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DNA annealing by Red β is insufficient for homologous recombination and the additional requirements involve intra- and inter-molecular interactions

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Single strand annealing proteins (SSAPs) like Red β initiate homologous recombination by annealing complementary DNA strands. We show that C-terminally truncated Red β , whilst still able to promote annealing and nucleoprotein filament formation, is unable to mediate homologous recombination. Mutations of the C-terminal domain were evaluated using both single- and double stranded (ss and ds) substrates in recombination assays. Mutations of critical amino acids affected either dsDNA recombination or both ssDNA and dsDNA recombination indicating two separable functions, one of which is critical for dsDNA recombination and the second for recombination *per se*. As evaluated by co-immunoprecipitation experiments, the dsDNA recombination function relates to the Red α -Red β protein-protein interaction, which requires not only contacts in the C-terminal domain but also a region near the N-terminus. Because the nucleoprotein filament formed with C-terminally truncated Red β has altered properties, the second C-terminal function could be due to an interaction required for functional filaments. Alternatively the second C-terminal function could indicate a requirement for a Red β -host factor interaction. These data further advance the model for Red recombination and the proposition that Red β and RAD52 SSAPs share ancestral and mechanistic roots.

In living cells the genome is constantly being damaged by environmental agents, oxidative stress and replication errors¹. Amongst an arsenal of repair pathways, double strand break repair initiated by DNA annealing proteins is central to the maintenance of genomic integrity^{2,3}. By mechanism, DNA annealing proteins are divided into ATPases capable of strand invasion (RecA, RAD51) and single strand annealing proteins (SSAPs) that do not utilize ATP⁴. Until recently SSAPs were classified into at least three separate groups each named after the most prominent member; RAD52, Red β and Erf⁵. Of these, RAD52 is best understood because it is highly conserved in all eukaryotes and involved in cancer mechanisms^{6–9}. Red β from λ phage is also notable because it mediates the very useful DNA engineering technology termed recombinering^{10–12}. In 2009, using deep bioinformatic tools to identify a distant signature motif, we proposed that RAD52 and Red β are members of an SSAP superfamily¹³. This proposition has been supported by the application of different bioinformatic methods^{14,15}.

As opposed to single strand (ss) DNA binding proteins such as SSB and RPA, which protect and occlude ssDNA from recombination, RAD52/Red β SSAPs promote recombination and share several biochemical

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similarities. They are weak ssDNA binding proteins with no affinity for double-stranded (ds) DNA. In the absence of DNA *in vitro*, they multimerize into rings or chains at high concentrations ($>0.5 \mu\text{M}$). Also, they share a similar protein architecture based on an N-terminal ssDNA binding domain of ~ 180 amino acids and a C-terminal extension that in the case of RAD52 is required for homologous recombination (HR) through specific protein-protein interactions⁶. These shared biochemical, protein sequence and functional similarities suggest the existence of an ancestral annealing mechanism involved in HR.

RAD52 is the best characterized SSAP due, in part, to two crystal structures of the N-terminal DNA binding domain of human RAD52^{16,17}. Both crystal structures revealed that the N-terminal domain of ~ 200 amino acids forms a mushroom-shaped undecameric ring with an external groove lined with positive charges, which probably binds the phosphodiester backbone of ssDNA. Although full length RAD52 forms a heptameric, not undecameric, multimer¹⁸, a RAD52 homolog from a *Lactococcus* phage, SakRad52, also forms undecameric rings without DNA *in vitro*¹⁹. Along with undecameric/dodecameric rings formed by Red β ²⁰, the recurrence of these beautiful ~ 11 mer rings shared amongst various SSAPs has promoted ring-based models for homology searching and DNA annealing^{17,20–23}. The ring models have been challenged by our recent experiments, which indicate that Red β homology searching occurs by a monomer to monomer random hit mechanism at concentrations below the *in vitro* threshold concentration for formation of the ~ 11 mer rings^{13,24}.

Whether mediated by monomers or rings, four lines of evidence indicate that annealing by Red β initiates recombination on the lagging strand template at the replication fork. The first indication arose from strand bias observed using ss oligonucleotides (oligos). The ss oligos that can act as Okazaki-like primers for lagging strand synthesis consistently delivered more recombination than their complementary oligos^{25,26}. Second, Red recombination requires ongoing replication at the moment of recombination and not merely to amplify the recombination product²⁷. Third, dsDNA substrates are processed into full length ssDNA intermediates by Red α before annealing by Red β into the replication fork^{27,28}. Fourth, host mutations that enlarge the ssDNA loop on the lagging strand template at the replication fork increase the frequency of Red recombination²⁹.

In addition to roles in genome maintenance, SSAPs are also found in phages and viruses with a 5' to 3' exonuclease as “SynExo” pairs³⁰. The Red (recombination deficient) operon in λ phage is a SynExo paradigm pairing Red α , a 5' to 3' exonuclease that is a toroidal homotrimer^{31,32}, with Red β . The Red α /Red β SynExo pair interacts through a specific protein-protein interaction³³ that is required for efficient homologous recombination using ds³⁴ but not ss²⁵ DNA insertions. To date, neither the molecular detail nor the function of this Red α /Red β protein-protein interaction has been defined, partly due to the lack of a Red β crystal or NMR structure.

Here we further characterize the Red α -Red β protein-protein interaction and examine Red β structure and function. This information is integrated with existing data into a new model for concerted action by Red α and Red β .

Results

Both N- and C-terminae of Red β are essential for recombination. Red β encompasses three parts; a central region that is defined by its conservation with other SSAPs⁵ flanked by N- and C-terminal regions of 47 and 83 amino acids respectively (Fig. 1a). The conserved region is required for both DNA binding and annealing whereas the C-terminal region is dispensable for annealing^{15,35} and its function remains undefined. Whether the N-terminus is required for DNA binding, annealing or recombination has not been determined. To further dissect Red β function, we generated two N-terminal truncations, N1Red β (20–261) and N2Red β (38–261) and three C-terminal truncations, C1Red β (1–237), C2Red β (1–217) and C3Red β (1–185). All deletions were well expressed as evaluated by Western blotting (Fig. 1b; Supplementary Fig. 1a). Functional testing for recombination activity in *E. coli* was evaluated using either a single strand oligonucleotide repair (ssOR) assay in a BAC (bacterial artificial chromosome; Fig. 1c) or a Beta recombination assay³⁶ based on a dsDNA substrate with one 5' end protected against exonuclease digestion by a pair of phosphothioate bonds (Fig. 1e). All Red β truncations disabled recombination in both assays (Fig. 1d,f; data not shown) except for the least C-terminally truncated construct C1Red β (1–237), which retained approximately 25% of the wt level in the ssOR assay as well as the expected bias between lagging and leading strands. These results indicate that the recombination functions of Red β rely on amino acids that lie outside of the conserved region at both ends of the protein and the very C-terminus is required for dsDNA but not ssDNA recombination.

DNA annealing is not sufficient for *in vivo* recombination. To determine whether the Red β deletion mutants lost recombination because they lost the ability to anneal DNA, we purified N1Red β and C3Red β . The wild type and two mutant proteins were all well expressed and soluble with similar secondary structural properties as evaluated by circular dichroism (Supplementary Fig. 1b). Then we evaluated their annealing capacities by gel shift. N1Red β failed to promote annealing (Supplementary Fig. 1c,d), which provides a straightforward explanation for the lack of recombination and also indicates that the annealing domain includes the poorly conserved sequences at the N-terminus. In contrast, the biggest C-terminal deletion, C3Red β , showed qualitatively similar DNA binding and annealing properties as wt Red β , including weak ssDNA binding and formation of the annealed nucleoprotein filament upon sequential addition of complementary strands (Fig. 2a). These observations confirm previous work with a C-terminal truncation (Red β 1–177), which demonstrated normal annealing activity upon sequential addition of complementary oligonucleotides³⁵.

Quaternary structures of Red β truncations. In the absence of DNA, wt Red β has been shown to form rings²⁰ and/or a shallow right-handed open helix equated to a ‘split-lock washer’¹³. Using atomic force microscopy, we observed that C3Red β retained this property whereas N1Red β appeared as balls rather than rings (Fig. 2b).

Wt Red β , C3Red β and N1Red β were compared by size exclusion chromatography (SEC) at an input concentration of $350 \mu\text{M}$ (1 mg/ml). Red β eluted as a single peak at ~ 480 kDa apparent size (Fig. 2c), which is greater than calculated for a complex of 11 to 12 monomers ($11/12 \times 29 = \sim 335$ kDa) but consistent with the expectation that

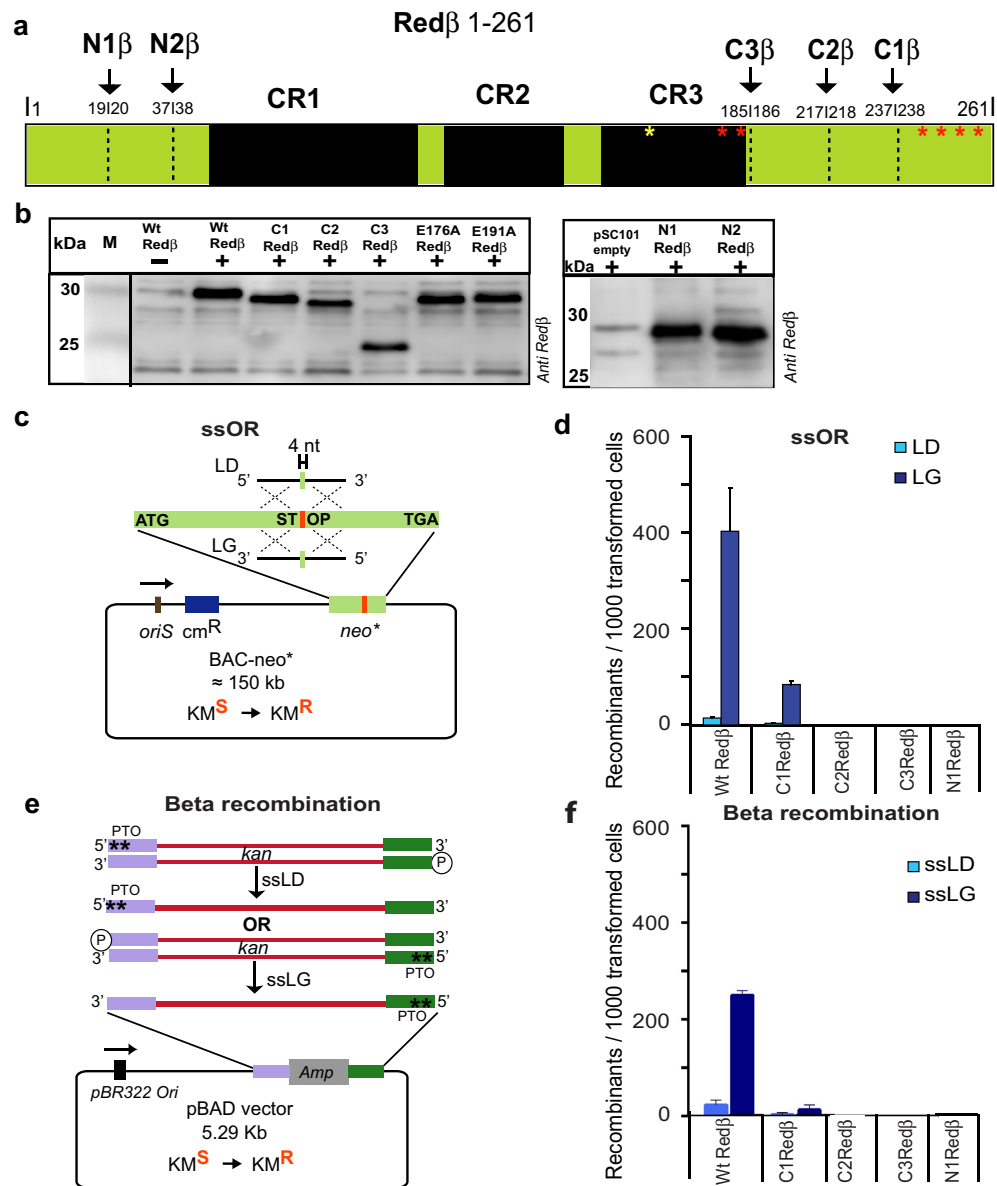


Figure 1. Redβ requires both N- and C-terminae for homologous recombination. (a) Diagram of Redβ depicting the CR1–3 regions conserved with other phage SSAPs⁵ as well as the deletions and point mutations used in this work. (b) Expression of Redβ from pSC101BAD-Redβ induced by arabinose and evaluated by Western using a Redβ antibody. Lane Wt Redβ-; uninduced with arabinose; lane pSC101 empty; induced with arabinose but without Redβ in the expression plasmid. (c) Schematic representation of the ssOR (single strand Oligonucleotide Repair) assay. The neomycin resistance gene was mutated to introduce a central stop codon (neo*) and inserted into a BAC. Repair of the mutation by incorporation of an oligonucleotide restores kanamycin resistance. The complementary single strand oligonucleotides can either anneal to the lagging strand template and act as an Okazaki-like fragment primer (LG) or not (LD). The BAC resides in an *E. coli* host, which is induced for expression of wt and mutant Red proteins from a pSC101 plasmid by addition of arabinose 45 minutes before electroporation of the oligonucleotide followed by quantitating the acquisition of kanamycin resistance by counting colonies on kanamycin plates. (d) Performance of wt and truncated Redβ proteins in the ssOR assay using either the LD oligo (light blue) or LG oligo (dark blue). Experiments were performed in triplicates and data were normalized to the number of cells transformed with a control plasmid and are represented as mean ± SD. (e) Schematic representation of the Beta recombination assay. Two PCR products were generated to carry the neo (kanamycin resistance) gene. Both products carried identical DNA sequence however differed according to the position of two consecutive phosphothioate bonds at opposing 5'-ends. In both PCR products, the other 5' end was phosphorylated. After electroporation, only one strand will be digested by Redα into full length single strands as illustrated. The strand whose 3' end can serve as a primer for Okazaki-like fragment synthesis is termed ssLG and the other strand is termed ssLD. The kanamycin resistance gene (kan) is flanked by 50 bp homology arms (purple and green) that flank the ampicillin resistance gene in pBAD. (f) Performance of the wt and truncated Redβ proteins in the Beta recombination assay. Experiments were performed as described in (d).

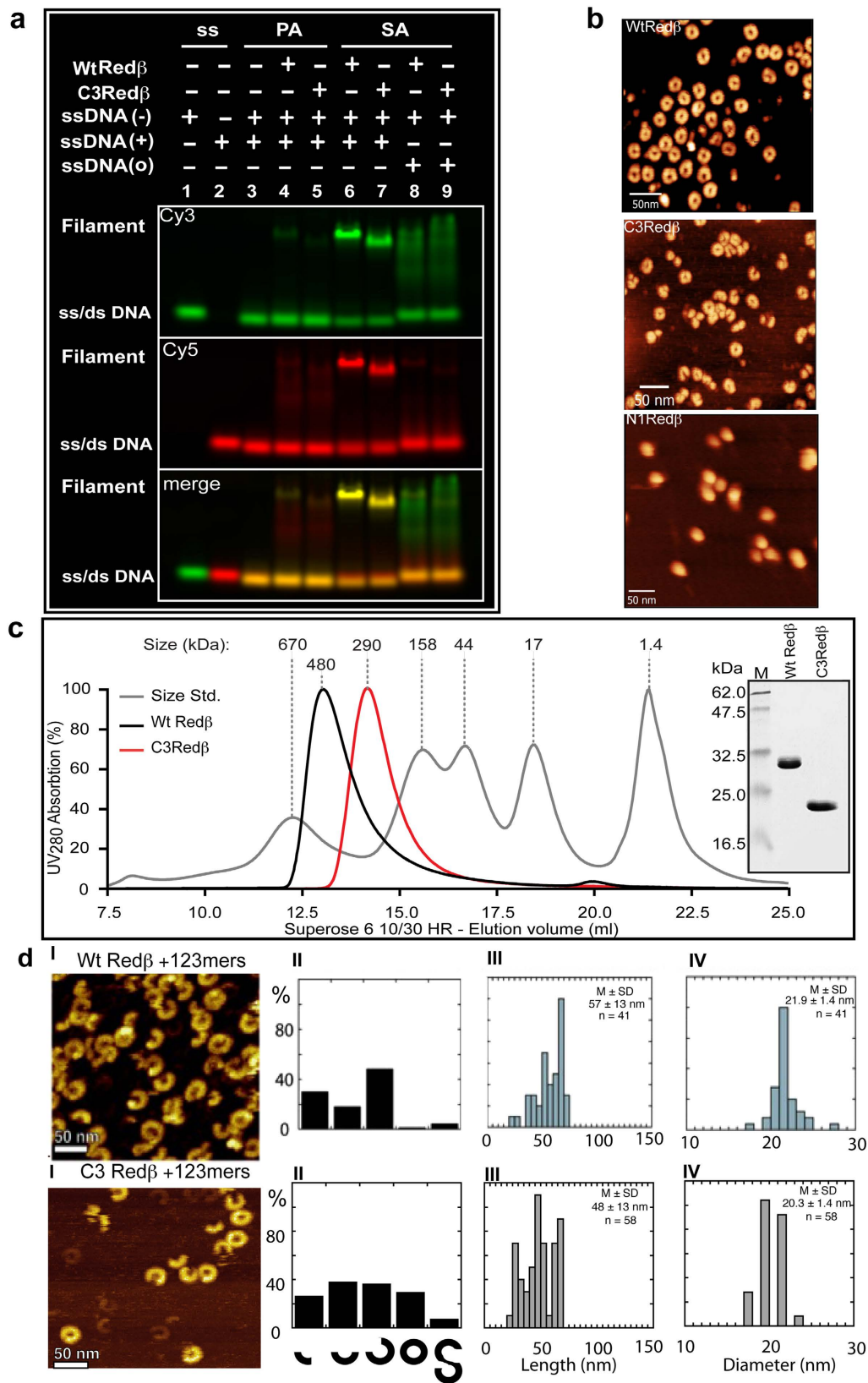


Figure 2. C3Red β forms rings and can anneal DNA to form the nucleoprotein filament. (a) Electrophoretic shift mobility assay (EMSA) for Red β -DNA complexes (wt Red β and C3Red β) using Cy3/Cy5 labeled complementary 50 nt ssDNA strands. All lanes have the Cy3 labeled oligo (–) except lane 2. All lanes have the Cy5 labeled complementary oligo (+) except lanes 1, 8 and 9. Lanes 8 and 9 have a non-complementary Cy5 labeled oligo (o). PA; pre-annealed (lanes 3–5) – the complementary oligos were pre-annealed before adding the indicated proteins. SA; sequentially added (lanes 6–9) – the Cy3 labeled oligo was incubated with the indicated

protein before addition of the Cy5 labeled oligo. The presence of a faint band shift and slight red smear in lanes 4 and 5 indicates that the oligo preannealing did not go to completion, which Red β completed (green band) and there was excess Cy5 oligo (red smear). The annealed nucleoprotein filament complex can be seen in lane 6 (wt Red β) and the C3-truncation (lane 7). In lanes 8 and 9, the smear shows the weak ssDNA binding of both wt and C3-truncated Red β to the Cy3 labeled oligo. Oligonucleotide sequences are shown in Suppl. Fig. 5. **(b)** Atomic Force Microscopy (AFM) images of wt Red β , C3Red β and N1Red β imaged without DNA. To achieve a reasonable distribution of adherent complexes on the mica surface, input concentrations of 0.7, 2.0 and 2.6 μ M respectively were used. **(c)** Analytical size exclusion chromatography of purified wt Red β (black line) and C3Red β (red line) on a Superose 6 column. The grey curve shows the molecular standards with respective sizes. Inset: Coomassie-stained SDS-PAGE gel (15%) showing purified recombinant C-terminal StrepII tagged wt Red β and C3Red β proteins. **(d)** AFM images of wt Red β and C3Red β . Panels I: representative images of the filaments formed by wt Red β (upper) and C3Red β (lower) annealing two complementary 123 mers; Panels II: histograms of the different filament species illustrated below. Panels III: histograms of filament length (nm). Panels IV: histograms of filament diameters (nm). M, mean; SD, standard deviation; n, number of particles analyzed.

these rings/shallow helices should produce a larger Stokes radius than predicted by their molecular mass. C3Red β eluted as a single peak at \sim 290 kDa again larger than expected for 11 to 12 monomers ($11/12 \times 20 = \sim$ 230 kDa) with approximately the same proportional discrepancy as wt Red β ($480/335 = 1.4$; $290/230 = 1.3$). Additionally the half-maximum peak widths of wt Red β and C3Red β were almost identical suggesting comparable oligomeric states and degrees of heterogeneity. However N1Red β eluted at an apparently larger size than wt Red β (Supplementary Fig. 1e), which is consistent with its altered appearance in AFM.

To extend this analysis, we examined the SEC peaks after elution using dynamic and static light scattering (DLS, SLS; Table 1). Wt Red β presented two well separated species one corresponding to the monomer (Species 3) and the other to the apparent 11/12-mer complex (Species 1; Table 1). This observation indicates that wt Red β dissociates from the multimeric complex upon dilution. In contrast, for both C3Red β and N1Red β , less than 10% of the populations were apparently monomeric and the samples were more broadly dispersed than wt Red β displaying additional intermediate-sized multimers (Table 1). Notably N1Red β showed the least propensity to dissociate. These observations indicate that both the N- and C-terminae modulate Red β intra-molecular interactions in the absence of DNA by potentially destabilizing the multimeric forms. Removal of either the N- or C-terminae apparently alters and stabilizes the homomeric interactions.

AFM comparison of the wt and truncated Red β nucleoprotein filaments. Wt Red β and C3Red β were analyzed by AFM imaging after annealing two 123 mer oligonucleotides as described previously¹³. The images revealed that C3Red β also forms left-handed nucleoprotein helical filaments like Red β , however distinct differences were apparent (Fig. 2d). The Red β filaments were quite uniform in presentation, length and diameter, whereas the C3Red β filaments were more heterogeneous and shorter with smaller diameters (Fig. 2d), again indicating that whereas the C-terminus is not essential for formation of the nucleoprotein filament, it modulates filament properties.

Point mutations at Red β C-terminus that selectively debilitate beta recombination. To investigate the properties of the Red β C-terminus in more detail, we created a series of alanine point mutations in the terminal C1 deletion region and tested them in the ssOR and Beta recombination assays (Fig. 3). As controls we employed alanine point mutations around the C-terminal end of the annealing domain (E176A, E187A, E191A). All point mutations were similarly expressed under recombination conditions (Fig. 3a). Mutations E176A and E187A did not impair recombination in either assay whereas Q240A strongly reduced it in both. In contrast, like the C1Red β deletion (Fig. 1d,f), mutations E191A, Q252A, E256A and K258A affected beta recombination more than ssOR. Indeed K258A and C1Red β presented a similar profile suggesting that the phenotype of the C1 deletion is largely due to the removal of this lysine. Also notable was the fact that all debilitating mutations either reduced both ds and ss recombination or mainly reduced ds recombination alone. These results indicate that although the C-terminus is not required for annealing, its contribution to recombination can be partially separated into (i) a function(s) required for both ds and ss recombination and (ii) a function(s) required for ds but not ss recombination.

Red β interactions with Red α . Recombination initiated by the Red proteins can utilize either dsDNA or ssDNA substrates. For recombination with dsDNA, the specific protein-protein interaction between Red α and Red β ³³ is important³⁴. However recombination using ssDNA oligonucleotides does not require Red α ^{25,26}. Consequently impaired Red α -Red β protein-protein interaction is a likely explanation for the C-terminal mutations that impaired ds recombination more than ssOR. To test this idea, we first determined the equilibrium dissociation constant for the Red α -Red β protein-protein interaction using isothermal titration calorimetry (ITC; Fig. 4a). Red α (150 μ M) was injected into a cell containing 15 μ M Red β or buffer with injection volumes of 2 μ l at 3 minute intervals. A K_D of $7.9 \pm 0.8 \mu$ M was determined. Next we performed co-immunoprecipitation experiments using Red α and Red β antibodies with good properties (Fig. 4b–d; Supplementary Figs 2 and 3). Immunoprecipitations by the Red α antibody of Red α interacting with the C2 and C3 deletions were impaired but the C1Red β deletion and all the single point mutations tested, except Q240A, were efficiently immunoprecipitated. Assuming that single point mutations may not be sufficient to debilitate the protein-protein interaction in a biochemical assay, we made several combinations and found that Q252A/E256A, E191A/Q252A/E256A and E191A/Q240A/Q252A/E256A/K258A indeed showed decreased Red α interaction (Fig. 4c). Also notable is the observation that the interaction was not abolished in any of the mutant proteins but only weakened. Consistent

Protein	Species 1				Species 2				Species 3			
	Rh (nm)	Dist (%)	~Mw (kDa)	No. of Subunits	Rh (nm)	Dist (%)	~Mw (kDa)	No. of Subunits	Rh (nm)	Dist (%)	~Mw (kDa)	No. of Subunits
WtRed β	13.5 \pm 4.4	31.8	490	11–12	—	—	—	—	4.5 \pm 0.6	68.2	29 \pm 4	1
C3Red β	15.7 \pm 3.6	45.1	520	12–13	8.3 \pm 1.5	45.6	58	2–3	4.0 \pm 1.4	9.3	26 \pm 1	1
N1Red β	15.1 \pm 0.6	84.3	512	12–13	9.3 \pm 0.6	8.3	56	2–3	3.9 \pm 0.8	7.4	22 \pm 3	1

Table 1. Mass distributions for wt, C3 and N1Red β after size exclusion chromatography. The wt Red β , C3Red β and N1Red β peaks fractionated by size exclusion chromatography (Fig. 2c), were analyzed by DLS and SLS, which revealed two (wt) and three (C1, N1) species as summarized with hydrodynamic radius (Rh) and distributions (Dist) as percentages together with estimated molecular weights and the corresponding number of Red β molecules per species.

with this, co-immunoprecipitation of N-terminal deletions revealed an interaction site between amino acids 20 and 38, whose deletion again reduced but did not abolish the interaction (Fig. 4d).

Neo-tail assay as a tool to decipher the mechanism of Red α β interactions. To test the functional significance of the Red α -Red β interaction, we devised a new assay termed “neo-tail”, which exploits the fact that the neomycin phosphotransferase protein does not tolerate any additional amino acids at its C-terminus³⁷. We mutated the stop codon of the neomycin phosphotransferase gene so that it does not convey kanamycin resistance (Fig. 5a). The stop codon and consequently kanamycin resistance is restored by recombination using ssDNA or dsDNA substrates that all share exactly the same 5' and 3' homology arms even when they are separated by different length inserts. Using the same homology arms to restore the same antibiotic resistance reduces the number of variables in the assay thereby permitting a very accurate evaluation of increasing insert length on recombination efficiency. Using the neo-tail assay and 35 nt/bp 5' and 3' homology arms, we evaluated a range of inserts from 10 to 200 corresponding to 80 (35 + 10 + 35) to 270 (35 + 200 + 35) nucleotide or base pair substrates. The substrates were prepared for electroporation as either 5' phosphothioated ssDNA or asymmetrically 5' phosphothioated dsDNA (Supplementary Fig. 4). In both cases, the recombinogenic ssDNAs, either the provided ssDNAs or the Red α product after digestion of the dsDNAs *in vivo*, are identical and the homology arm at the 3' end will serve as an Okazaki fragment primer when annealed to the lagging strand template.

To characterize the neo-tail assay, we compared the ssDNA and dsDNA substrates in recombination mediated by wild type Red α β γ , which revealed two differences (Fig. 5b). First, overall recombination with ssDNA substrates was at least an order of magnitude less efficient than with dsDNA substrates. Second, recombination with ssDNA showed a precipitous drop in efficiency at lengths above the 80 nucleotide substrate and all substrates longer than 100 were equally inefficient. Recombination efficiency with dsDNA also fell with increasing lengths, however the decrease was more uniform.

We attribute these two differences to the action of Red α with the following model. For dsDNA substrates, Red α and Red β act in concert through their specific protein-protein interaction that loads Red β onto the ssDNA emerging from Red α exonuclease digestion. This processive loading of Red β not only promotes recombination by preventing collapse of the ssDNA onto itself but also may facilitate the exploration for complementary sequences by Red β bound ssDNA. These advantages are not available when ssDNA substrates are provided. In particular, the folding of the ssDNA upon itself occludes Red β binding thereby reducing recombination. In this experiment, the 80 mer ssDNA appears to be reasonably accessible for Red β binding whereas longer lengths adopted occluding conformations.

To test this interpretation and to evaluate functionally the Red α -Red β protein-protein interactions identified in Fig. 4, we compared the Red β point mutation combinations Q252A/E256A, E191A/Q252A/E256A and E191A/Q240A/Q252A/E256A/K258A with wt Red β in the same assay. Consistent with the Q240A mutation, E191A/Q240A/Q252A/E256A/K258A mutations inactivated Red β (data not shown). The other two point mutation combinations had a mild effect on ssDNA recombination regardless of substrate length whereas they had a stronger impact on dsDNA recombination (Fig. 5c) indicating that the contribution of the Red α -Red β protein-protein interaction to recombination had been impaired by the mutations.

Discussion

Due to similar biochemical and physical properties, including weak ssDNA but no dsDNA binding affinity, multimerization to form rings and a similar protein architecture based on an N-terminal DNA annealing domain with C-terminal extension, we proposed that Red β and RAD52 are members of an ancestrally related SSAP superfamily. Here we add further evidence to support the proposition that SSAPs share an ancestral and functional relationship. Like RAD52⁶, Red β includes a poorly conserved N-terminus that is nevertheless required for annealing as well as protein-protein interactions C-terminal to the DNA annealing domain that are required for recombination.

In a search for the role of the C-terminal domain in Red β recombination, three issues arose involving (i) the protein-protein interaction between Red α and Red β ; (ii) an additional activity required for both ds and ssDNA recombination and (iii) an intrinsic contribution to Red β multimerization. These three will be discussed in turn.

Because they co-purify, the protein-protein interaction between Red α and Red β , has been known for at least 30 years³³. However the functional relevance of this interaction has not been deeply investigated. In a search for a way to use homologous recombination in *E. coli* to engineer recombinant DNA, we discovered the very useful

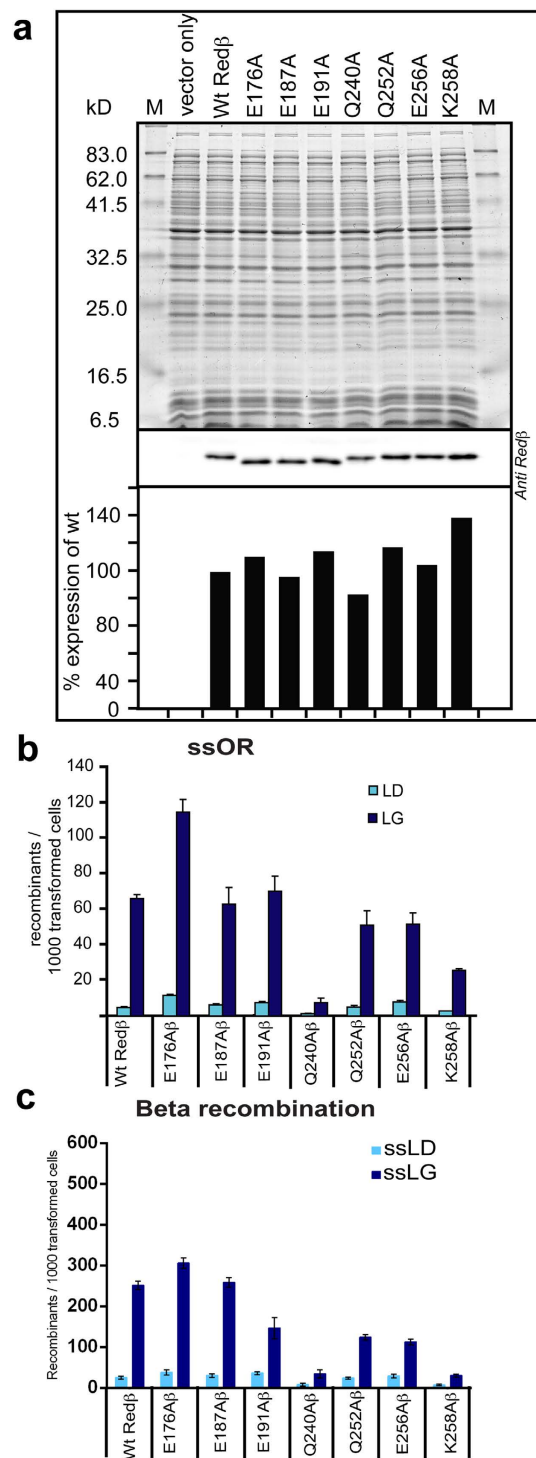


Figure 3. Point mutations of Red β C-terminus show reduced recombination efficiencies. (a) Western blot analysis showing equivalent protein expression levels of the seven Red β point mutants. The upper panel shows the 12% SDS-PAGE stained with Coomassie Brilliant Blue above the Western probed with an anti-Red β antibody, which is quantified below (wt Red β = 100%). (b) The indicated Red β point mutations, expressed from pSC101-BAD-Red β plasmids, were evaluated using the ssOR assay described in Fig. 1c,d. (c) The indicated Red β point mutations, expressed from pSC101-BAD-Red β plasmids, were evaluated using the Beta recombination assay described in Fig. 1e,f.

properties of the SynExo pairs, RecE/RecT from λ phage and Red α /Red β from λ phage^{10,11,38}, which initiated recombinering technology^{12,39} and also prompted the question: can these related phage SynExo pairs swap partners? That is, can the RecE exonuclease co-operate with Red β SSAP and vice versa, can the Red α exonuclease

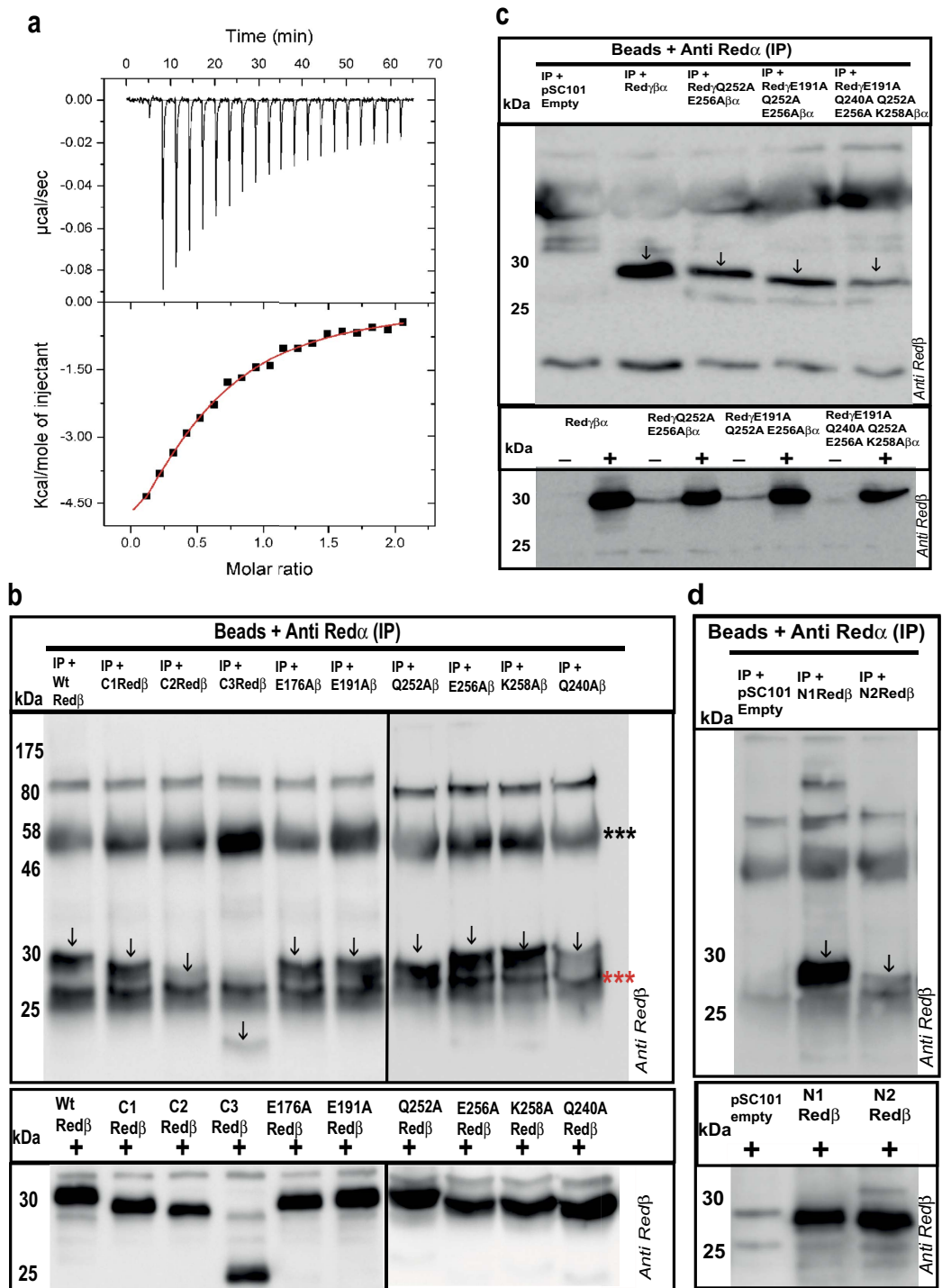


Figure 4. Evaluation of the Red α -Red β interaction. (a) Isothermal titration calorimetry determined by injecting Red α (2 μ l; 150 μ M; 3 minute intervals) into the cell containing Red β (15 μ M) and the resulting heat signal is plotted against time (*upper panel*) with the binding isotherm plotted in the lower panel including the theoretical fit to a single class of binding site (red line). (b) Upper panel: Co-IP of Red β mutations using anti-Red α antibody. The Western blots of the immunoprecipitates were probed with anti-Red β antibody. Vertical arrows indicate the Red β protein. The three red and black asterisks indicate the immunoglobulin light and heavy chains respectively. Lower panel: Western blots showing the expression of different Red β protein truncations and point mutants, which were used as input for the Co-IPs. (c) As for (b) except the left most lane on the upper panel shows a Co-IP from cells containing pSC101 without any inserted Red genes; and the lower panel shows Red β protein expression without (–) and with (+) arabinose induction of Red protein expression. (d) As for (b,c). In accordance with journal policy, uncropped versions of these Western blots are presented in Supplementary Fig. 4.

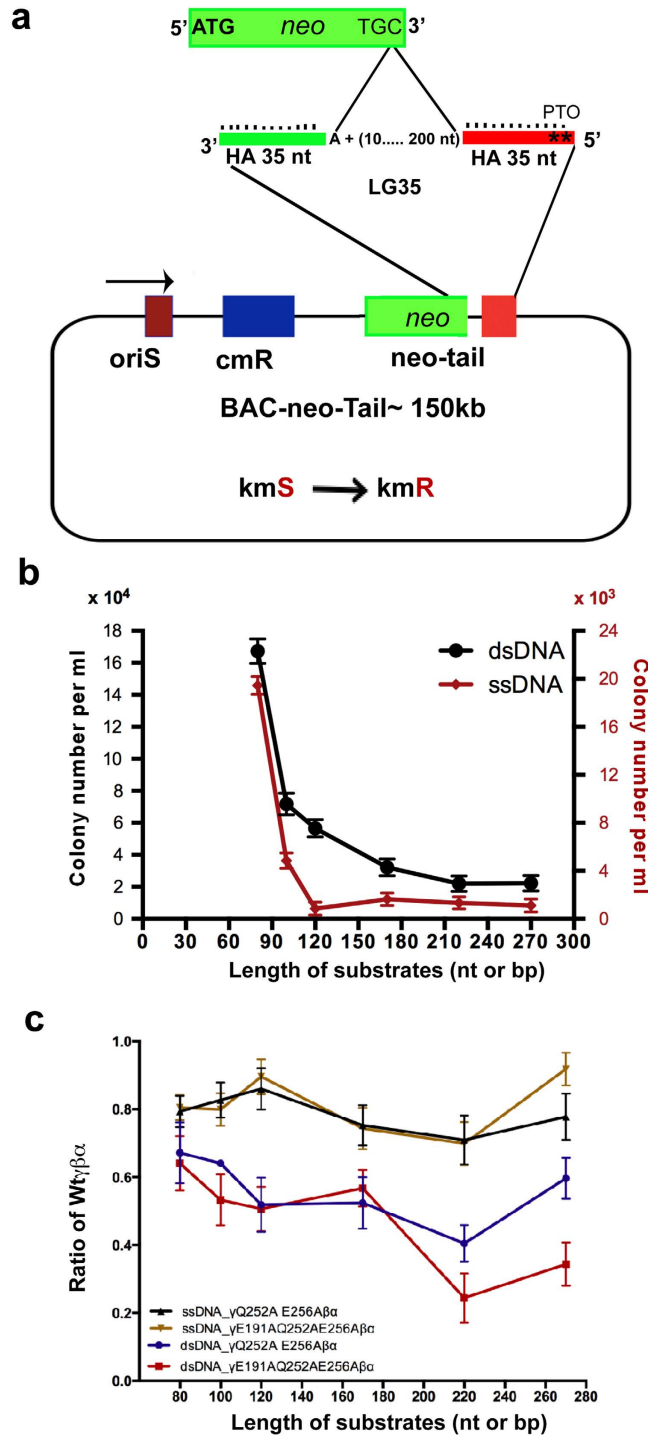


Figure 5. Red β C-terminal point mutations that decrease the Red α interaction impair Beta recombination but not ssOR. (a) Schematic representation of the *neo*-tail assay. The stop codon of the *neo* gene was mutated to TGC. Homologous recombination to revert the stop codon to TGA will restore kanamycin resistance. Six dsDNA substrates were prepared, each including the same 35 bp homology arms (green and orange) and a pair of phosphothioate bonds at the 5' end of the strand whose 3' end can serve to prime Okazaki-like fragment synthesis. The six substrates differed by the lengths between the homology arms (10 + 70 = 80, 30 + 70 = 100, 50 + 70 = 120, 100 + 70 = 170, 150 + 70 = 220 and 200 + 70 = 270 bp) and were electroporated as either ds or ssDNA after *in vitro* Red α digestion. (b) *Neo*-tail assay comparison of the six substrates as ds (black) or ss (red) DNA in homologous recombination mediated by wild type Red β . Red β was either expressed from pSC101-BAD-Red β (ss) or pSC101-BAD-Red $\gamma\beta\gamma\alpha$ (ds) and the results are from three independent experiments each performed in triplicate. Data were normalized to the number of cells transformed with a control plasmid and are represented as mean \pm SD. (c) Recombination efficiencies for Red β Q252A, E256A and Red β E191A, Q252A, E256A expressed as a ratio compared to wt Red β using the *neo*-tail assay with ss and dsDNA substrates. Error bars indicate the mean \pm SD of an experiment performed in triplicate.

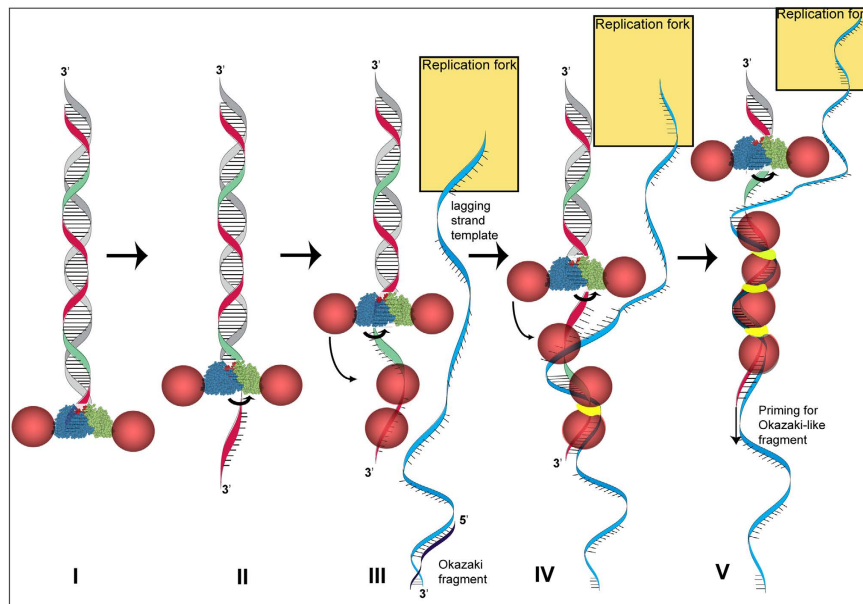


Figure 6. Model for Red $\alpha\beta$ molecular crosstalk. (I) Red α toroids (blue and green cones) thread onto the 3' end of a dsDNA break (I) and digest nucleotides from the 5' end, extruding the 3' end as single stranded (red strand; (II)). Red β monomers (pink spheres) bound to Red α are promoted to bind to the emerging ssDNA (III). Upon locating complementary sequence on the lagging strand template (blue strand) at the replication fork, a Red β dimer undergoes a conformational change (yellow band) to establish a DNA clamp (IV) that nucleates nucleoprotein filament growth and allows, possibly promotes, the 3' end (red strand) to serve as a primer for Okazaki-like fragment synthesis (V). The replication fork is depicted as a golden rectangle.

co-operate with RecT SSAP? The answer was conclusively negative. The heterotypic combinations do not mediate homologous recombination between dsDNA substrates suggesting the protein-protein interactions are specific and required for function. Supporting this suggestion, we showed that RecE and RecT, like Red α and Red β , also specifically interact³⁴.

Hence the requirement for the C-terminal domain in dsDNA recombination could be readily explained if the Red α interaction lies in this region. Mutagenesis of the C-terminal domain provided support for this explanation. Several mutations (C1; E191A; Q252A; E256A; K258A) impaired dsDNA recombination more than ssDNA recombination. However, in co-IP experiments, none of these mutations alone impaired the Red α -Red β interaction. Potentially these data indicate a discrepancy between the mutagenic impact of the single mutations on recombination *in vivo* compared to the biochemical protein-protein interaction. This discrepancy may be due to the relative insensitivity of co-IP experiments compared to functional recombination assays. However it may also be due to the complexity of the protein-protein interaction. In particular we discovered that the protein-protein interaction also depends upon amino acids at the N-terminus between residues 20 and 37. Also selected combinations (Q252A/E256A; E191A/Q252A/E256A) both impaired the protein-protein interaction and debilitated dsDNA recombination more than ssDNA recombination. These findings indicate that either Red β is folded to juxtapose amino acids 20–37, 191 and 252–261 to compose the Red α interaction surface or that the Red α -Red β interaction has a dynamic component potentially employing different aspects of the interaction during ongoing recombination. We favor the latter possibility because it lends an explanation for the role of the Red α -Red β interaction in dsDNA recombination. A dynamic component in the protein-protein interaction is also indicated by the relatively modest affinity ($K_D = 7.9 \mu\text{M}$), which is sufficient to provide specificity but weak enough for easy disassembly.

The identification of Red α interaction with the C-terminal domain does not explain why ssDNA recombination also requires the C-terminal domain. In this regard, the Q240A mutation is notable because it debilitates both ss and dsDNA recombination. This mutation slightly impairs expression (Fig. 3a) so possibly perturbs folding of the C-terminal domain. Alternatively the drastic mutagenic impact on both ss and dsDNA recombination may indicate the existence of another function required for recombination, such as a protein-protein interaction with a host factor. Our recent experience exporting Red recombination into other prokaryotes suggests that Red-host protein-protein interactions are significant^{40,41}. The proposition that the C-terminal domain not only interacts with Red α but also another factor required for recombination is also supported by the C1 deletion, which debilitates recombination (Fig. 1d,f) but does not reduce the interaction with Red α (Fig. 4a).

A third contribution from the C-terminal domain involves its effect on multimerisation, annealing and formation of the nucleoprotein filament. Both in the absence of DNA and upon annealing, the properties of the multimeric forms of truncated Red β differed from wt Red β , as did the balance between monomeric and multimeric forms. The ability of the C-terminal domain to modulate the multimeric properties of the DNA binding domain may have a functional impact on recombination, possibly by tuning the balance between monomeric and

multimeric forms to optimize the homology search. However, further studies are required to evaluate the merits of this proposition. Notably, our observations on the Red β C-terminal domain are concordant with the recent publication by Smith and Bell⁴². Deletion of the N-terminus leads to similar deductions. Like the C3 deletion, the N1 deletion showed a greatly reduced propensity to dissociate to monomers (Table 1). Additionally as visualized by AFM, the multimeric forms of the N1 deletion without DNA are balls rather than rings (Fig. 2a). This suggests that although the N1 deletion retains part of the specific homomeric interaction, the N-terminus is required for both an orderly interaction and the instability of this interaction.

Previously we unraveled the mechanism of Red-mediated dsDNA recombination, which occurs on the lagging strand template at the replication fork, to explain a part of its remarkable efficiency and usefulness²⁷. Here we address other aspects related to Red β recombination functions and the role of the protein-protein interaction with Red α . Combining the data presented here with previous knowledge permits the following model for dsDNA recombination (Fig. 6). Red recombination begins with Red α exonuclease digestion. Red α is a homotrimeric toroid, which loads onto the 3' end and removes nucleotides from the 5' end of the complementary strand whilst extruding the 3' ended single strand through the center of the toroid^{31,32}. Red α binds Red β at 1:1 stoichiometry³³. We propose that Red α promotes Red β loading onto the emerging ssDNA before it can fold onto itself and occlude Red β binding. Red β binds weakly to ssDNA and promotes annealing⁴³ by random collision promoted by bound monomers to search for candidate sequence matches, which are converted into very stable dimeric Red β DNA clamps upon successful identification of complementarity^{13,24}. These clamps nucleate the zipping-up of complementary strands by growth of a Red β nucleoprotein filament. This process occurs at the replication fork as its progression exposes single stranded DNA regions predominantly on the lagging strand template ahead of Okazaki fragment synthesis. Once annealed into the replication fork, the 3' end of the incoming DNA strand serves as an Okazaki-like replication primer for lagging strand synthesis. Then a mutation or recombinant strand will be incorporated if Red β has also annealed complementary sequences from the 5' end of the incoming ssDNA to the lagging strand template^{27–29}.

Methods

All experiments were performed in *E. coli* strain GB2005 (F-mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 recA1 endA1 araD139 Δ (ara, leu) 7697 galU galK l rpsL nupG fhuA::IS2 recET red α , phage T1-resistant). For *in vivo* assays, Red α , Red β , N1Red β (20–261), C1Red β (1–237), C2Red β (1–217) and C3Red β (1–185) were inserted into pSC101, which depends on the temperature sensitive oriR101⁴⁴, and expressed from the L-arabinose inducible pBAD promoter⁴⁵.

Recombination assays. For the beta recombination assay, the dsDNA was prepared by PCR amplification using the Phusion HF-DNA polymerase with one primer carrying two phosphothioate (PTO) bonds at the 5' end and the other primer having a 5' phosphate as described²⁷. PCR amplified dsDNA was purified using Invisorb Fragment CleanUp (Stratec Molecular GmbH) and 100 pmol was electroporated for each data point. For the neo-tail assay, the Red γ β α operon (accession codes: NP_040616, NP_040617 and NP_040618) was similarly cloned in pSC101. The point mutations Red β E176A, Red β E187A, Red β E191A, Red β Q240A, Red β Q252A, Red β E256A and Red β K258A and combinatorial point mutants (Red γ β Q252A, E256A α), (Red γ β E191A, Q252A, E256A α) and (Red γ β E191A, Q240A, Q252A, E256A, K258A α) were made using synthesized gene segments (GeneArt, Life Technologies) and linear plus linear recombineering with RecE/RecT⁴⁶. All mutations were confirmed by sequencing. The insert size series (10, 30, 50, 100, 150 and 200 bp) was cloned in p15A. The inserts plus 35 bp flanking homology arms were PCR amplified and purified as described above for the beta recombination assay. To generate ssDNA, the PCR products were digested with Red α (purified from pASK expression) and the reaction was cleaned up using ssDNA/RNA clean-up concentrator kit (Zymo Research, USA). The purified dsDNA and ssDNA were checked by gel analysis (data not shown). 100 pmol of either dsDNA or ssDNA were used for electroporation. For counting colonies to estimate recombination efficiency, the low number of colonies obtained from the empty pSC101 control was subtracted from all other values in the same experiment.

Protein expression. For *in vitro* studies, Red α and SSAP proteins were produced using one step Strep-tag II/Strep-Tactin affinity chromatography, which allows mild protein purification under physiological conditions⁴⁷. Initially, the ORFs of Red α and Red β were cloned individually into pASK-IBA2 (IBA Göttingen, Germany) under transcriptional control of the tetracycline promoter⁴⁸. The eight amino acid long Strep-tag II (WSHPQFEK) was fused to the Red α C-terminus and Red β N-terminus separated from the respective end by a Ser-Ala spacer. Overexpression of the tagged proteins and purification was described previously¹³.

Affinity and preparative gel filtration chromatography. The cleared lysate was loaded onto a gravity flow Strep-Tactin Superflow Sepharose column with a bed volume of 4.0 ml, which was equilibrated with Buffer W. Washing steps were carried out extensively for 40 column volumes. The recombinant protein was eluted by addition of the Strep-tag II specific competitor D-desthiobiotin to Buffer W at a final concentration of 2.5 mM. Recombinant protein containing fractions were pooled, dialyzed against storage buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 30% (v/v) glycerol) in a small volume using disposable dialysis tubes of the Mini Dialysis Kit 8 kDa cut-off (GE Healthcare), concentrated by ultrafiltration using Vivaspinn 6 ml concentrators with twin vertical membranes (Vivascience) and the protein concentration was determined using either Bradford protein reagent (Sigma) or UV quantification. Quality and quantities of the purification process were monitored by analytical SDS-PAGE analysis. Purified Strep-tag II tagged proteins were generally more than 95% pure at a concentration range of 0.5–10.0 mg/ml.

To obtain highly homogenous protein preparations, the affinity chromatography eluates were injected in to Superdex 200 column (column volume 120 ml) connected to an ÄKTAexplorer 10S system (GE Healthcare).

The column was equilibrated with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM MgCl₂. The flow rate was kept constant at 1.0 ml/min all through the procedure. The eluent protein concentration was constantly monitored by spectroscopic UV detection at 280 nm. Desired peaks were pooled, concentrated and quantified on a Nanodrop 1000 (ThermoScientific GmbH).

Electrophoretic mobility shift assays. Protein-DNA complexes were generated by incubation of 10 μM protein with 1 μM DNA in Tris-buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl₂) at 37 °C for 30 min, followed by addition of the complementary oligo and incubation for a further 30 minutes (when relevant). Samples were then mixed with 10x orange gel loading dye (Li-Cor Biosciences) and separated on 1.2 to 1.6% agarose gels in 2x TBE buffer. DNA was fluorescently-labelled by ordering oligonucleotides with 5'-fluorescent Cy3 or Cy5 dyes. Fluorescent signals were detected and recorded using Typhoon 9410 Variable Mode Imager (GE Healthcare).

Analytical size exclusion chromatography. Purified Strep-Tag II C-terminally fused Red α and SSAP proteins were run in high-resolution Superose 6 10/30 HR column (GE Healthcare) under native solution conditions. An exclusion limit of 4000 kDa was used, with an optimal separation range from 5 to 500 kDa and a bed volume of 24 ml was connected to an ÄKTAexplorer 10S system (GE Healthcare). Column equilibrations, size calibrations and sample analysis were performed in 25 mM Hepes pH 8.0, 150 mM NaCl, 1 mM MgCl₂ and 0.5 mM DTT buffer at a flow rate of 0.3 ml/min and a backpressure of 1.2 MPa. The column was calibrated using a gel filtration standard (Bio-Rad), composed of five molecular weight markers namely thyroglobulin (670 kDa), bovine gamma-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B12 (1.35 kDa). Protein samples of 1.0 mg were loaded into a 100 μl injection loop and subsequently injected onto the column. The eluent protein concentration was constantly monitored by spectroscopic UV detection at 280 nm. For reproducibility each protein was injected at least two times using a 100 μl injection loop.

Size exclusion chromatography (SEC) combined with dynamic light scattering (DLS) and static light scattering (SLS). To determine apparent molecular weight of different Red β preparations, we employed batch light scattering applications using DLS and SLS/MALS (WYATT, USA) with a Dawn Heleos II 18-angle-detector in combination with SEC. We performed the SEC using Sephadex200 column (GE Life) plugged to HPLC system with autosampler (Agilent), dRI, UV and SLS/MALS and injected purified Red β proteins at 1 mg/ml concentration. Every injection was repeated twice and analysed using Astra 5 (Wyatt) for molar mass distribution calculations.

Atomic Force Microscopy (AFM) imaging and data analysis. AFM Imaging was done as described previously¹³ except for the AFM images of Fig. 2b (wt Red β , C3Red β , N1Red β), which were recorded dry using an Agilent 5100 AFM and HQ:NSC18/Al BS cantilevers (MikroMasch) in intermittent contact mode. All data analysis and data fitting was done in Igor Pro version 5.04B (WaveMetrics Inc.). The refined models (Fig. 2D) were generated employing computer-aided design (CAD) construction software SolidWorks 2005 SPO.0 (SolidWorks Corporation) and by the support of Quindium GmbH & Co. KG.

Isothermal titration calorimetry. ITC experiments were performed at 25 °C on an iTC200 (GE Healthcare). Red α and Red β were dialyzed overnight in buffer containing 20 mM HEPES, 150 mM sodium chloride, 1 mM magnesium chloride, 0.5 mM TECP, pH 8, using 5000 MWCO Spectra/Por DispoLyzers (Spectrum Laboratories, Rancho Dominguez, CA). 150 μM Red α was injected into the cell containing 15 μM Red β . An automatic injection syringe was used with injection volumes of 2 μl at 3 min intervals, rotating at 750 rpm. Data acquisition and subsequent nonlinear regression analysis were performed using ORIGIN software supplied with the instrument. Experimental data were fitted to a single-site binding model to extract binding stoichiometry (n), the dissociation constant (K_D) and the enthalpy change (ΔH) as previously described⁴⁹. Thermodynamic parameters were calculated from the Gibbs free energy equation, $\Delta G = \Delta H - T\Delta S = -RT \ln(1/K_D)$, where ΔG and ΔS are the changes in free energy and entropy of binding, respectively and ΔG was -7.0 kcal/mol. ΔH was -9.84 ± 1.12 kcal/mol; $-T\Delta S = 2.87$ kcal/mol ($\Delta S = -9.64$ cal/mol/deg). T is the absolute temperature and R = 1.91 cal/mol/K. Control experiment were performed by titrating Red α in buffer and corrected for the heat of dilution.

Immunoprecipitation and co-immunoprecipitation. GB2005 carrying pSC101 encoding Red $\gamma\beta\alpha$ with or without various mutations in Red β was freshly diluted from an overnight culture in LB media plus 3 μg/ml tetracycline and grown at +37 °C until OD₆₀₀ ~0.4. Protein expression was then induced with L-arabinose 0.3% for 45 minutes at +37 °C. Cells were cooled on ice for 5 minutes and centrifuged at 13,200 rpm for 1 minute at +4 °C. Following washing two times with ice cold 1xPBS, cells were resuspended in the lysis buffer (100 mM Tris-HCl pH 8.0; 150 mM NaCl and 1 mM EDTA) supplemented with complete bacterial protease inhibitor cocktail (Sigma) and sonicated using a sonicating water bath (Diagenode GmbH) with 30 second on and off cycles for 5 minutes. The cell debris was removed by centrifugation at 16, 100 g, 30', 4 °C.

Immunoprecipitation reactions used 50–60 μl protein-G sepharose beads (GE Healthcare) in 20% ethanol per 1.5 ml Eppendorf tube. The beads were washed twice with 1 ml of 1xPBS followed by centrifugation at 0.8 g for 30 seconds at room temperature. Following this, beads were washed 3 times in 1 ml of immunoprecipitation buffer (IP buffer) comprising (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 10% glycerol, 1% triton X-100) supplemented with complete bacterial protease inhibitors cocktail (Sigma). Immunoprecipitation was initiated by adding 2 μl of anti-rabbit anti-Red α or anti-Red β sera to the beads in 500 μl of IP buffer followed by immediate addition of the cell lysates from the protein expression. IP reactions were carried out at room temperature either

for 20 minutes or 1 hour. Following the IP reaction, the beads were washed 4 times with 1 ml of IP buffer then suspended in SDS-PAGE Laemmli loading buffer, then incubated +95 °C for 5 minutes. After centrifugation, the supernatant was loaded on to a 12% reducing gel and electrophoresed at 130 V for 90 minutes before Western blotting. Immunoprecipitation reactions were confirmed by using anti-Red α antibody while the Co-IP reactions were monitored using anti-rabbit anti-Red β antibody. The membranes were analyzed with chemiluminescent detection method using Luminata Forte Western HRP Substrate. The images were documented with Image quant LAS machine 4000 (GE Healthcare) and AIDA software, version 3.20 (Raytest).

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Author Contributions

S.S., A.E., J.F., A.K., M.G., A.K., G.G., D.M., M.S. and A.F.S. designed the experiments. S.S., A.E., M.G. and S.R. performed the experiments. J.F. and J.T. made the Red expression plasmids. S.S. and A.F.S. wrote the paper.

Additional Information

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