via unpaired two-tailed Student's t-test. Source data are provided as a Source Data file.

**Figure 7. Model of intrinsic chromatin features regulating orientation-specific joining of CSR for generating productive antibodies in different species.**

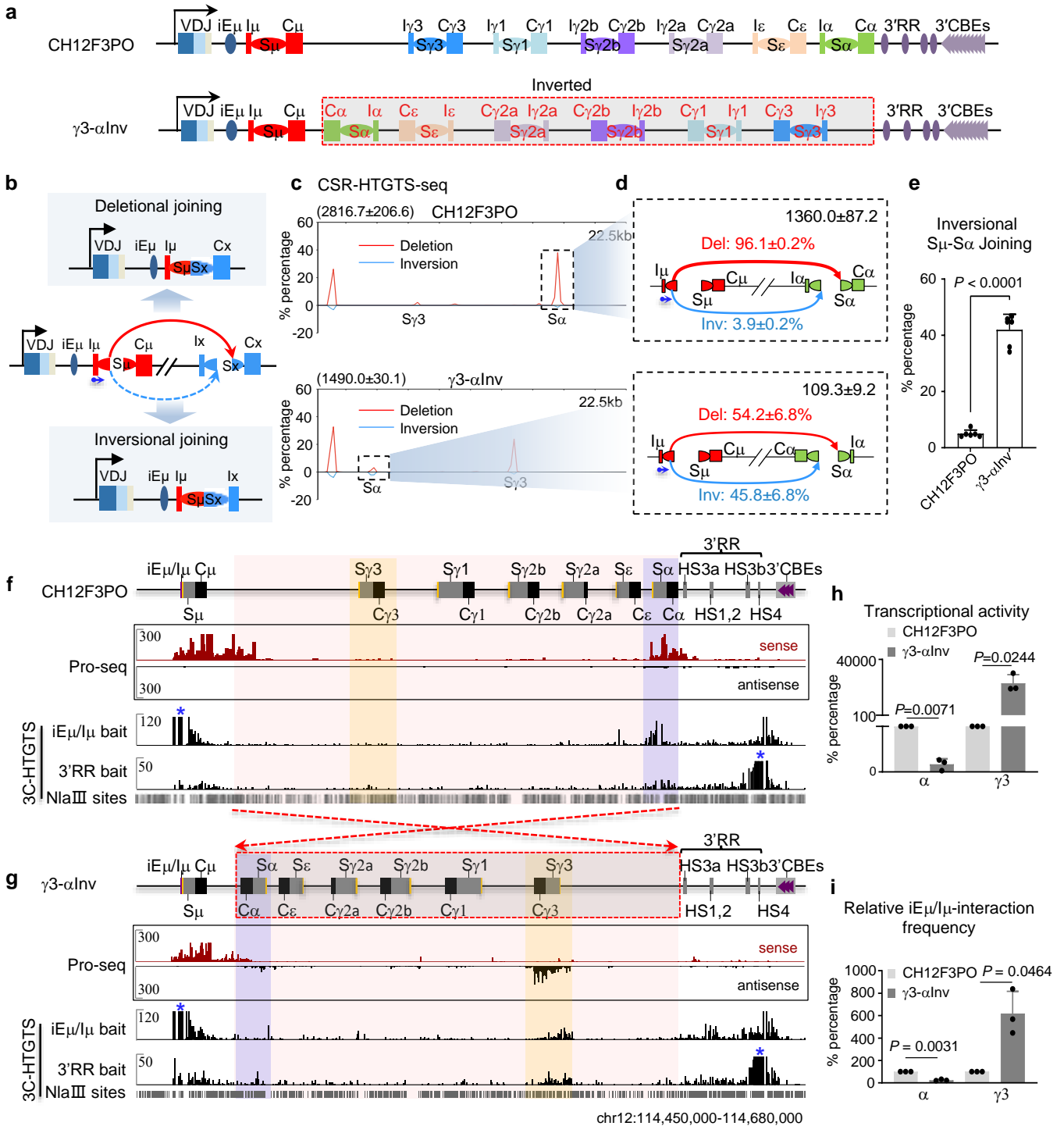
**a** Chromatin features of *Igh* constant region in different species (such as *Ginglymostoma cirratum*, *Danio rerio*, *Xenopus laevis*, *Alligator sinensis*, *Gallus gallus*, *Mus musculus*, and *Homo sapiens*) throughout evolution. The columns with different colors indicate different C<sub>H</sub> units. The green arrows indicate the transcription orientation. **b** long-distance C<sub>H</sub>S under the same transcriptional orientation within *Igh* domain promotes orientation-biased deletional joining of CSR for generating productive antibodies in some species such as *Mus musculus*. Chromatin

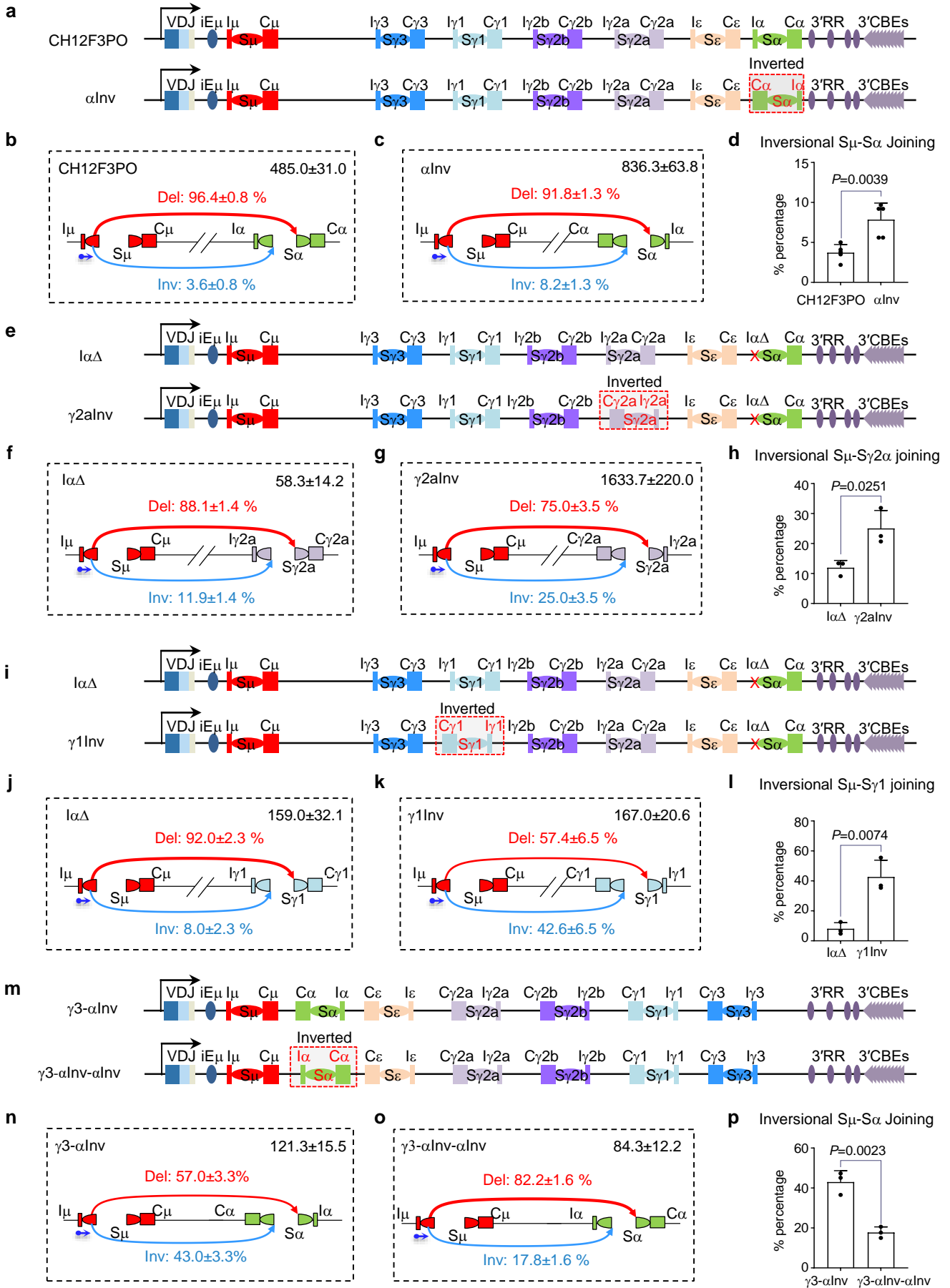
loop extrusion might promote this orientation-biased end joining for high-efficient CSR. **c** Opposite transcribed  $C_{HS}$ , short-distance  $C_{HS}$ , or different chromatin domain-isolated  $C_{HS}$  promote inversional joining-mediated productive CSR in some species such as *Gallus gallus*. Short-range diffusion might promote this relatively less-efficient CSR. The red bricks indicate the anchors for mediating TAD formation. The blue circles indicate the cohesin rings. The light red circle indicates the CSR center. The pink circle indicates the short-range diffusion area. The black arrows indicate the transcription orientation. The blue arrows indicate chromatin loop extrusion-mediated pulling force. The dotted arrows indicate the potential joining events between donor  $S\mu$  and acceptor  $Sx$  breaks. The key elements, such as donor  $S\mu$  (red oval), activated acceptor  $Sx$  (green oval),  $iE\mu$  (atrovirens oval), and  $3'RR$  (blue oval), are also indicated.

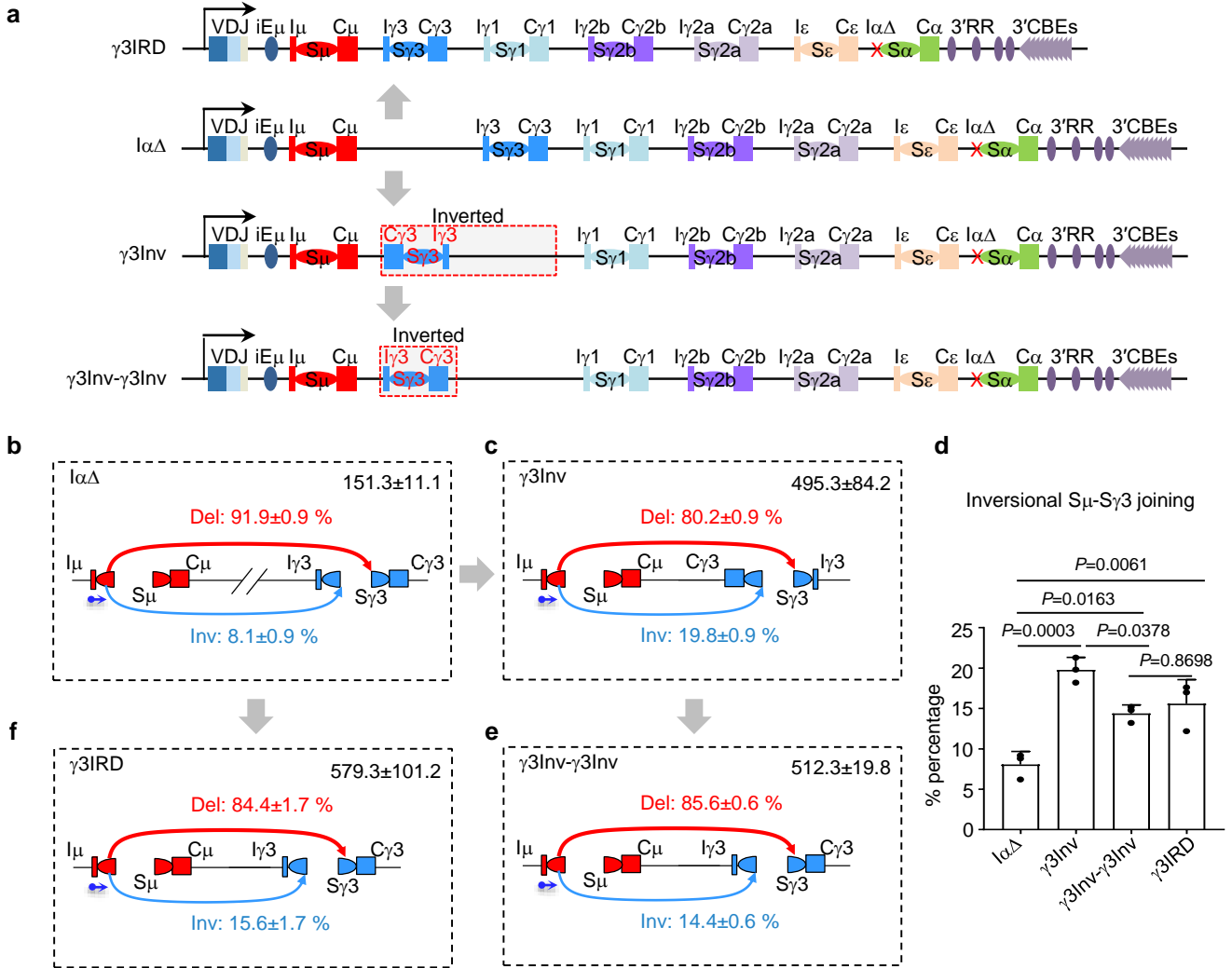
### Editor's Summary

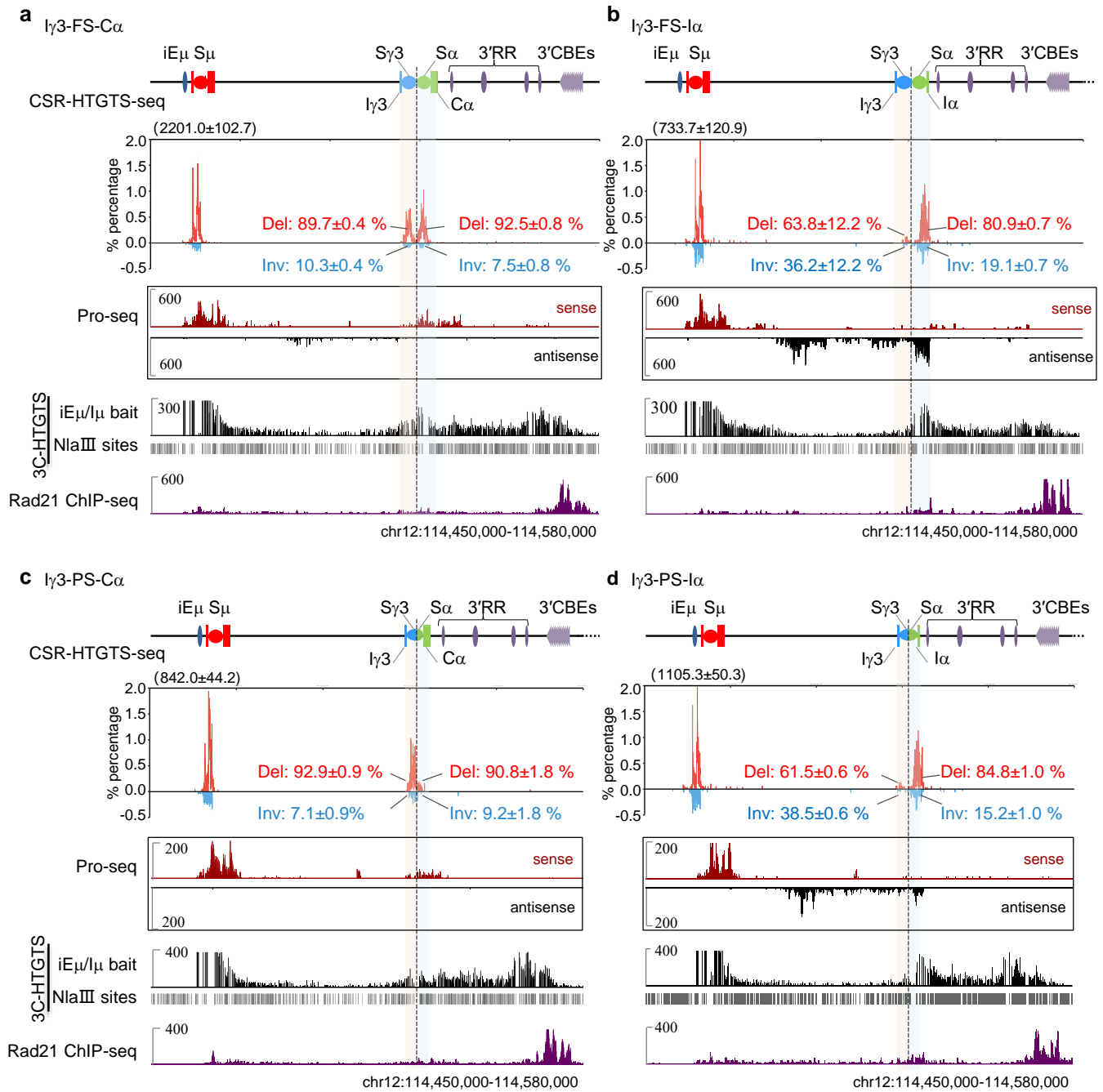
How chromatin architecture enforces productive class switching is not fully understood. Here, the authors demonstrate that transcriptional orientation, genomic distance, and Igh domain organization bias AID-initiated breaks toward deletional joining required for productive CSR.

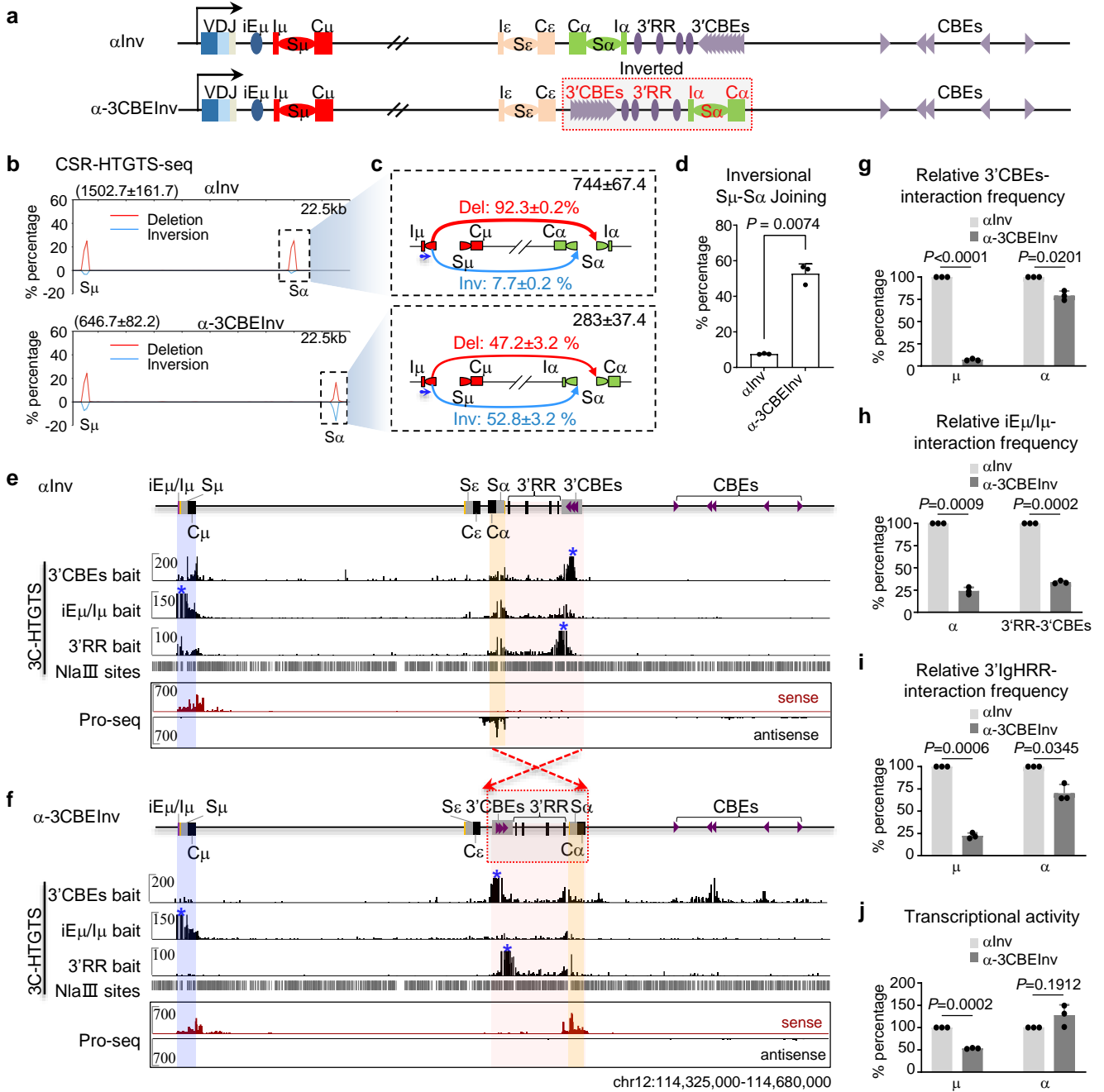
**Peer Review Information:** *Nature Communications* thanks the anonymous reviewer(s) for their contribution to the peer review of this work. A peer review file is available.

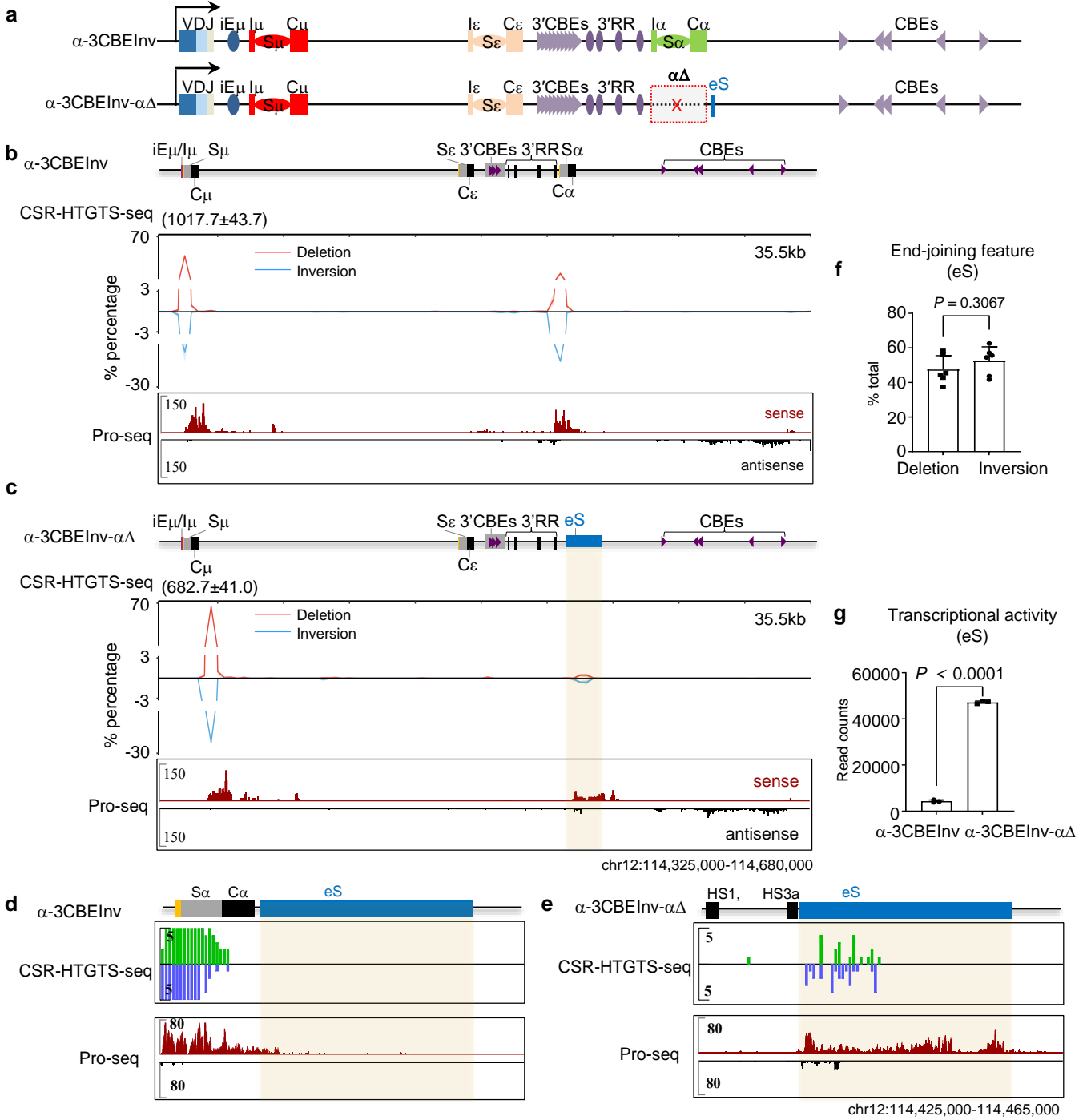






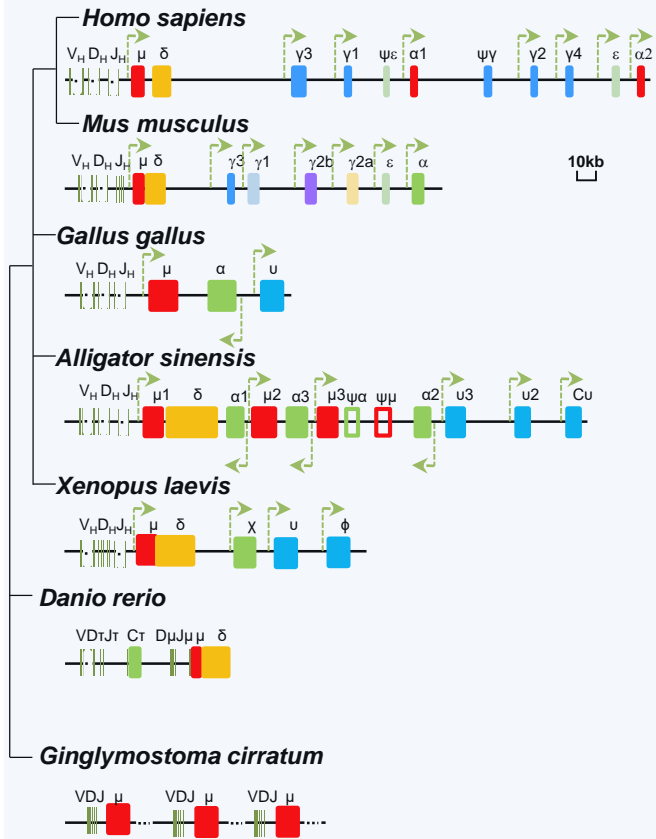




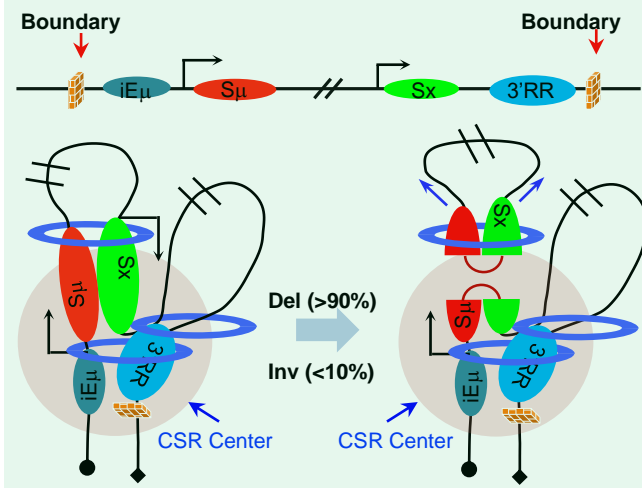


**Intrinsic chromatin features determine orientation-specific end joining for productive CSR in different organisms during evolution**  
(Transcription orientation, chromatin distance, TADs et al)

**a Chromatin features of Igh locus in different species during evolution**



**b i) Same transcription orientation between  $S_\mu$  and  $S_x$**   
**ii) Long distance between  $S_\mu$  and  $S_x$**   
**iii)  $S_\mu$  and  $S_x$  locate in the same TAD**



**c Opposite transcription orientation between  $S_\mu$  and  $S_x$**

