Supplemental Information

Determination of RAG binding affinities to 12RSS.

Binding affinities were determined from fluorescence anisotropy measurements using end-labeled TAMRA-12RSS.

A 20 nM solution of DNA probe was titrated with increasing concentrations of RAG, and the fluorescence anisotropy (r) was recorded in each condition. The fraction of bound DNA (f_B) was calculated from the experimental r value as

$$f_B = \frac{r - r_F}{r_B - r_F} \tag{S1}$$

where r_F is the fluorescence anisotropy of the free probe (TAMRA-12RSS), and r_B is the fluorescence anisotropy of the 12RSS-RAG complex. This equation assumes that the fluorescence quantum efficiency of TAMRA does not change upon binding to RAG, which was verified experimentally in all titrations.

Based on previous report (1), we assume a 1:2 stoichiometry for the RSS-RAG complex, and therefore

$$K_d = \frac{[P]^2[D]}{[DP_2]} \tag{S2}$$

Where [P] represents the molar concentration of free protein in solution, [D] is the molar concentration of free DNA in solution, and [DP_2] is the molar concentration of bound DNA in solution.

The fraction of bound DNA can be expressed as

$$f_B = \frac{[DP_2]}{[D]+[DP_2]} \quad \text{(S3)}$$

and from (S2) and (S3) we obtain

$$\frac{K_d f_B}{(1-f_B)} = [P]^2$$

Mass balances for the protein and DNA give $P_0 = [P]+2[DP_2]$ and $D_0 = [D]+[DP_2]$ where P_0 and D_0 are the total concentrations of protein and DNA in solution. Therefore,

$$\frac{K_d f_B}{(1-f_B)} = (P_0 - 2 f_B D_0)^2 \quad \text{(S4)}$$

Equation S4 is cubic in f_B, and the real solution was found in *Mathematica* 7.0. Plots of f_B (obtained from eq.S1) as a function of P_0 were fitted with the real solution of S4 using $D_0 = 20$ nM, and leaving K_d as the fitting parameter. Note that the K_d defined in eq.S2 has units of M^2. Here, we report $(K_d)^{1/2}$, which has units of concentration, to facilitate the comparison with other values in the literature. Results are shown in the table below and figure S3 for WT-RAG. All values are expressed in units of nM.

c/cRAG + Ca^{2+}	c/cRAG + Mg^{2+}	c/cRAG + Mn^{2+}	e/fsRAG + Ca^{2+}	e/cRAG + Ca^{2+}
13.5 ± 2.3	14.5 ± 2.4	3.9 ± 3.0	3.9 ± 3.5	14.7 ± 5.4

Reference

1. Ciubotaru, M., L. M. Ptaszek, G. A. Baker, S. N. Baker, F. V. Bright, and D. G. Schatz.2003. RAG1-DNA binding in V(D)J recombination. Specificity and DNA-induced conformational changes revealed by fluorescence and CD spectroscopy. *J Biol Chem* 278:5584-5596.

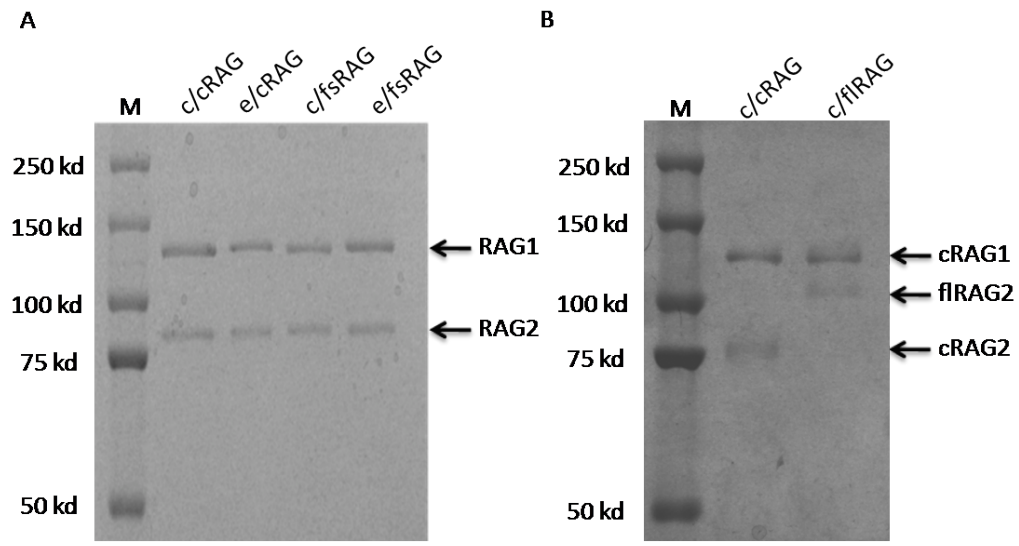
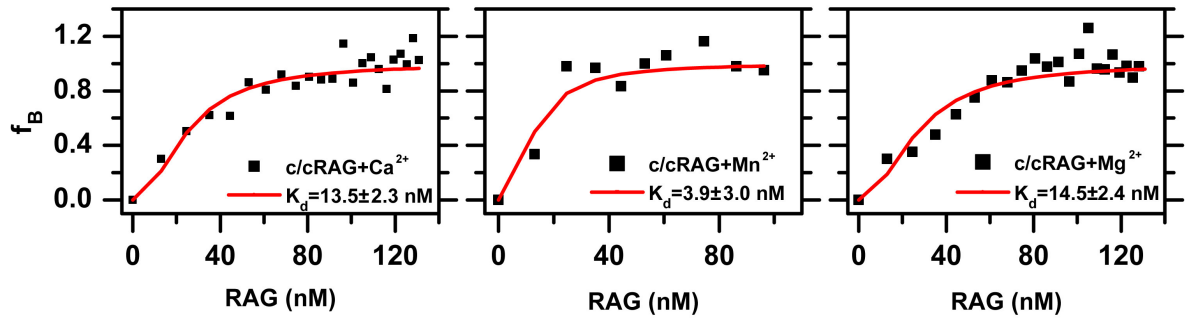


Figure S1.Visualization of various RAG preparations on SDS-PAGE, revealed by Coomassie blue staining. (A) Different core RAG combinations, including cRAG1, cRAG2, eRAG1, fsRAG2. (B) cRAG1 paired with either cRAG2 or flRAG2.

A



B

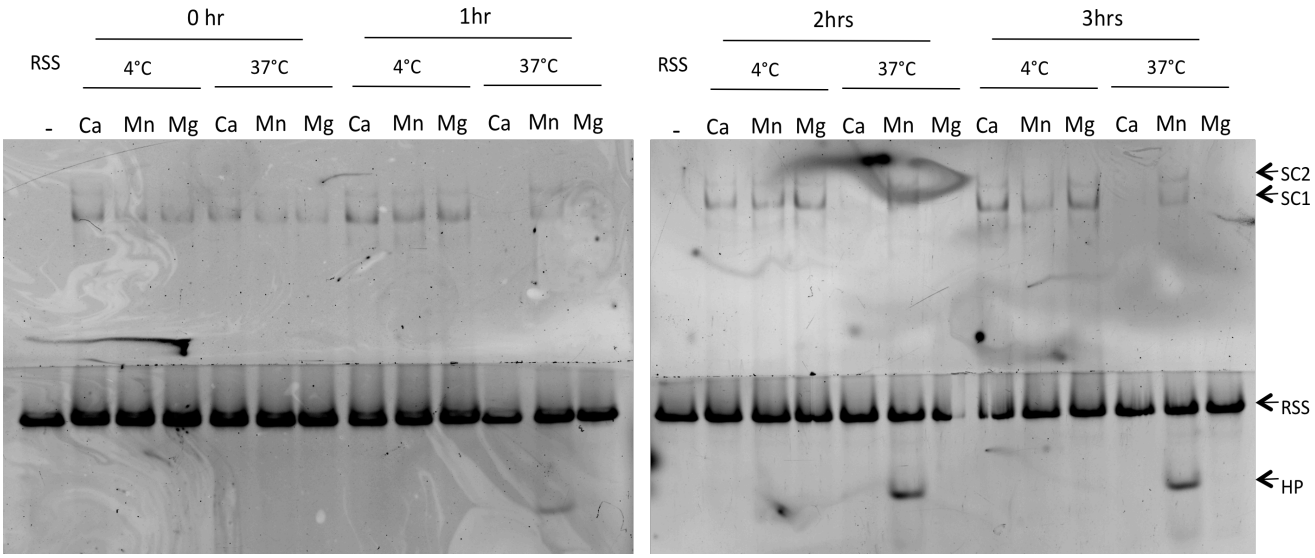


Figure S2. (A) Determination of the binding affinity (K_d) between c/cRAG and 12RSS in the presence of Ca^{2+} (left), Mn^{2+} (middle), and Mg^{2+} (right). The fraction of bound 12RSS (f_B) was calculated from fluorescence anisotropy data obtained with 5'-labeled TAMRA 12RSS as described in the supplemental information. The red lines represent the fit to a model that assumes a 1:2 binding stoichiometry, from which we obtained the dissociation constant of the complex (see supplemental information). (B) RAG-RSS interactions analyzed by Electrophoretic Mobility Shift Assay (EMSA). c/cRAG was incubated with TAMRA-12RSS at 4 °C or 37 °C for the time indicated in the figure, in the presence of either Ca^{2+} or Mn^{2+} or Mg^{2+}. The first lane in each gel

shows the intact 12RSS without RAG. The arrows point to the bands corresponding to the DNA-protein complexes (SC1 and SC2), the free 12RSS probe (RSS) and the hairpin (HP). Note the presence of DNA-protein complexes at 37°C after 3 hrs of incubation in the presence of Mn^{2+}.

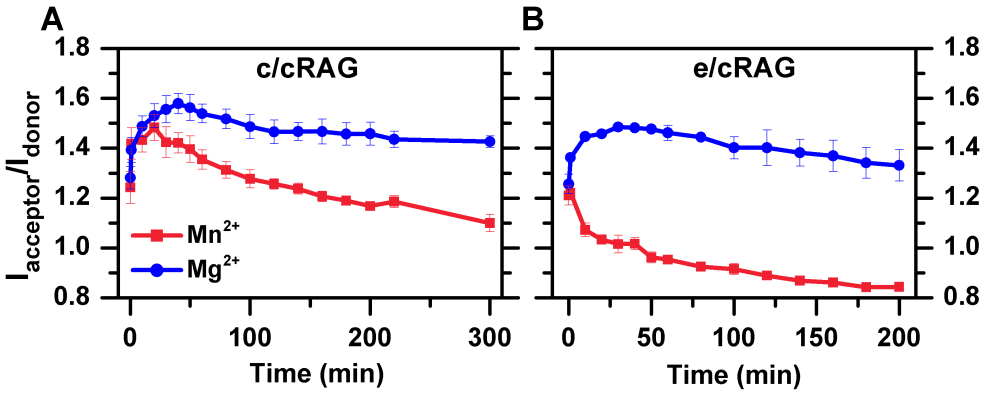


Figure S3. FRET profiles of coupled cleavage reactions catalyzed by (A) c/cRAG or (B) e/cRAG in the presence of HMGB1 and unlabeled 23RSS under Mg^{2+} (blue) or Mn^{2+} (red).

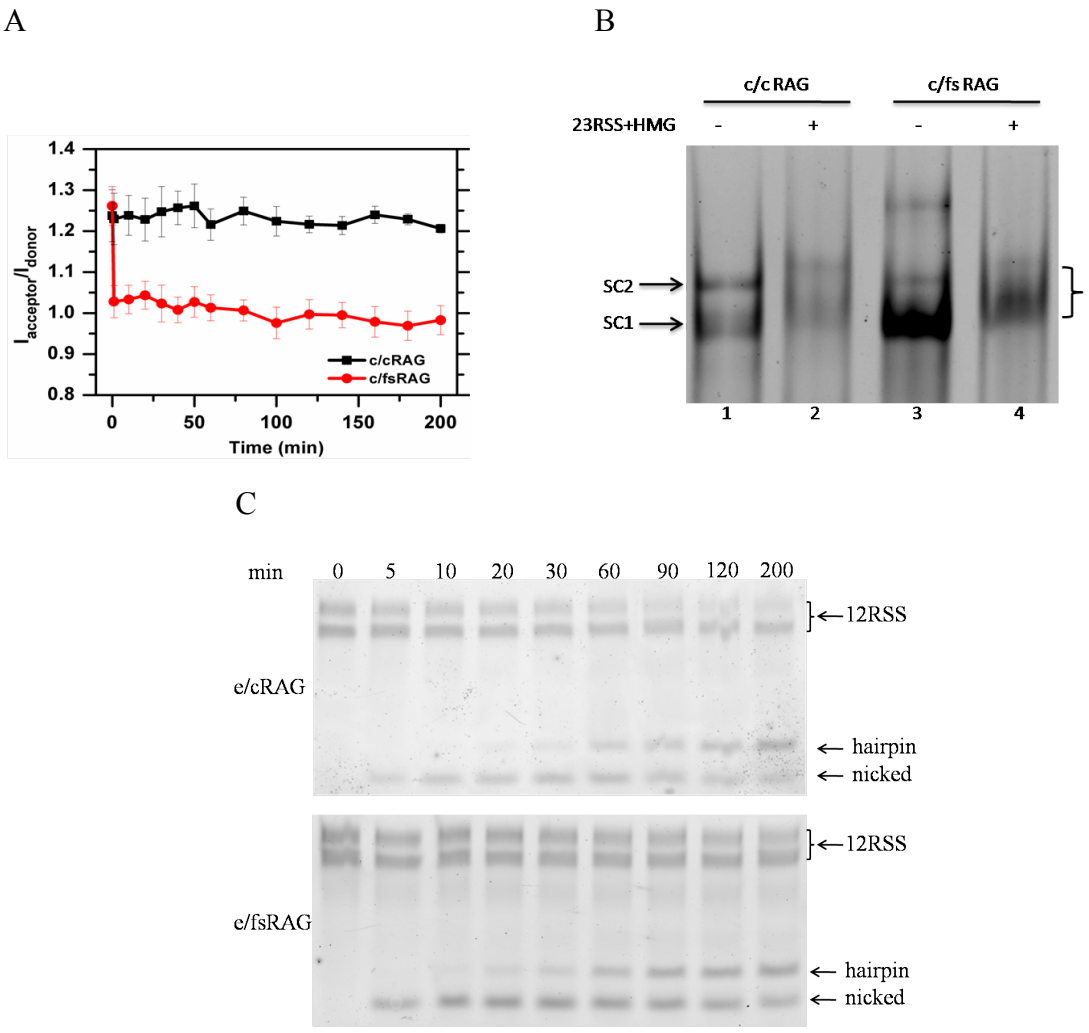


Figure S4. (A) FRET profiles of the doubly-labeled 12RSS in reactions mediated by c/cRAG (black) and c/fsRAG (red) in the presence of Mg^{2+}, showing a steep initial decrease in FRET for c/fsRAG. (B) EMSA analysis of RAG-12RSS interactions in the absence or presence of partner 23RSS and HMGB1. The major shifted bands present in lanes 2 and 4 (marked with bracket), are at the position higher than the SC1, reflecting synaptic complexes. (C) Comparison between e/cRAG and e/fsRAG on the kinetics of recombination cleavage reaction made at the TAMRA-ATTO 12RSS.

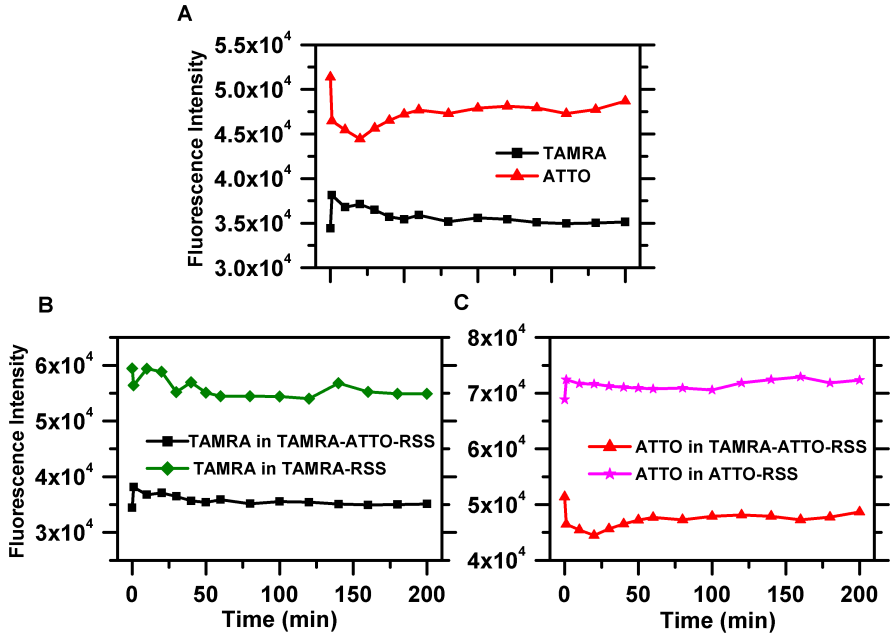


Figure S5. (A) Emission intensity of TAMRA (black squares) and ATTO (red triangles) in the doubly-labeled 12RSS in the presence of e/fsRAG and Ca^{2+} showing an initial rapid decrease in FRET. (B) Fluorescence intensity of TAMRA in the doubly-labeled probe (black squares) and singly-labeled 12RSS (green diamonds) in the presence of e/fsRAG and Ca^{2+}. (C) Fluorescence intensity of ATTO in the doubly-labeled probe (red triangles) and singly-labeled 12RSS (magenta stars) in the same reaction conditions. The fluorescence intensities of TAMRA-12RSS and ATTO-12RSS were rather constant throughout the incubation, indicating that the changes observed with the doubly-labeled RSS samples are due to FRET change.

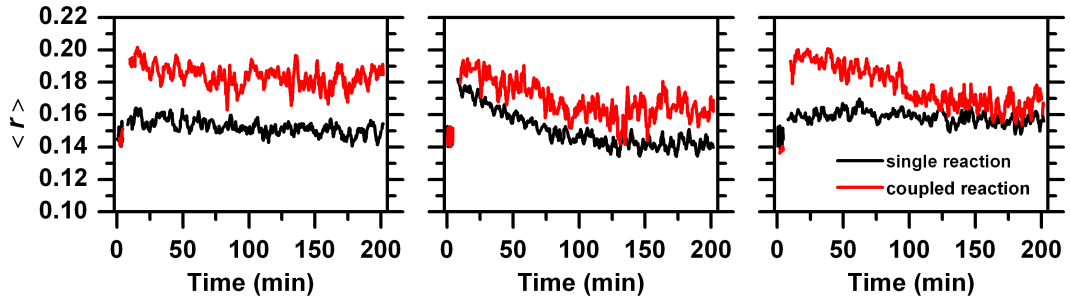


Figure S6. Fluorescence anisotropy of TAMRA-12RSS in the presence of c/cRAG in either single (black line) or coupled reactions (red line) in the presence Ca^{2+} (left panel), Mn^{2+} (middle panel) or Mg^{2+} (right panel).

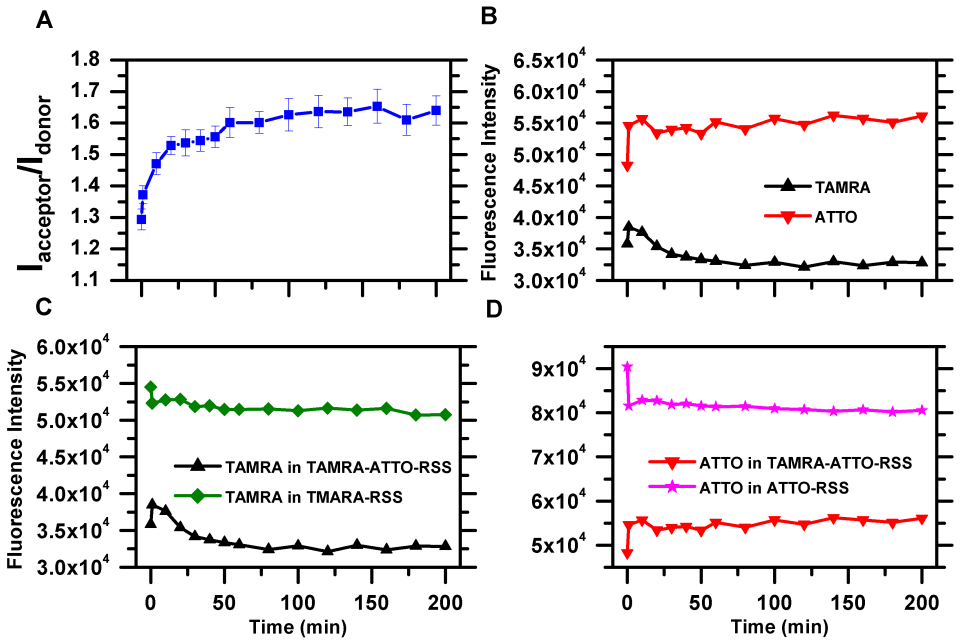


Figure S7. (A) Increase of $I_{acceptor}/I_{donor}$ was detected in d/cRAG-mediated coupled reaction in the presence of Mg^{2+}, where no cleavage occurs. (B) Emission intensity of TAMRA (black) and ATTO (red) from the above reaction, based on which the ratio, $I_{acceptor}/I_{donor}$, displayed in (A) was derived. (C) Fluorescence intensity of TAMRA in the doubly-labeled probe (black triangles) and singly-labeled 12RSS (green diamonds) under the same reaction condition. (D) Fluorescence intensity of ATTO in the doubly-labeled probe (red triangles) and singly-labeled 12RSS (magenta stars) under the same reaction condition

A B

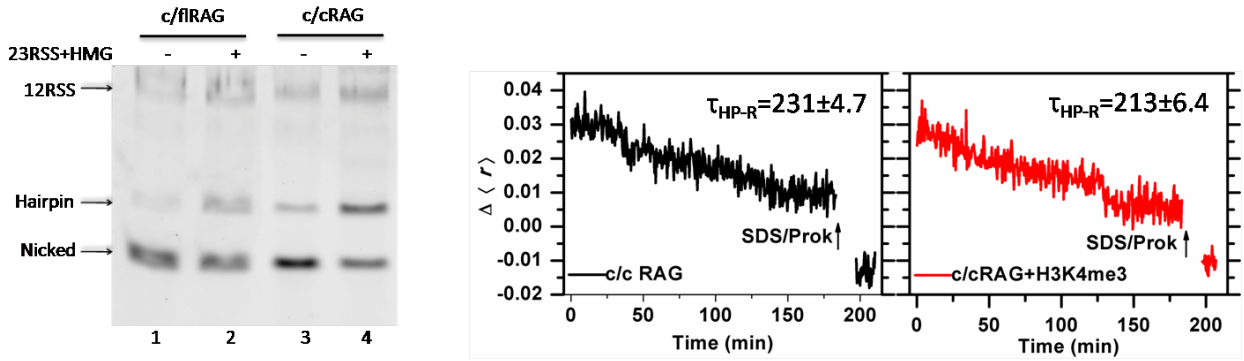


Figure S8. (A) *In vitro* cleavage reactions mediated by c/fl RAG or c/cRAG, in the absence (lanes 1 and 3) or presence of partner 23RSS and HMGB1 (lanes 2 and 4). (B) Effect of H3K4me3 peptide on c/cRAG-mediated coupled cleavage reaction, analyzed by fluorescence anisotropy. Left, w/o H3K4me3 peptide. Right, with H3K4me3 peptide. The lifetime $\tau_{HP\text{-}R}$ is indicated in each gragh.