ORIGINAL ARTICLE

CrossMark

Escherichia coli AlkB interacts with single-stranded DNA binding protein SSB by an intrinsically disordered region of SSB

Richa Nigam[1] · Monisha Mohan[1] · Gururaj Shivange[1] · Pranjal Kumar Dewangan[1] · Roy Anindya[1] (ORCID)

Abstract

Intrinsically disordered regions (IDRs) of proteins often regulate function through interactions with folded domains. *Escherichia coli* single-stranded DNA binding protein SSB binds and stabilizes single-stranded DNA (ssDNA). The N-terminal of SSB contains characteristic OB (oligonucleotide/oligosaccharide-binding) fold which binds ssDNA tightly but non-specifically. SSB also forms complexes with a large number proteins via the C-terminal interaction domain consisting mostly of acidic amino acid residues. The amino acid residues located between the OB-fold and C-terminal acidic domain are known to constitute an IDR and no functional significance has been attributed to this region. Although SSB is known to bind many DNA repair protein, it is not known whether it binds to DNA dealkylation repair protein AlkB. Here, we characterize AlkB SSB interaction and demonstrate that SSB binds to AlkB via the IDR. We have established that AlkB-SSB interaction by in vitro pull-down and yeast two-hybrid analysis. We mapped the site of contact to be the residues 152–169 of SSB. Unlike most of the SSB-binding proteins which utilize C-terminal acidic domain for interaction, IDR of SSB is necessary and sufficient for AlkB interaction.

Keywords Fe(II)/2-oxoglutarate-dependent dioxygenase · AlkB · SSB · DNA repair

Introduction

Escherichia coli SSB is a tetrameric protein consisting of four identical subunits. Each SSB monomer is consists of 178 amino acid residues and has three domains: the N-terminal OB-domain (Oligonucleotide/oligosaccharide Binding) [1] that binds ssDNA (residues 1–115), intrinsically disordered linker region (residues 116–169) and [2] C-terminal

Richa Nigam and Monisha Mohan—equally contributed.

✉ Roy Anindya
  anindya@iith.ac.in

  Richa Nigam
  bo14resch01003@iith.ac.in

  Monisha Mohan
  bo15resch11003@iith.ac.in

  Gururaj Shivange
  bo11p002@iith.ac.in

  Pranjal Kumar Dewangan
  bo13m1007@iith.ac.in

1 Department of Biotechnology, Indian Institute of Technology Hyderabad, Kandi, Sangareddy, Telangana 502285, India

acidic domain (residues 170–178) that binds several proteins involved in DNA metabolism [3]. The IDR of SSB is not observable in crystal structures, suggesting that these C-terminal tails are intrinsically disordered. Indeed, the IDR displayed low sequence complexity and enriched with large number of proline, glycine, and glutamine residues, resulting highly disordered/unstructured characteristics. Computational analysis revealed that the IDR is essential for highly cooperative binding of SSB to ssDNA [4]. Beside this, no other functional importance of IDR is known. C-terminal acidic domain of SSB act as protein interaction platform and bind various replication and repair proteins including base excision repair pathway enzyme uracil DNA glycosylase [5], translesion DNA polymerases [6, 7], RecJ [8], recombination mediator RecO [9], repair protein RadD [10], Exonuclease-1 [11, 12] and RecQ helicase [13].

As many DNA repair proteins are known to interact with SSB, we wanted to know if DNA dealkylation repair protein AlkB also intercts with SSB. AlkB repairs various alkyl-adducts including N^1-methyladenine (N^1meA), N^3-methylcytosine (N^3meC) present in DNA by oxidative demethylation. Previous studies showed that AlkB is 2-oxoglutarate (2-OG) and iron (Fe^{II}) dependent dioxygenase that preferentially

removes alkyl-adducts from ssDNA. Although the molecular mechanism of AlkB-mediated catalysis is well-understood, it is not clear whether SSB is involved in interaction with AlkB. Herein, we present an analysis of the SSB-AlkB interaction to address whether SSB can bind to AlkB. For this we generated several truncated SSB construct and studied the SSB-AlkB interaction by yeast-two hybrid and pull-down analysis. We report the surprising result that binding of SSB to AlkB involves the IDR of SSB. The C-terminal acidic domain of SSB, which is widely involved in protein–protein interaction, is dispensable for this interaction.

Materials and methods

Purification of recombinant proteins

Protein expression was performed following published procedure [14, 15].

In vitro binding assay

For GST pull-down experiments, full-length SSB and different truncated mutants of SSB were expressed as GST-fusion proteins and AlkB was expressed as His-tag protein. 100 µg of GST-tagged SSB proteins bound to 50 µl glutathione Sepharose beads (Thermo Scientific) were incubated with approximately 100 µg of free His-tagged AlkB in 500 µl binding buffer containing 10 mM Tris–HCl pH 7.4, 100 mM NaCl and 5% glycerol at room temperature for 2 h. For ssDNA-dependent interaction, 10 µM of 70-mer oligonucleotide was added along with His-tagged AlkB and GST-SSB. Protein complexes were then pulled down and analyzed by western blot with anti-His antibody as described previously [16].

Isothermal titration calorimetry (ITC)

For ITC experiments, synthetic peptide corresponding to amino acid residues 152–169 (AQSRPQQSAPAAPSNEP) were obtained (GM Research Foundation Pvt. Ltd). All the ITC analysis were carried out in Microcal ITC 200 instrument at 25 °C while stirring at 750 rpm in 20 mM Tris–HCl, pH 8.0 containing 100 mM NaCl and 1 mM β-Mercaptoethanol. The tag-less AlkB (50µM) protein was filled in the cell (280 µL). The syringe was loaded with peptide 250 mM in the same buffer as that of protein. The titrant peptides was injected in the cell in 20 successive injections of 2 µL each at interval of 180 s. The collected data was processed using origin 7.0 software.

CD-spectroscopy

The CD experiments were conducted on a JASCO J-1500 instrument. A quartz cell of 1 mm path length was used for all the experiments. To examine the effect of NaCl on the AlkB and SSB conformation, SSB (20 µM) and AlkB (20 µM) were dialysed in low salt buffer (20 mM Tris–HCl, pH 7.4, 0.1 M NaCl) or high salt buffer (20 mM Tris–HCl, pH 7.4, 1.0 M NaCl). Spectra were obtained at room temperature. CD spectra of SSB-152–169 peptide (0.25 mM) was measured in buffer containing 20 mM Tris–HCl, pH 8.0, 50 mM NaCl.

Yeast two-hybrid analysis

The pACT2-AlkB (activation domain) plasmid was co-transformed with pGBKT7-SSB, pGBKT7-SSB-116-177 and pGBKT7-SSB-1-115 (binding domain) plasmid into yeast strain pJ69-4A [17] to generate strain J69RA1 (pACT2-AlkB + pGBKT7-SSB), J69RA2 (pACT2-AlkB + pGBKT7-SSB-116-177), J69RA3 (pACT2-AlkB + pGBKT7-SSB-1-115), J69RA4 (pACT2-AlkB + pGBKT7-SSB-1-169), J69RA5 (pACT2-AlkB + pGBKT7-SSB-1-151), J69RA6 (pACT2-AlkB + pGBKT7-SSB-152-169) and J69RA7 (pACT2-AlkB + pGBKT7-SSB-170-178). Experiments were carried away as described previously [18].

Results and discussion

In order to characterize AlkB-SSB interaction, several deletion constructs were generated (Fig. 1a). First, we separated the DNA binding (OB) domain (SSB-1-115) from the disordered C-terminal domain (SSB-116-177). While characterizing SSB-χ protein interaction, C-terminal 26-residues of SSB were shown to be important [19]. Hence, we created SSB-1-151 lacking C-terminal 26 residues. However, a later crystal structure of the χ-SSB interaction site revealed the C-terminal 8 residues of SSB was the true interaction domain [20]. Therefore we generated SSB-116–169 which retains the entire structurally disordered region except the terminal 8 acidic amino acid rich segment and SSB-116–151 which lacks C-terminal 26 amino acid (Fig. 1a). In order to further confirm if SSB actually interacts with via the C-terminal 8 amino acids or the remaining 18 residues, we have also created SSB-170–178 and SSB-152–169, respectively (Fig. 1a).

To assess whether AlkB can directly interact with SSB without the involvement of any intermediate proteins, yeast two-hybrid analysis was performed. *E. coli ssb* and *alkB*

Fig. 1 Yeast two hybrid analysis of SSB AlkB interaction. **a** Schematic diagram of SSB deletion constructs. **b** SSB-AlkB interaction analysis by yeast two-hybrid system. Yeast cells carrying plasmid pACT2, pACT2-AlkB, pGBKT7, pGBKT7-SSB, and other SSB deletion constructs were spotted on plates with appropriate media. Positive interactions are indicated by growth on media lacking histidine and expression of β-galactosidase
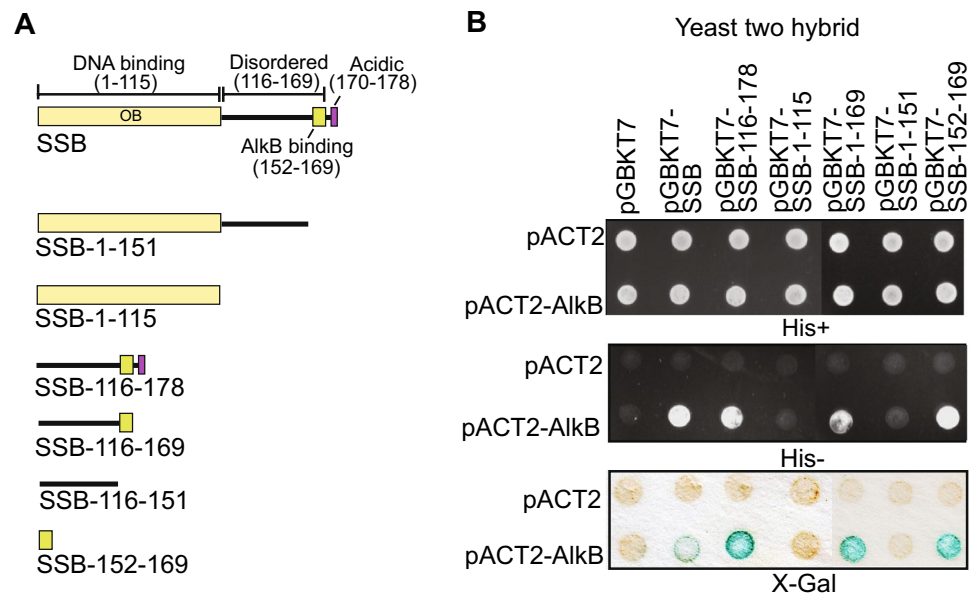
genes were cloned into vectors pGBKT7 (*TRP1* marker) and pACT2 (*LEU2* marker) rendering SSB fusion with DNA binding domain and AlkB fusion with transcription activation domains of the Gal4 transcription factor. The direct interactions of fusion partners were investigated by expression of all the reporter genes (*HIS3*, and *lacZ*). PJ69-4A cells carrying plasmid pair pACT2-AlkB/pGBKT7-SSB grew on media lacking leucine, tryptophan and histidine and showed a blue colour on media supplemented with X-gal, suggesting direct AlkB-SSB interaction (Fig. 1b). This result indicated that AlkB and SSB directly interact with each other in the cellular context. We were also curious to know if the N-terminal DNA binding OB domain or the C-terminal domain of SSB interacts with SSB. To address this, SSB-116–178 and SSB-1–115 constructs were expressed. As shown in Fig. 1b, only pACT2-AlkB/pGBKT7-SSB-116–178 grew well on media lacking histidine and showed a blue colour on media containing X-gal, suggesting AlkB interaction domain is located within the 116–178 amino acid residues of SSB and not within the N-terminal OB domain. Within this C-terminal half of SSB, there are two domains, (1) IDR (residues 116–169) and (2) acidic region (residues 170–178). If the acidic domain is involved in binding AlkB, then a truncated form of SSB without these residues will not be able to interact with SSB. PJ69-4A cells expressing AlkB and SSB-1–169 which lacks the last 9 amino acids grew on media lacking histidine, suggesting that the acidic region is not involved in binding. However, SSB-1–151 which lacks the last 27 amino acids failed to grow on media lacking histidine, suggesting that the region involved in binding must be located between the amino acid 152 and 169. To confirm this interaction domain more precisely, we examined the growth of pACT2-AlkB/SSB-152–169 on media lacking histidine.

We observed that SSB-152–169 supported the growth, suggesting that the 18 amino acid residues, corresponding to amino acid residues 152–169 and located in the IDR, could be involved in the interaction.

To independently verify the SSB-AlkB direct interaction in vitro, the GST-pull down assay was performed. In this experiment, AlkB protein was expressed with His-tag and SSB protein was produced as a GST-fusion, which could be pulled down using glutathione-Sepharose beads. The in vitro pull down results shows that his-tagged AlkB protein was bound to GST-SSB (Fig. 2a, lane 2), but not to GST alone (Fig. 2a, lane 3). These results suggest that AlkB and SSB made direct contact in vitro. Having established
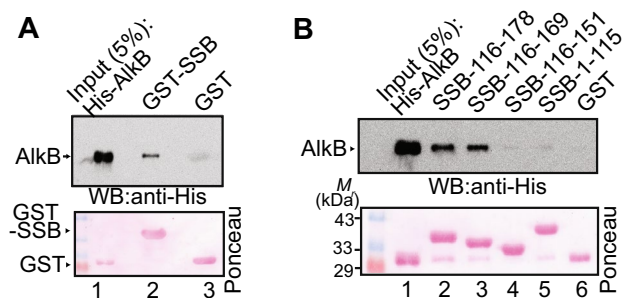
Fig. 2 GST pull down experiment of SSB AlkB interaction. **a** His-tag AlkB and GST-SSB bound to glutathione Sepharose beads was mixed together and interacting proteins were pull down by glutathione Sepharose. Top: inputs and pull downs were separated by SDS-PAGE and analyzed by western blot with anti-His antibody. Bottom: ponceau-S staining. Position of His-tag AlkB and GST-SSB is shown with an arrow. **b** GST-SSB fusion proteins used for mapping the AlkB-interacting regions on SSB. Top: inputs and pull downs were separated by SDS-PAGE and analyzed by western blot with anti-His antibody. Bottom: ponceau-S staining

in vitro interaction of SSB with AlkB, we wanted to know which part of the unstructured C-terminal region of SSB is involved in interaction. We expressed the entire C-terminal disordered domain (SSB-116–177), or the same domain lacking C-terminal 8 amino acids (SSB-116–169) or 26 amino acids (SSB-116–151) as GST-fusion protein. We have also generated a SSB containing only DNA binding OB domain (SSB-1–115) (Fig. 2c). To study in vitro interaction, purified his-tagged AlkB was mixed with GST-SSB-116–169, GST-SSB-116–151, and GST-SSB-115 and pull-down experiments were performed as before. Western blot analysis revealed that AlkB could indeed bind to GST-SSB-116–177 and GST-SSB-116–169 (Fig. 2c, lane 2 and 3, respectively) but not with GST-SSB-116–151 and SSB-1–115 (Fig. 2c, lane 4 and 5, respectively). Based on this in vitro interaction result together with the yeast two-hybrid data, we were able to map the residues 152–169 of SSB as the AlkB-interacting region.

We have also examined the strength of SSB-AlkB interaction by performing pull-down experiment in the presence of increasing concentration of NaCl. As shown in Fig. 3a the association of AlkB with SSB was not salt-stable and NaCl interrupted the binding. However, when ssDNA was included in the interaction mixture, SSB-AlkB interaction

was more stable (Fig. 3b). SSB-AlkB interaction remained intact at 0.5 M NaCl suggesting that ssDNA may render the complex more resistant to high salt washes.

We have also examined the effect of NaCl on the conformation of AlkB and SSB by using CD spectroscopy. As shown in Fig. 3c, the CD spectra of SSB remained almost unchanged in the presence 1.0 M NaCl. This result is in agreement with earlier report suggesting that tetrameric SSB is resistant to NaCl-dependent conformational changes [21]. We have also analyzed conformation of AlkB protein using CD spectroscopy. *E. coli* AlkB CD spectrum was reported to have characteristic negative peak at 214 nm [22]. We have also observed this negative peak at 100 mM NaCl (Fig. 3d). However, at higher salt concentration (1.0 M NaCl) this negative peak was absent indicating some conformational changes in the AlkB structure (Fig. 3d). Taken together, these results suggest that SSB-AlkB interaction is facilitated by the presence of ssDNA in low ionic strength condition.

To further characterize the strength of the interaction between residues 152–169 of SSB and AlkB, ITC was used. First, we confirmed the intrinsically disordered nature of the peptide by CD analysis. The peptide corresponding to residues 152–169 peptide exhibited a negative minimum close to 200 nm and a relatively low ellipticity above 210 nm

Fig. 3 Effect of increasing ionic strength on SSB-AlkB interaction. **a** GST pull-down experiments with His-tag AlkB and GST-tagged SSB proteins were carried out in the presence of increasing concentration of NaCl. **b** Effect of ssDNA on SSB-AlkB interaction monitored by pull-down experiment as described in **a**. Top: western blot with anti-His antibody. Bottom: ponceau-S staining. **c** Circular dichroism (CD) analysis of SSB in the presence of low (0.1 M) and high (1.0 M) concentration of NaCl. **d** CD analysis of AlkB in the presence of low (0.1 M) and high (1.0 M) concentration of NaCl
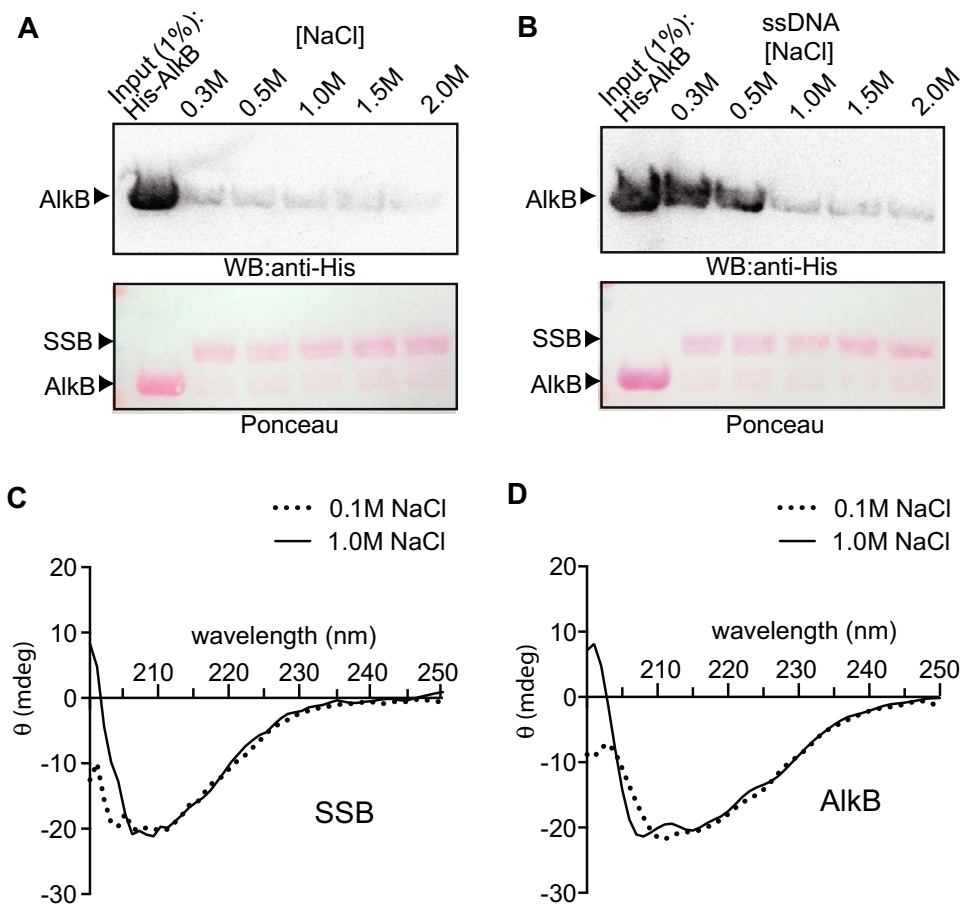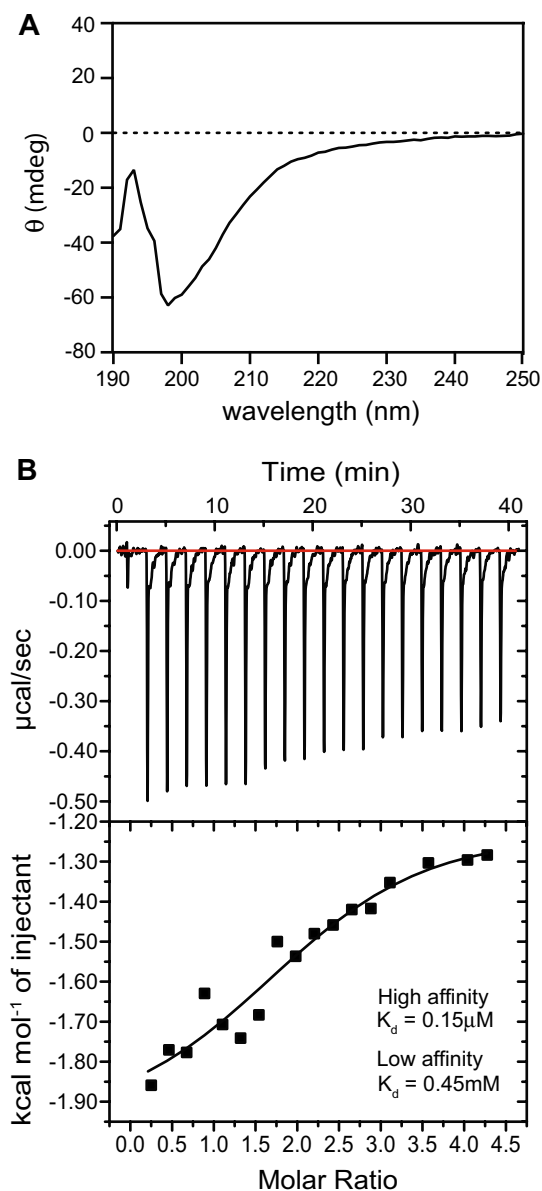
Fig. 4 Conformational analysis of SSB peptide 152–169 by circular dichroism (CD) and binding analysis by isothermal titration calorimetry (ITC). **a** CD spectra for SSB peptide 152–169. Shown are the spectra between 190 and 250 nm. **b** Binding of SSB peptide 152–169 to recombinant AlkB was carried out using ITC. The top panel represents the raw data from the titration as a series of peaks corresponding to the heat change (µcal/s) with each injection. The bottom panel is heat change on ligand addition (kcal/mole) against the peptide:AlkB molar ratio

(Fig. 4a), a typical observation for disordered proteins [23, 24]. Subsequently, ITC experiment was performed with this SSB peptide (residues 152–169) and AlkB. The best fit obtained with a two-site binding model, suggesting a high-affinity site (K_d = 0.15 µM) and a very low-affinity site (K_d = 0.45 mM) (Fig. 4b). This result demonstrates that AlkB binds to SSB-152–169 peptide, albeit weakly.

In conclusion, this study identifies a new binding site and a new binding partner for *E. coli* SSB protein. We showed that an 18 amino acids (152–169) region of SSB located within IDR is involved in the AlkB interaction. The role of C-terminal acidic domain of SSB as a platform for recruitment of DNA repair and recombination proteins is well established. Considering the site of AlkB-SSB interaction being at IDR of SSB, we speculate that C-terminal acidic domain of SSB would remain free for other protein–protein interaction. Identification of IDR of SSB as protein interaction site opens up the possibility that many other proteins might also interact with SSB via this site.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This work was conducted in compliance with ethical standards.

References

1. Raghunathan S, Kozlov AG, Lohman TM, Waksman G (2000) Structure of the DNA binding domain of *E. coli* SSB bound to ssDNA. Nat Struct Biol 7(8):648–652. https://doi.org/10.1038/77943

2. Savvides SN, Raghunathan S, Futterer K, Kozlov AG, Lohman TM, Waksman G (2004) The C-terminal domain of full-length *E. coli* SSB is disordered even when bound to DNA. Protein Sci 13(7):1942–1947. https://doi.org/10.1110/ps.04661904

3. Costes A, Lecointe F, McGovern S, Quevillon-Cheruel S, Polard P (2010) The C-terminal domain of the bacterial SSB protein acts as a DNA maintenance hub at active chromosome replication forks. PLoS Genet 6(12):e1001238. https://doi.org/10.1371/journal.pgen.1001238

4. Kozlov AG, Weiland E, Mittal A, Waldman V, Antony E, Fazio N, Pappu RV, Lohman TM (2015) Intrinsically disordered C-terminal tails of *E. coli* single-stranded DNA binding protein regulate cooperative binding to single-stranded DNA. J Mol Biol 427(4):763–774. https://doi.org/10.1016/j.jmb.2014.12.020

5. Handa P, Acharya N, Varshney U (2001) Chimeras between single-stranded DNA-binding proteins from *Escherichia coli* and *Mycobacterium tuberculosis* reveal that their C-terminal domains interact with uracil DNA glycosylases. J Biol Chem 276(20):16992–16997. https://doi.org/10.1074/jbc.M100393200

6. Arad G, Hendel A, Urbanke C, Curth U, Livneh Z (2008) Single-stranded DNA-binding protein recruits DNA polymerase V to primer termini on RecA-coated DNA. J Biol Chem 283(13):8274–8282. https://doi.org/10.1074/jbc.M710290200

7. Furukohri A, Nishikawa Y, Akiyama MT, Maki H (2012) Interaction between *Escherichia coli* DNA polymerase IV and single-stranded DNA-binding protein is required for DNA synthesis on SSB-coated DNA. Nucleic Acids Res 40(13):6039–6048. https://doi.org/10.1093/nar/gks264

8. Han ES, Cooper DL, Persky NS, Sutera VA Jr, Whitaker RD, Montello ML, Lovett ST (2006) RecJ exonuclease: substrates, products and interaction with SSB. Nucleic Acids Res 34(4):1084–1091. https://doi.org/10.1093/nar/gkj503

9. Umezu K, Kolodner RD (1994) Protein interactions in genetic recombination in *Escherichia coli*. Interactions involving RecO and RecR overcome the inhibition of RecA by single-stranded DNA-binding protein. J Biol Chem 269(47):30005–30013

10. Chen SH, Byrne-Nash RT, Cox MM (2016) *Escherichia coli* RadD protein functionally interacts with the single-stranded DNA-binding protein. J Biol Chem 291(39):20779–20786. https://doi.org/10.1074/jbc.M116.736223

11. Genschel J, Curth U, Urbanke C (2000) Interaction of *E. coli* single-stranded DNA binding protein (SSB) with exonuclease I. The carboxy-terminus of SSB is the recognition site for the nuclease. Biol Chem 381(3):183–192. https://doi.org/10.1515/BC.2000.025

12. Lu D, Myers AR, George NP, Keck JL (2011) Mechanism of Exonuclease I stimulation by the single-stranded DNA-binding protein. Nucleic Acids Res 39(15):6536–6545. https://doi.org/10.1093/nar/gkr315

13. Shereda RD, Bernstein DA, Keck JL (2007) A central role for SSB in *Escherichia coli* RecQ DNA helicase function. J Biol Chem 282(26):19247–19258. https://doi.org/10.1074/jbc.M608011200

14. Shivange G, Kodipelli N, Anindya R (2014) A nonradioactive restriction enzyme-mediated assay to detect DNA repair by Fe(II)/2-oxoglutarate-dependent dioxygenase. Anal Biochem 465C:35–37. https://doi.org/10.1016/j.ab.2014.07.003

15. Nigam R, Anindya R (2018) *Escherichia coli* single-stranded DNA binding protein SSB promotes AlkB-mediated DNA dealkylation repair. Biochem Biophys Res Commun 496(2):274–279. https://doi.org/10.1016/j.bbrc.2018.01.043

16. Shivange G, Monisha M, Nigam R, Kodipelli N, Anindya R (2016) RecA stimulates AlkB-mediated direct repair of DNA adducts. Nucleic Acids Res 44(18):8754–8763. https://doi.org/10.1093/nar/gkw611

17. James P, Halladay J, Craig EA (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144(4):1425–1436

18. Shivange G, Kodipelli N, Monisha M, Anindya R (2014) A role for *Saccharomyces cerevisiae* Tpa1 protein in direct alkylation repair. J Biol Chem 289(52):35939–35952. https://doi.org/10.1074/jbc.M114.590216

19. Shereda RD, Kozlov AG, Lohman TM, Cox MM, Keck JL (2008) SSB as an organizer/mobilizer of genome maintenance complexes. Crit Rev Biochem Mol Biol 43(5):289–318. https://doi.org/10.1080/10409230802341296

20. Marceau AH, Bahng S, Massoni SC, George NP, Sandler SJ, Marians KJ, Keck JL (2011) Structure of the SSB-DNA polymerase III interface and its role in DNA replication. EMBO J 30(20):4236–4247. https://doi.org/10.1038/emboj.2011.305

21. Kozlov AG, Lohman TM (1998) Calorimetric studies of *E. coli* SSB protein-single-stranded DNA interactions. Effects of monovalent salts on binding enthalpy. J Mol Biol 278(5):999–1014. https://doi.org/10.1006/jmbi.1998.1738

22. Bleijlevens B, Shivarattan T, Flashman E, Yang Y, Simpson PJ, Koivisto P, Sedgwick B, Schofield CJ, Matthews SJ (2008) Dynamic states of the DNA repair enzyme AlkB regulate product release. EMBO Rep 9(9):872–877. https://doi.org/10.1038/embor.2008.120

23. Kelly SM, Jess TJ, Price NC (2005) How to study proteins by circular dichroism. Biochim Biophys Acta 1751(2):119–139. https://doi.org/10.1016/j.bbapap.2005.06.005

24. Kelly SM, Price NC (2000) The use of circular dichroism in the investigation of protein structure and function. Curr Protein Pept Sci 1(4):349–384