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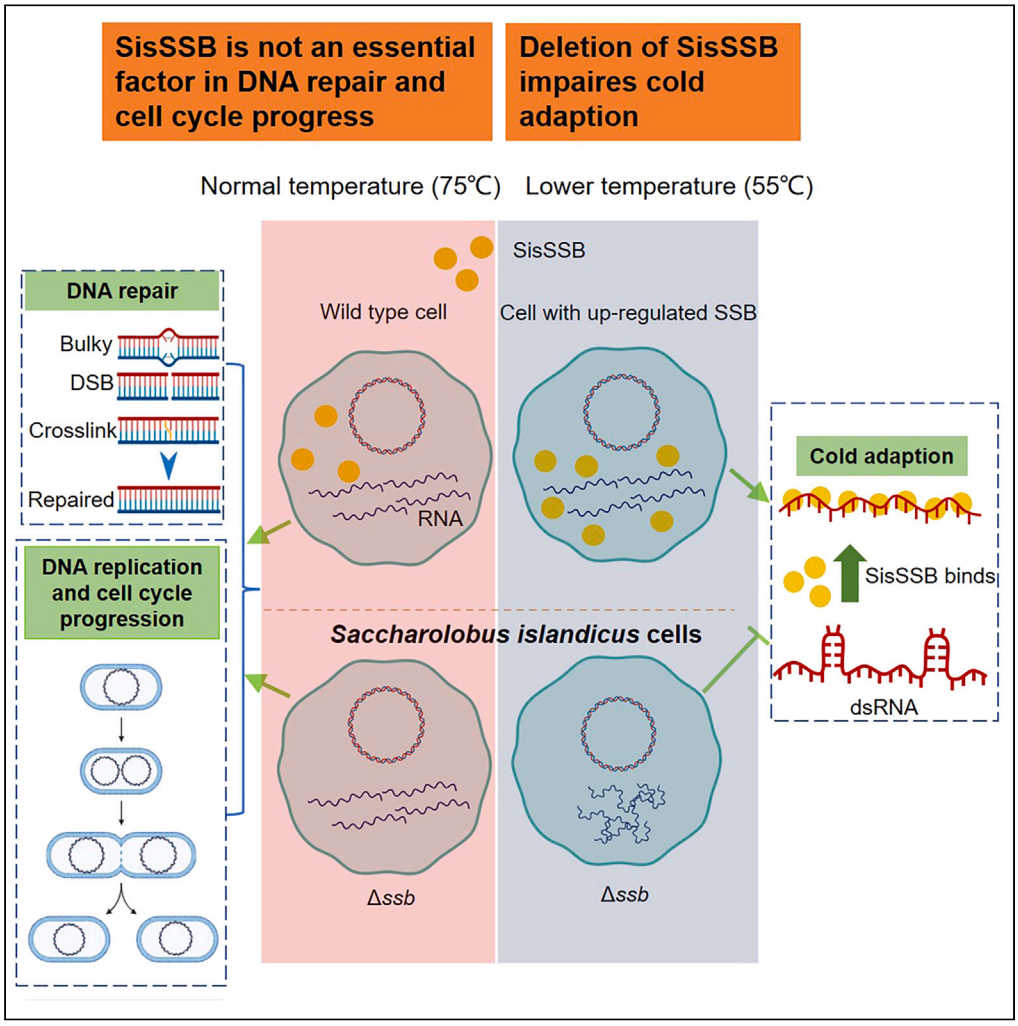

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Highlights

Two known single-stranded DNA-binding proteins are not essential in and archaeon

SisSSB exhibits melting activity dsRNA *in vitro* and unwinds RNA hairpin in *E. coli*

SisSSB acts as a cold-shock protein, facilitating temperature downshift adaption

The dsRNA melting activity is conserved in Crenarchaea that lack Csp homolog

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The canonical single-stranded DNA-binding protein is not an essential replication factor but an RNA chaperon in *Saccharolobus islandicus*

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SUMMARY

Single-stranded DNA-binding proteins (SSBs) have been regarded as indispensable replication factors. Herein, we report that the genes encoding the canonical SSB (SisSSB) and the non-canonical SSB (SisDBP) in *Saccharolobus islandicus* REY15A are not essential for cell viability. Interestingly, at a lower temperature (55°C), the protein level of SisSSB increases and the growth of Δ*Sisssb* and Δ*Sisssb*Δ*Sisdbp* is retarded. SisSSB exhibits melting activity on dsRNA and DNA/RNA hybrid *in vitro* and is able to melt RNA hairpin in *Escherichia coli*. Furthermore, the core SisSSB domain is able to complement the absence of cold-shock proteins in *E. coli*. Importantly, these activities are conserved in the canonical SSBs from Crenarchaeota species that lack bacterial Csp homologs. Overall, our study has clarified the function of the archaeal canonical SSBs which do not function as a DNA-processing factor, but play a role in the processes requiring melting of dsRNA or DNA/RNA hybrid.

INTRODUCTION

Single-stranded DNA-binding proteins (SSBs) are conserved and ubiquitous in bacteria, archaea, and eukaryotes. SSBs play vital roles in DNA replication, homologous recombination, and DNA damage repair.[1–3] Canonical SSBs share a structural fold called oligosaccharide/oligonucleotide-binding (OB)-fold that is responsible for ssDNA binding.[4] In replication, SSBs protect ssDNA from degradation and prevent secondary structure formation by binding to ssDNA in the replisome. SSBs also recruit replication proteins for the initiation and elongation of DNA replication.[5–7] SSBs interact with a variety of proteins for DNA transactions and are believed to play irreplaceable roles in genomic stability maintenance.[8–10]

In eukaryotes, the major single-stranded DNA-binding factor is the heterotrimeric replication protein A (RPA), which is made up of three subunits RPA1, RPA2, and RPA3 and contains six OB-folds in total.[11] Four of the six OB-folds (DBD-A, DBD-B, DBD-C, and DBD-D) are responsible for ssDNA binding, while DBD-E is the structural center of RPA heterotrimeric and DBD-F works for protein-protein interaction.[12] In bacteria, the *Escherichia coli* SSB (EcoSSB) comprises an OB-fold at the N terminal for ssDNA binding and a disordered C-terminal tail that mediates protein-protein interactions. EcoSSB always forms a tetramer in solution.[13] Whereas the archaeal SSBs are diverse. Some archaeal species, those of Euryarchaeota in particular, such as *Thermococcus kodakarensis*[14] and *Pyrococcus furiosus*,[15] encode eukaryotic RPA-like heterotrimer SSBs. Many crenarchaeal SSBs, for example, SSB from *Saccharolobus solfataricus* (formerly *Sulfolobus solfataricus*), SsoSSB, comprises an N-terminal OB-fold domain and a flexible C-terminal tail like EcoSSB. However, the N-terminal OB-fold structure of SsoSSB resembles more to that of eukaryotic RPA DBD-B domain than the EcoSSB OB-fold domain.[16] Further, it was found that Thermoproteales of the phylum Crenarchaeota lack any recognizable canonical SSB but possess a non-canonical SSB called ThermoDBP. It was thus assumed that ThermDBP displaces the canonical SSB for ssDNA binding.[17]

The model hyperthermophilic crenarchaeon *Saccharolobus islandicus* (a close relative of *S. solfataricus*) encodes both SsoSSB and ThermoDBP homologs, which we named as SisSSB (SiRe_0161) and SisDBP (SiRe_1003).[18,19] *Sisssb* was identified as an essential gene in *S. islandicus* M.16.4.[20] Surprisingly, different from RPA in human, the gene coding for the canonical SSB could be deleted in *Sulfolobus acidocaldarius*.[21] These raise interesting questions such as whether the crenarchaeal DBP is able to function as a replication factor and whether the canonical SSB could have a different function. In addition, the interactome of canonical SSB is poorly understood in archaea. In short, the exact physiological role of crenarchaeal SSBs remains ambiguous and unclear.

In this study, we performed genetic, biochemical, and transcriptomic studies on both canonical SSB and ThermoDBP in the hyperthermophilic archaeon *S. islandicus* REY15A.[17,22] We found that either *ssb* or *dbp* or both can be deleted. The deletion did not cause apparent physiological changes nor the genome instability, but led to growth retardance at lower temperatures. A 2-fold overexpression of SisSSB resulted

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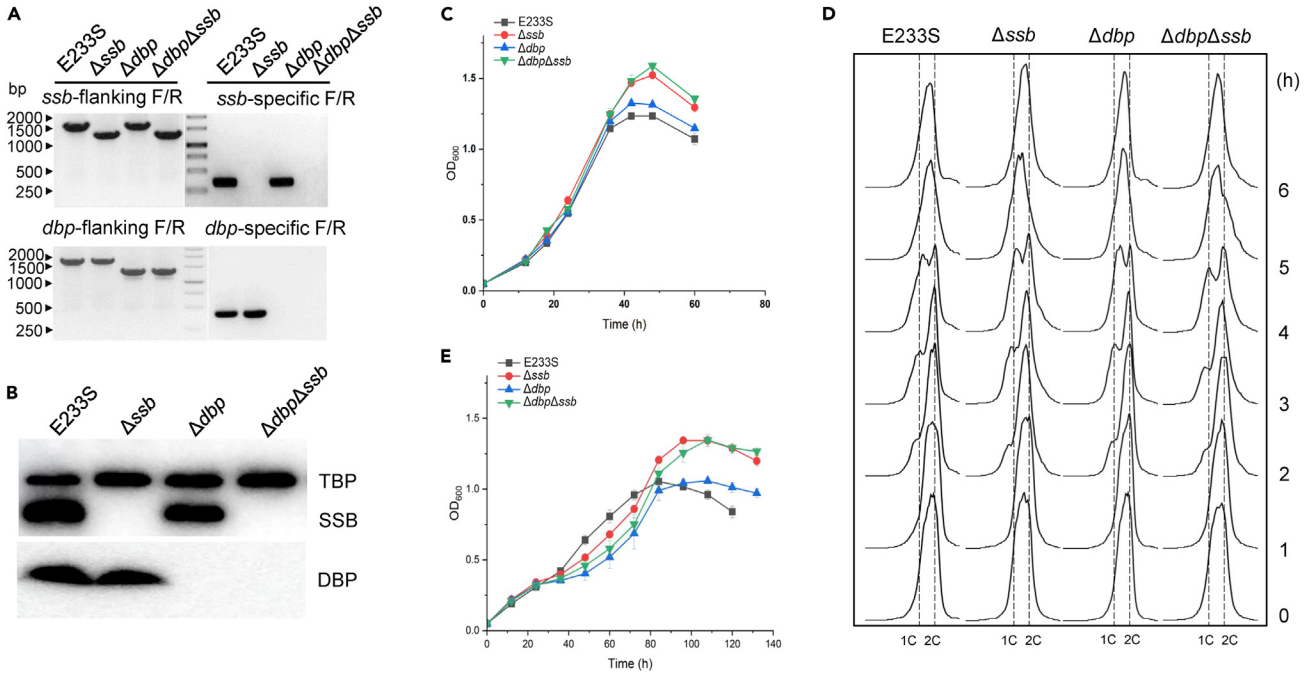

Figure 1. Growth and flow cytometry analysis of the knockout strains of *Sisssb* and *Sisdbp*

(A) PCR verification of the knockout strains Δ*ssb*, Δ*dbp*, and Δ*dbp*Δ*ssb*. Genomic DNA and two primer pairs, flanking F/R and gene specific F/R, were used for the analysis.

(B) Western blotting analysis of the knockout strains using whole-cell lysate. Cells were collected at OD_{600} = 0.5–0.8, disrupted by sonication. Primary antibody of SisDBP was added alone, while those of SisTBP and SisSSB were incubated simultaneously. Anti-SisTBP was used as the loading control.

(C) Growth curves under normal conditions. Cells were cultured with shaking at 110 rpm and 75°C in STVU medium with an initial OD_{600} 0.05. The values were calculated based on three biological repeats.

(D) Flow cytometry analysis of the knockout strains. Acetic acid (6 mM) was added into the culture medium when the OD_{600} reached 0.15–0.2. Samples were taken at different time (0, 1, 2, 3, 4, 5, 6, 7, and 8 h) and analyzed as described in the materials and methods. "1C" and "2C" indicate one and two copies of chromosomes.

(E) Growth curves of cells treated with 4-NQO. Cells were cultured as in (C) except that 4-NQO (3 μM) was added into the culture medium when the OD_{600} reached 0.2 (at 12 h). The values were calculated based on three biological repeats. See also Figure S2.

in overall elongation of the cell cycle, and high overexpression was lethal to the cell. SisSSB was further demonstrated to function as a cold-shock protein in *S. islandicus*. As SisSSB exhibited RNA unwinding and anti-transcriptional termination abilities, we conclude that the canonical SSB in *S. islandicus* REY15A is not an essential replication factor but functions as an RNA chaperon and a cold-shock protein.

RESULTS

Sisssb and *Sisdbp* can be deleted in *S. islandicus* REY15A

The hyperthermophilic archaeon *S. islandicus* REY15A genome encodes two annotated SSBs, the canonical SSB that contains an OB-fold (SisSSB) and the non-canonical Thermoproteales DBP (ThermoDBP) homolog (SisDBP).[16–19] SSB and RPA have long been known as essential replication factors in bacteria and eukaryotes, respectively.[2,6,23,24] Although the OB-fold structure of the crenarchaeal SSB resembles more the eukaryotic RPA OB-fold, the role of *Sulfolobus* SSB in DNA replication has never been verified. Recently, the gene encoding SSB was reported to be dispensable in *Sulfolobus acidocaldarius*,[21] which implies that *ssb* is not an essential gene in archaea. To address whether the SisDBP was able to complement the loss of *ssb* and what is the real function of SSB, we performed genetic analysis on the two SSBs. Using the endogenous CRISPR-Cas-based method,[25] we firstly obtained Δ*ssb* and Δ*dbp* (Figure S1A). To our surprise, the strain Δ*dbp*Δ*ssb* with double deletion of the two genes was also obtained by transforming the pGE-*dbp*-Knockout plasmid into Δ*ssb* cells and subsequent mutant screening. The deletion was confirmed by PCR and western blotting (Figures 1A and 1B). Intriguingly, the growth of the deletion strains at 75°C in the liquid STVU medium did not show apparent difference (Figure 1C); in fact, the cell density of strains lacking *ssb* even increased slightly (Figure 1C). We found that the cell morphology was the same as the control E233S (Figure S2F).

Deletion of the *ssb* does not impair cell cycle progression and the sensitivity to DNA damage agents

According to previous reports, ssDNA needs to be protected during DNA replication.[5,6] We employed flow cytometry to check whether the S phase (DNA synthesis) was affected or not. Cells were synchronized at G2 phase (Figures 1D, 0 h), and then the cell cycle was restarted by removing the acetic acid in the medium following a method as described in our previous report.[26] The flow cytometry profiles showed

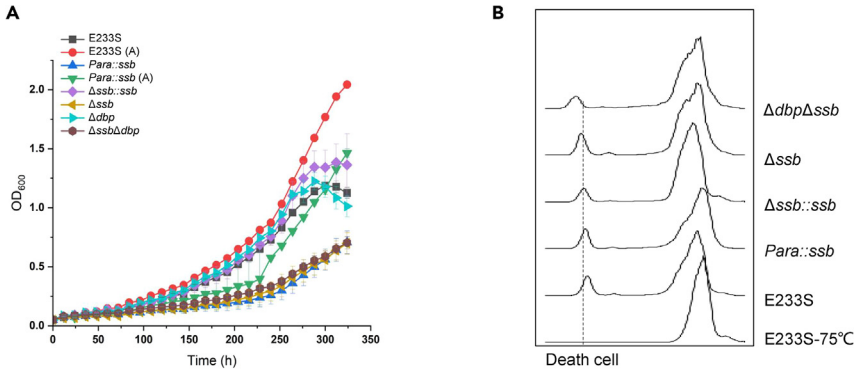

Figure 2. SSB-deficient cells exhibited slow growth at lower temperature

(A) Growth curves of SSB-deficient strains at 55°C. Cells were cultured in liquid STVU at 75°C to OD_{600} = 0.6–0.8 and then used as inoculates with an initial OD_{600} of 0.05. The cultivation was continued in STVU medium or in the arabinose-containing medium ATVU (indicated with "A") at 55°C with shaking. The optical density was monitored and the values were calculated based on measurements of three biological replicates.

(B) Cytometry profiles of the strains grown at 55°C. Samples were taken at 72 h and analyzed. Cells of E233S cultivated to middle logarithmic phase (OD_{600}~0.4) at 75°C was used as a control. See also Figure S3.

that the wild-type E233S exhibited an obvious peak of 1C (cells with 1 copy of chromosome) at 2 h, and the same 1C peaks also appeared at 2 h in Δ*ssb*, Δ*dbp*, and Δ*dbp*Δ*ssb* cells. It took about 3 h for the appearance of two copies of chromosomes (2C) peak (from 2 to 5 h) as the wild type. It seems that deletion of either *ssb*, *dbp*, or both did not affect the DNA replication efficiency.

It is generally accepted that ssDNA is generated after DNA damage and is involved in DNA damage response (DDR) and DNA repair.[27–29] To check if SSB and DBP are involved in DNA repair, the growth of the deletion strains was followed by measuring the optical density of their cultures in the presence of each of the four different DNA damage agents, 4-nitroquinoline N-oxide (4-NQO), cisplatin, hydroxyurea, and methyl-methanesulfonate, which induce DNA bulky adduct, intra-strand crosslink, stalling of the DNA replication fork, and nucleotide alkylating, respectively. We found that these DNA damage agents had no apparent more detrimental effect on the growth of these deletion strains compared with E233S (Figure 1E; Figures S2A–S2C).

In *S. islandicus* REY15A, a DDR network centered by Orc1-2 plays an important role in response to and repair of damaged DNA.[30,31] In the presence of DNA damage agent NQO or UV treatment, Orc1-2 upregulates the expression of genes coding for components of pili, DNA exchange system, and the homologous recombination repair (HRR) systems. When *S. islandicus* cells encounter extensive DNA damage, they form aggregates in which intercellular DNA exchange is believed to occur, providing undamaged DNA templates for HRR in recipient cells. To explore whether SSB and DBP in *S. islandicus* are involved in DNA damage signaling, cell morphology and cell aggregation were examined. As shown in Figures S2D and S2E, cell aggregation occurred more rapidly in Δ*ssb* than in E233S. 3 h after NQO treatment, 65% of Δ*ssb* cells formed aggregation, while almost no cell aggregation was observed in E233S. In addition, 12 h after NQO treatment, Δ*ssb* exhibited higher cell aggregation than E233S (89% vs. 59%). During DDR, Δ*ssb* displayed a higher cell aggregation ratio than E233S. Nevertheless, cell aggregation in Δ*ssb*, as in E233S, decreased gradually after 12 h, and finally disappeared. The results suggest that the DNA damage response in Δ*ssb* is more prompt than that of the wild type. The deletion of DBP did not increase the cell aggregation ratio after NQO treatment. These results indicate that the two ssDNA-binding proteins are not directly involved in DNA damage response and repair, but play other roles independent of DNA replication, DNA damage response, and DNA repair. The exact mechanism underlying *ssb* deletion-induced quicker DDR response remains unknown; however, loss of SSB may make more ssDNA exposed which could initiate DDR more quickly.

SSB is elevated at a lower growth temperature

In *E. coli*, *ssb* mutant strain *ssb-1* is temperature sensitive and displays lethal phenotype at 30°C.[32] In the hyperthermophilic crenarchaeon *S. acidocaldarius*, *ssb* deletion resulted retarded growth at lower temperatures.[21] We then tested the growth of the *ssb* deletion and the overexpression strains of *S. islandicus* REY15A at lower temperatures 65°C, 60°C, and 55°C. In agreement with phenotype in *S. acidocaldarius*,[21] deletion of *ssb* led to much slower growth than strain E233S at 55°C. Interestingly, the *dbp* and *ssb* double-deletion strain and the *ssb* knockdown strain *Para::ssb* in which the native *ssb* promoter is replaced with an arabinose-inducible promoter (*ParaS*-SD, see the following section for details) exhibited the same growth phenotype with the *ssb* deletion strain in the non-inducible medium (Figure 2A; Figures S3A and S3B). As temperature decreased, the growth inhibition became more pronounced. However, in the presence of 0.2% arabinose, the 2-fold SSB overexpression strain *Para::ssb* exhibited better growth than Δ*ssb* and the knockdown strains at 55°C, although it still grew slower than E233S (Figure S3). The results suggest that the archaeal SSBs function in cold adaption. The *E. coli* SSB has a much weaker affinity to ssRNA than ssDNA.[33] Human RPA also has a high affinity for ssDNA and low affinity for ssRNA; the difference is at least three orders of magnitude.[34] Previous studies showed that SSB from the hyperthermophilic crenarchaeon *S. solfataricus* binds to both ssDNA and ssRNA with almost equal affinity.[22] This extraordinary nucleic acid-binding feature of archaeal SSB implies that they could have other unknown functions.

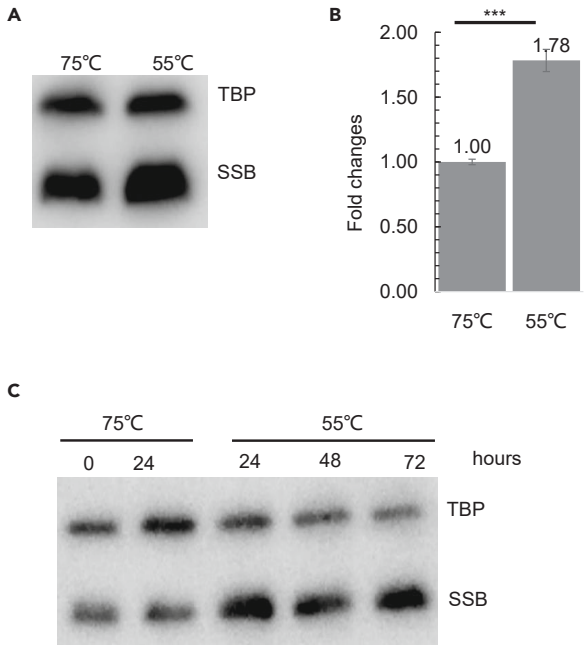

Figure 3. The protein level of SisSSB increases at lower temperature

(A) Western blotting analysis of SisSSB of cells cultured at 75°C and 55°C. E233S cells were cultured at 75°C to middle logarithmic phase (OD_{600}~0.4) then the cultures were moved to a sharker at 55°C. After cultivated for 24 h at 55°C, the cells were taken and disrupted by sonication. The cell lysates were subjected to SDS-PAGE and western blotting analysis with antibodies against SisSSB and SisTBP (loading control).

(B) Quantitative analysis of the results in (A). The values were calculated based on three replicates. Error bars indicated the standard deviation.

(C) Western blotting analysis of SisSSB of cells cultured for different times at 55°C. E233S cells were cultured at 75°C to middle logarithmic phase (OD_{600}~0.4). The samples were taken and divided evenly into culture flasks. One was incubated and cultured at 75°C and the other was at 55°C. Cells were taken from the cultures after 24, 48, and 72 h, respectively.

It is known that the levels of cold-shock proteins (Csps) in bacteria increase in response to temperature downshift.[35] Csps are supposed to function as RNA chaperones which prevent mRNA secondary structure formation so as to facilitate translation at low temperature.[36,37] Csps are required for bacteria to adapt to ambient temperatures, and deletion of Csps results in significant growth deficiency at low temperature in bacteria.[38] Given that deletion of *ssb* also caused a cold-sensitivity phenotype of *S. islandicus*, we assume that SisSSB may function as an RNA chaperon as the bacterial Csps. To test this hypothesis, we detected the protein level of SisSSB in E233S at 75°C and 55°C, respectively. The cells were cultured in liquid STVU medium at 75°C to middle logarithmic phase (OD_{600} ≈ 0.4), and then cultured at 55°C for 24 h. As expected, the SisSSB protein level increased to 1.8-folds when the culture was shifted from 75°C to 55°C (Figures 3A and 3B). In addition, this higher SisSSB level maintained for further 3 days (Figure 3C). These observations suggest that SSB plays an import role in cold-shock response.

SisSSB has anti-transcription termination activities *in vivo* and can unwind dsRNA *in vitro*

To investigate if SSB has RNA chaperon activity *in vivo*, we used the *E. coli* RL211 strain for analysis.[39,40] This strain harbors a chloramphenicol resistance gene (chloramphenicol acetyltransferase, CAT) as a reporter located downstream of a strong *trpL* terminator.[41] A pINIII vector carrying IPTG-inducible SisSSB was transformed into RL211, and upon the terminator is melted, the CAT gene will be expressed, enabling the strain to grow in the presence of chloramphenicol. *E. coli* CspA, CspE, and a confirmed RNA chaperone Sis10b in *S. islandicus* REY15A were used as positive controls. As shown in Figure 4A, same as CspA and CspE, cells carrying pIN-SisSSB grew well on chloramphenicol-containing agar plate even without IPTG induction, and SisSSB exhibited a stronger ability than Sis10 b at 37°C. These results suggest that SisSSB exhibited stronger anti-transcriptional termination ability than Sis10b *in vivo*. While with the addition of 0.2 mM IPTG, overexpression of SisSSB affected the growth of *E. coli* RL211 cells (Figures S4A and S4B).

To test if SisSSB has RNA chaperone activity *in vitro*, we used a molecular beacon assay.[40] As shown in Figure 4B, a 40 nt 5′-FAM-labeled ssRNA was annealed with another ssRNA labeled with a fluorescence quencher (BHQ1) at the 3′-end. The RNA chaperon Sis10b was used as a positive control. After annealing, the fluorescence of the partial duplex RNA decreased to ~5% of that of the heat-denatured dsRNA substrate (Figure 4B). In the presence of SisSSB, the fluorescence intensity increased to ~33% at 37°C which was higher than that of Sis10b (~17%), consistent with the result of the anti-transcription termination assay *in vivo*. The previously described results demonstrate that SisSSB is able to unwind RNA *in vivo* in *E. coli* and *in vitro*. It should be noted that this activity is passive. SisSSB binds to ssNA regions and then as the duplex region breathes, more SisSSB binds resulting in the eventual strand separation.

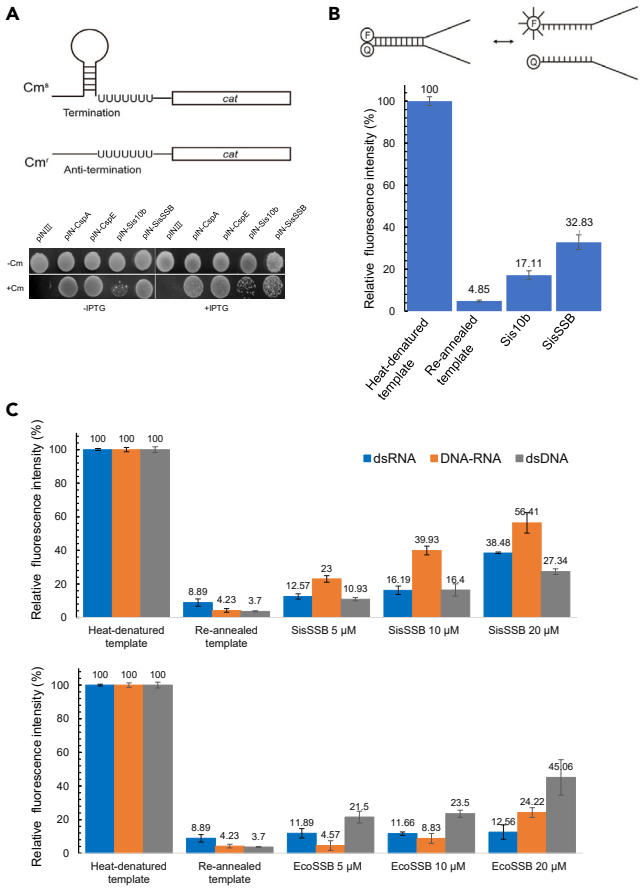

Figure 4. SisSSB has RNA chaperon activity *in vivo* and *in vitro*

(A) SisSSB has antitermination activity in *E. coli*. Upper panel, schematic of the *in vivo* antitermination assay using *E. coli* strain RL211. A chloramphenicol resistance gene *cat* cassette located downstream of the *trpL* terminators is used as a reporter. The hairpin RNA structure terminates the expression while its unwinding allows expression of the reporter. Lower panel, spot assay showing the antitermination activity of SisSSB. Cells were transformed with empty pINIII vector or pINIII carrying *cspA*, *cspE*, *Sis10b*, or *SisSSB* were cultured. The cultures were taken and adjusted to an OD_{600} with the medium. Aliquot (6 μL) of each culture was plated onto an LB plate containing 100 μg/mL ampicillin, with or without 30 μg/mL chloramphenicol (+/−Cm) or 0.2 mM IPTG (+/−IPTG).

(B) SisSSB is able to melt dsRNA. Upper panel, schematic showing the substrate and melted products. Lower panel, quantification of melting activity of SisSSB. The FAM-labeled ssRNA was defined as 100% and annealed dsRNA template was used as a negative control. Sis10b was used as a positive control. The values were calculated based on three replicates. Error bars indicated the standard deviation.

(C) Analysis of the melting activity on dsRNA, RNA-DNA hybrid, and dsDNA of SisSSB (upper panel) and EcoSSB (lower panel). The protein concentrations are 5, 10, and 20 μM, respectively. The values were calculated based on three replicates. See also Figure S4.

To further characterize the activities of SisSSB, we analyzed and compared the melting activity of SisSSB on dsRNA, RNA-DNA hybrid, and dsDNA with increasing concentrations of the protein. As shown in Figure 4C, the capacity of dsRNA unwinding was stronger than that of dsDNA. In contrast, EcoSSB had the optimal unwinding capacity on dsDNA (Figure 4C). Intriguingly, SisSSB, but not EcoSSB, exhibited the highest activity on DNA-RNA hybrid among the three substrates (Figure 4C). The difference in the unwinding activity on different substrates suggests that cellular roles of the two SSBs are different.

The OB-fold domain of SisSSB can complement *E. coli* Csps

To further analyze the RNA chaperone activity of SisSSB, we used a cold-sensitive *E. coli* strain BX04 in which the genes coding for the Csps (CspA, CspB, CspE, and CspG) were all deleted.[38,39] Plasmids pIN carrying IPTG-inducible CspE, Sis10b, SisSSB, or SisSSB mutants (Figure 5A) were transformed into BX04 and the transformants were grown in LB containing 100 μg/mL ampicillin. The cultures were diluted in gradience and spotted onto plates containing 100 μg/mL ampicillin with or without 0.2 mM IPTG. The plates were incubated for 3–5 days at 37°C and 22°C, respectively. As shown in Figures 5B and S5A, strain BX04 harboring the CspE overexpression plasmid exhibited an optimal growth at 22°C, while BX04 with empty pIN plasmid was sensitive to low temperature. Interestingly, cells expressing SisSSB ΔC29 containing the OB-fold and its C-terminal-structured segment showed similar growth to those expressing CspE at 22°C, while cells expressing SisSSB ΔC53 grew

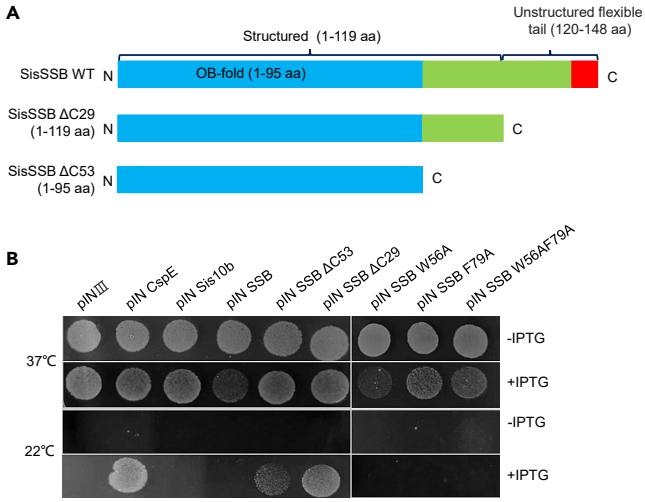

Figure 5. The OB-fold domain of SisSSB is able to complement the loss of CSP in *E coli*

(A) Schematic showing the domain structure of SisSSB and its C-terminal truncated mutants ΔC29 and ΔC53. The OB-fold was indicated in blue and the C terminal in green. The C-terminal tail that contains 8 acidic amino acids was indicated in red.

(B) Dot assay showing growth of strains at 37°C and 22°C, respectively, in the presence or absence of IPTG. *E. coli* BX04 cells carrying the pINIII plasmids for the expression of SisSSB and its C-terminal truncated or binding site deficient mutants were cultured and adjusted to an OD_{600} of 1.0 with LB medium containing 100 μg/mL ampicillin, serially diluted in 10-fold, and spotted onto LB plates supplemented with ampicillin and with or without IPTG. The plates were incubated for 2–5 days at 37°C and 22°C, respectively. See also Figure S5.

worse than those harboring plasmid expressing CspE or SisSSBΔC29 but better than those carrying the empty plasmid. Intriguingly, the growth of BX04 cells carrying plasmid overexpressing SisSSB(W56A), SisSSB(F79A), or SisSSB(W56AF79A) was impaired at 37°C and 22°C, similar to that overexpressing the wild-type SSB (Figures 5B and S5). EMSA assays showed that the two C-truncated mutants ΔC53 and ΔC29 of SisSSB have nearly the same nucleic acid-binding capacity as the wild-type SisSSB (Figure S5B). Additionally, we analyzed the nucleotide-binding activity of aromatic amino acid residue mutants W56A, F79A, and W56AF79A and found that the binding ability on ssDNA and ssRNA was all reduced (Figure S5B), in agreement with a previous report on SsoSSB.[16] The results demonstrate that the N-terminal OB-fold domain can function as Csp to destabilize the RNA secondary structures at low temperature. We assume that SisSSB having the C-terminal flexible tail rich in negatively charged residues of SisSSB is harmful to *E. coli* and this effect seems to be independent of ssDNA binding.

Overexpression of SisSSB leads to cell cycle elongation

To further understand the *in vivo* function of SisSSB, we attempted to overexpress SisSSB in *S. islandicus* REY15A. First, we tried to construct an overexpression strain carrying the plasmid pSeSD-SisSSB (no tag), but failed to obtain any colony after several attempts. However, we successfully constructed a strain with the native *Sisssb* promoter region (200 bp upstream of the start codon of *sire_0161*, Figure S1B) in the genome being replaced with an arabinose-inducible promoter (*ParaS*-SD) by the CRISPR-Cas-based genome-editing method.[25] The substitution was confirmed by PCR (Figure S1C) and western blotting. As shown in Figures 6A and 6B, compared with E233S, the promoter substitution strain *Para::ssb* exhibited a low SSB expression (0.32-fold) in the sucrose-containing medium STVU and an elevated SSB protein level (2.11-fold) in the arabinose-containing medium. Interestingly, in the sucrose-containing medium, the strain grew almost the same as E233S (Figure S1D) at 75°C. However, in the arabinose-containing medium, the growth was significantly retardant (Figure 6C). The results indicate that although reduction of *ssb* expression has no apparent effect on the cell growth, the elevation of the expression by only about 2-folds has a drastic effect on the cell growth. On the other hand, overexpression of SSB using the pSeSD-based plasmid is lethal for the cells, perhaps due to even higher SSB expression. The results also indicate that the cellular function of SisSSB is different from that of EcoSSB.

In *E. coli*, it was reported that EcoSSB binds to ssDNA at the replication fork in different modes depending on the concentration of cations and the ratio of SSB/ssDNA.[42,43] EcoSSB interacts with replication proteins during DNA replication, such as DNA polymerase χ subunit and primase in the replisome.[44,45] We assumed that the growth inhibition caused by SisSSB overexpression might be due to SSB/ssDNA ratio change or interaction with DNA replication proteins in the replisome, so that it may take a longer time for the DNA synthesis in the S period. To clarify this, cell cycle synchronization was performed to examine whether DNA replication was impaired under the elevated SSB level. As shown in Figure 6D, for the wild type, the peak of 1C was observed at 2.5 h, and it took about 3.5 h for the appearance of 2C peak (from 2.5 h to 6 h) in the arabinose medium. In contrast, the 1C peak in the SSB overexpression strain appeared at 5.5 h, which was about 2 h later than that of E233S. It took about 6.5 h from the appearance of 1C to that of 2C (from 5.5 to 12 h). These results show that although elongated S period (about two-time long) occurred in the overexpression strain, the whole cell cycle period also doubled (6 h:12 h) (Figure 6E). This suggests that SSB overexpression affects the cell cycle globally, not just the DNA synthesis. The previously described results reinforce that SSB is not a DNA

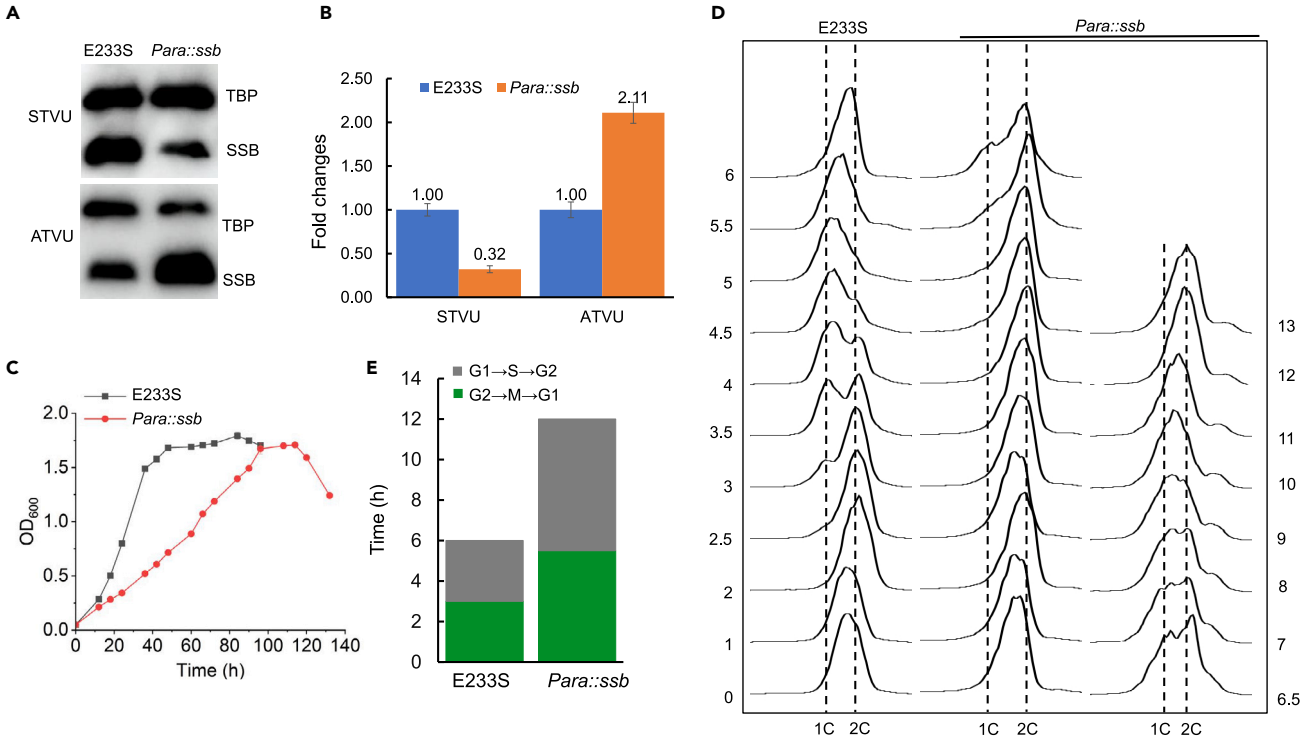

Figure 6. Overexpression of SisSSB has global effect on cell cycle progression and growth

(A) Assay of the expression levels in the promoter replacement strain *Para::ssb* under non-inducible and inducible conditions by western blotting. Cells were cultured in liquid STVU or ATVU medium and collected at OD_{600} 0.5–0.8. The cells were then disrupted by sonication and the cell lysates were subjected to SDS-PAGE and western blotting analysis with antibodies against SisSSB and SisTBP at the same time.

(B) Quantitative analysis of the results in (A). The values were calculated based on three replicates. Error bars indicated the standard deviation.

(C) Growth curves of the promoter substitution strain in comparison with the wild-type E233S. The cells were cultured in liquid ATVU medium with an initial OD_{600} 0.05 with shaking (110 rpm) at 75°C. OD_{600} was monitored at 6 or 12 h interval. The values were based on measurements of three biological replicates.

(D) Comparison of the cytometry profiles of the synchronized E233S and the promoter substitution stain. The cells were cultured in TSVU medium until the OD_{600} reached 0.15–0.2 when acetic acid (6 mM) was added into the culture. After cultured for 3 h, 0.2% D-arabinose was added to induce the expression of SisSSB. After incubation for further 6 h, samples were taken at different time points (0, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10, 11, and 12 h) and subjected to the flow cytometry analysis. "1C" and "2C" indicate the cells containing one and two copies of chromosomes, respectively.

(E) Comparison of the cell cycle periods of E233S and the SisSSB overexpression strain according to (D). G2→M→G1 (green), from the cell cycle release to 1C appearance; G1→S→G2 (gray): 1C appearance to 2C peak. See also Figure S1.

replication factor and suggest that the RNA chaperon function of SSB is different from bacterial Csps, the expression of which is induced in great magnitude and overexpression of Csps does not have detrimental or even lethal effect.

Transcriptomic analysis of the Δ*ssb* and Δ*dbp* and identification of the ssDNA interactome

To further probe the *in vivo* function and relationship of SisSSB and SisDBP at transcription level, we performed transcriptomic analysis and analyzed the global gene expression change caused by the deletion of *ssb* or *dbp*. Compared with E233S, 44 genes were upregulated (>2-folds) in Δ*ssb*, while only 3 of them are related to DNA replication process after DNA damage (Table 1; Table S3). Three genes encoding SiRe_0614, SiRe_0615, and SiRe_0616 were upregulated 2.27- to 3.25-folds after *ssb* deletion (Table 1). Among them, Dpo2 (SiRe_0615) has been identified as a functional eukaryotic pol zeta homolog responsible for DNA damage tolerance and plays an important role in archaeal DNA damage repair.[46,47] SiRe_0614 and SiRe_0616 are well conserved in Sulfolobales genomes at the *dpo2* gene locus,[48] and it was proposed that they code for Dpo2-associated factors and work in concert with Dpo2 in DNA translesion synthesis.[46] Besides, 13 genes associated with DDR were also upregulated, including Tfb3 (SiRe_1717) which is a transcription regulator of DDR, 3 genes involved in DNA transfer (SiRe_1316, SiRe_1879, and SiRe_1881), and the rest 9 genes which are Orc1-2-dependent NQO-inducible genes.[30] The rest 28 up-regulated genes and 18 downregulated genes were mostly related to cell metabolism (Table S3). In contrast, only 3 genes were upregulated and 5 genes were downregulated in Δ*dbp* (Table S3), none of them seems to be involved in DNA metabolism. These data suggest that SisSSB is involved in the protection of ssDNA. Deletion of *ssb* may lead to more ssDNA generation which could ignite DNA damage response. On the other hand, the data on Δ*dbp* do not give a clue to the function of SisDBP. In addition, it seems that deletion of *Sisssb* and *Sisdbp* did not affect each other at the transcription level, and transcription of one is not affected by deletion of the other.

Table 1. Changes of the transcription levels of genes involved in DNA replication and DNA damage response due to *ssb* deletion

Gene_ID	Fold change[a] (Δ*ssb* vs.E233S)	Description
DNA replication		
SiRe_0614	3.26	Uncharacterized protein associated with inactivated Dpo2
SiRe_0615	2.31	Dpo2
SiRe_0616	2.27	RecA/RadA recombinase, inactivated
Transcription factor		
SiRe_1717	2.52	TFB3
DNA transfer		
SiRe_1316	2.20	CedA1
SiRe_1879	2.48	UpsE
SiRe_1881	4.79	UpsA
Putative DDR related		
SiRe_0020	2.34	Uncharacterized protein
SiRe_0137	4.94	MFS family permease
SiRe_0187	4.08	Uncharacterized protein
SiRe_0589	3.47	Adenine-specific DNA methylase containing a Zn-ribbon
SiRe_0670	8.67	SWIM Zn-finger
SiRe_0269	3.85	Uncharacterized protein
SiRe_1957	3.76	Uncharacterized protein
SiRe_2100	6.09	Membrane protein involved in DNA uptake
SiRe_2101	2.01	Acyl-CoA dehydrogenase

[a]Genes with ≥2-fold change and p value ≤0.05 were listed. The ratios were based on the data from three independent repeats.

Since none of the proteins was found potentially capable of complementing the DNA-binding function of SSB through transcriptomic analysis, we then attempted to identify putative ssDNA-binding proteins by *in vitro* pull-down using ssDNA as a bait and subsequent mass spectrometric (MS) analysis. We followed a strategy reported by Paytubi et al.[17] A 5′-biotinylated 45 nt ssDNA was used as the bait. The ssDNA was bound to the agarose beads via the biotin-streptavidin interaction. The beads were incubated with the cell extracts of E233S, Δ*ssb*, and Δ*dbp*Δ*ssb* for 2 h at 55°C to prey the putative ssDNA-interacting proteins. The pull-down proteins were identified by western blotting and MS analysis. As shown in Figures 7A, 7SisSSB was a dominant protein in the ssDNA interactome from the E233S cell lysate. Unexpectedly, in the samples of Δ*ssb* and Δ*dbp*Δ*ssb*, there is no protein that had similar abundance as SSB pulled with ssDNA, although SisDBP appeared to increase in cell extracts. In addition to SisSSB and SisDBP, NusA (SiRe_1772) and an ATP-cone-containing protein (SiRe_2062) were also identified in all the samples, but not at a comparable level with that of SisSSB (Figure 7A). As shown in Table S4, SisSSB had the highest score from sample of E233S in the identified proteins, while SiRe_1772 and SiRe_2062 also had high scores in both Δ*ssb* and Δ*dbp*Δ*ssb*. However, proteins with ssDNA-binding activities, such as Sul7s,[49] RadA,[50] and GINS, did not appear in the ssDNA interactome even in Δ*ssb* and Δ*dbp*Δ*ssb* (Table S4). The results suggest that these proteins are probably not able to complement the ssDNA-binding function of SisSSB.

It was estimated that SsoSSB and ThermoDBP accounts for 0.08%–0.16% and 0.07%–0.13% of the total soluble protein of *S. solfataricus* and *Thermoproteus tenax*, respectively.[17] Consistently, SisSSB and SisDBP were estimated as 0.07%–0.12% and less than 0.01% of the *S. islandicus* proteins, respectively (Figure 7B). The fact that there is no significant increase of SisDBP in Δ*ssb* suggests that SisDBP is unable to displace SisSSB as an ssDNA-binding protein.

ssb deletion did not affect genome stability

To find more evidence that SSB does not participates in DNA replication, genome sequencing of Δ*ssb* and Δ*dbp*Δ*ssb* was performed. The retardant growth of Δ*ssb* at lower temperature could be due to replication defect lacking SSB at the replication fork. In *Saccharomyces cerevisiae*, deletion of the gene encoding Rtt105, an RPA chaperone which facilitates the nuclear localization of RPA and stimulates RPA loading at the replication forks,[51] led to deletions, duplications, and chromosome loss in genome DNA, which resembles replication slippage caused by the absence of pol32.[52] If SSB is involved in replication, growth of Δ*ssb* and Δ*dbp*Δ*ssb* at lower temperature may also result in genome instability. Strains Δ*ssb* and Δ*dbp*Δ*ssb* were grown at 55°C for 45 days in liquid STVU medium together with a reference strain Δ*ssb*::*ssb*. Average sequencing depth of Δ*ssb*::*ssb*, Δ*ssb*, and Δ*dbp*Δ*ssb* genome is 518, 493, and 464 magnification and the map rate of the three strains

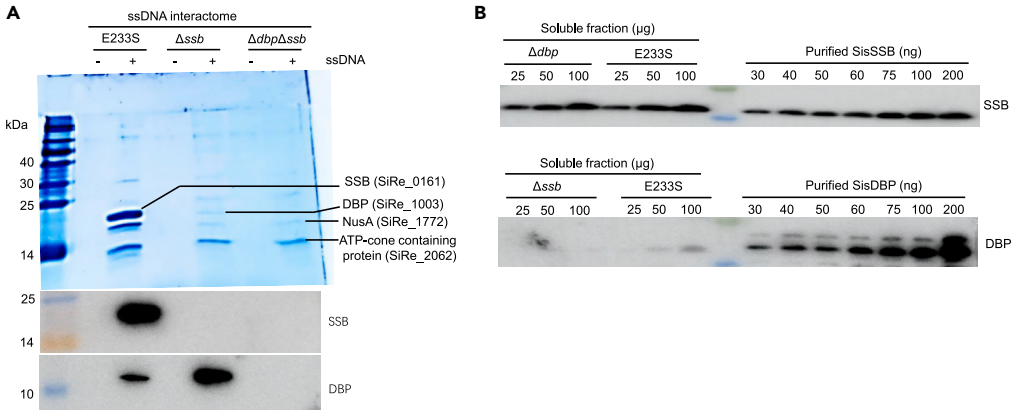

Figure 7. Identification of the ssDNA interactome and estimation of the cellular levels of SisSSB and SisDBP in E233S

(A) Identification of putative ssDNA-binding proteins using cell extracts of E233S, Δ*ssb*, and Δ*dbp*Δ*ssb*. The soluble extracts were separated by SDS-PAGE and analyzed by western blotting with the antibodies against SisSSB and SisDBP. The gel was stained with Coomassie brilliant blue G250. "-": streptavidin beads without ssDNA; "+": streptavidin beads with ssDNA.

(B) Estimation of the cellular levels of SisSSB and SisDBP. The levels of SisSSB and SisDBP in the cell lysates were analyzed by SDS/PAGE and western blotting with SisSSB and SisDBP antibodies. Purified SisSSB and SisDBP proteins in gradient were used for the quantification. See also Table S4.

is 99.28%, 99.28%, and 98.52%, respectively. Compared to Δ*ssb*::*ssb* genome, only few mutations were detected in Δ*ssb* and Δ*dbp*Δ*ssb* genome (Table 2). Both Δ*ssb* and Δ*dbp*Δ*ssb* genomes contain 22 SNPs, but they are not exactly the same (Table 2; Table S5). For the insertion and deletion of small fragments (<50 bp), Δ*ssb* has no insertion and 1 deletion, Δ*dbp*Δ*ssb* has 1 insertion and 2 deletions, and one smaller deletion is the same with that in Δ*ssb* (Table S5). No large segment insertion was found in both Δ*ssb* and Δ*dbp*Δ*ssb* genomes, while 1 and 2 large segments deletions were found in Δ*ssb* and Δ*dbp*Δ*ssb*, respectively (Table S5), which are engineered gene knockouts. Because there are only few random mutations in Δ*ssb* and Δ*dbp*Δ*ssb* genome (Table 2), we speculate that the occurrence of these variations is independent of the deletion of *Sisssb* and *Sisdbp*. These results indicate that the genome integrity and high-fidelity DNA replication were not compromised in the absence of *ssb* and *dbp*. In addition, flow cytometry of the cells showed that the DNA contents of SSB-deficient strains have no significant difference with E233S or Δ*ssb*::*ssb* (Figure 2B). These data strongly support that SSB is not a player at the replisome.

The RNA chaperon activity of the SSBs is conserved among archaea which lack Csp homologs

Given that the SisSSB does not function in DNA replication but plays a role as an RNA chaperon and Csp, next we want to know whether the RNA chaperon and Csp function is conserved in archaea. For this, we performed bioinformatic and *in vitro* as well as *in vivo* analyses of SSB proteins from representative archaeal species. Based on amino acid sequence alignment, archaeal single OB-fold SSBs are grouped into three type, SSB-1, SSB-2, and SSB-3 (Figure S6A). SisSSB belongs to the SSB-1 type. We then analyzed the distribution of SSBs/RPA and Csps in the TACK superphyla of Archaea (Figure 8A). Interestingly, most of crenarchaeal species only have one type of SSB, SSB-1, and no other SSB/RPA or Csps (Figure 8A). To examine the functions of the SSB1 homologs, we selected four SSB1 homologs (Figure S6A) from species without bacterial Csp homologs (*S. acidocaldarius* DSM 639, *Acidianus hospitalis* W1, *Staphylothermus hellenicus* DSM 12710, and *Thermogladius calderae* 1633) and two SSB1 homologs from species having exclusively SSB1 and bacterial Csp homologs (*Candidatus Korarchaeota archaeon* isolate UWMA-0234 and *Candidatus Heimdallarchaeota archaeon* LC_2). As described previously, pIN plasmids containing these wild-type SSBs, SacSSB, AhoSSB, SheSSB, TcaSSB, EcoSSB, KorSSB, HemidallSSB, and their C-terminal truncated mutant SacSSBΔC28, SacSSBΔC51, AhoSSBΔC29, SheSSBΔC17, TcaSSBΔC16, and EcoSSBΔC33 (KorSSB and HemidallSSB do not have flexible tail at the C terminal, Figure S7A) were transformed into *E. coli* BX04 to determine their ability to complement the cold sensitivity of BX04. The results showed that SacSSBΔC51, AhoSSBΔC29, SheSSBΔC17, and TcaSSBΔC16 complemented the cold sensitivity of BX04 as SisSSBΔC29, while KorSSB and HemidallSSB did not, which is similar to that of *E. coli* SSB and *E. coli* SSBΔC33 (Figures 8B and S7B). On the other hand, heterologous expression of full length of SSBs including SacSSB, SheSSB, AhoSSB, and even SacSSBΔC28 is harmful to *E. coli*, similar to that of SisSSB. *In vitro*, SacSSB, AhoSSB, SheSSB, and TcaSSB were able to bind to ssDNA and ssRNA with almost same high affinity, but KorSSB and HemidallSSB have higher binding capacity for ssDNA than ssRNA like EcoSSB (Figure S6B). Those results suggest that in the archaea containing SSB1 but without bacterial Csp homolog, their SSB1 could act as Csps to destabilize RNA secondary structures at low temperatures and help cells adapt to environment temperature downshift. In contrast, the SSBs possibly lose their RNA chaperone activity when the cells obtained Csp homologs during evolution.

DISCUSSION

Although the gene encoding SisSSB was classified as essential in *S. islandicus* by genome-wide random transposon insertion and identification, it is proven non-essential in this study. The *ssb* and *ssb/dpb* knockout strains grow poorly on solid plates by dot assay (Figure S2G) and the colonies

Table 2. Numbers of mutation events in Δ*ssb* and Δ*dbp*Δ*ssb* at 55°C by genome sequence

	Δ*ssb*[a]	Δ*dbp*Δ*ssb*
SNP	22	22
Insertion(<50 bp)	0	1
Deletion(<50 bp)	1	2
Insertion(≥50 bp)	0	0
Deletion(≥50 bp)	1	2

[a]SisSSB *in situ* complementary strain Δ*ssb::ssb* is used as the reference.

of those strains are much smaller than those of E233S (data not shown). This may explain the disagreement between our result and result by the genome-wide identification. Our result is in agreement with that by Suzuki and Kurosawa[21] who also found that cells of *S. acidocaldarius* lacking *ssb* were viable and exhibited robust growth in liquid media. Considering the crenarchaeal SSBs displayed similar ssDNA/ssRNA-binding activity and *in vivo* phenotypes (Figures 5B and 8B; Figures S5 and S6B), we propose that all the crenarchaeal SSBs are not essential as a DNA replication and DNA recombination factor. Our global transcription and microscopic analyses on cell aggregation of Δ*ssb* indicate that one function of SisSSB is to protect ssDNA in the cells. Its absence may produce more ssDNA, leading to ignition of the DDR network.

We have discovered another function of SSB, which is for the cells to adapt to ambient temperature shift. The protein level of SisSSB was upregulated at lower temperature, which is like the bacterial Csps, albeit with smaller magnitude (Figures 3A and 3B). SisSSB exhibits anti-transcriptional termination ability as Csps (Figure 4A) and is able to complement the loss of Csp in the cold-sensitive strain (Figure 5B; Figure S5B). In bacteria, expression of Csps is regulated at the post-transcriptional level through thermosensitive RNA elements, which are always located at the 5′ untranslated region (UTR) of mRNA.[53,54] However, the upregulation mechanism of SisSSB to adapt temperature downshift needs further exploration.

Sac10b is an abundant protein in *Saccharolobus* cells[55] and its homolog from *S. islandicus* Sis10b was confirmed as an RNA chaperone. However, how Sis10b functions *in vivo* is not clear. Genetic analysis suggested that Sis10b is an essential protein, as its knockdown caused reduced growth.[40] In our study, SisSSB exhibited a stronger *in vitro* RNA unwinding activity (Figure 4B) and anti-transcription termination in *E. coli* RL211 than Sis10b (Figure 4A). In addition, overexpression of Sis10b did not affect the growth of *E. coli* BX04 or rescue BX04 to grow at cold temperature. We think that Sis10b functions as a global RNA protector, while one of important roles of SisSSB is endowing the cells with adaption to the ambient temperatures downshift.

Archaeal TRAM proteins function as Csp via its RNA chaperone activity.[39] However, TRAM is not well conserved in Crenarchaeota (Figure 8A) and whether TRAM functions as Csp in Crenarchaeota remains unknown. Though TRAM homolog was found in *T. calderae* 1633, TcaSSB binds to ssRNA with high affinity and the N terminal of TcaSSB complements the *E. coli* Csps like SisSSB (Figure S6B; Figures 8A and 8B). In addition, bacterial Csp and archaeal TRAM homologs are present simultaneously in some archaea species (Figure 8A); it is not clear how these proteins are functionally distinguished.

An interesting feature for crenarchaeal SSB is that overexpression of SisSSB in *S. islandicus* REY15A caused a significant retardant growth, which is sharply different from bacterial Csps. We found the overexpression of SisSSB affects the cell cycle progression in a global manner (Figure 6D). In addition, SisSSB exhibited higher unwinding capacity of DNA/RNA hybrid than that of dsRNA and dsDNA in different protein concentration *in vitro* (Figure 4C); by contrast, *E. coli* SSB showed more effective ability to unwind dsDNA than dsRNA/DNA-RNA hybrid (Figure 4C). This strongly suggests that SisSSB may participate in the process that produces DNA-RNA hybrids, such as transcription. A previous investigation showed that SsoSSB had physical and functional interaction with RNA polymerase and it rescues transcription repression by reconstituted chromatin *in vitro*.[56] We assume that overexpression of SisSSB leads to a global transcriptional effect which results in retardant growth. However, the exact nucleic acid substrates and the roles of SisSSB in transcription or other processes *in vivo* also need further investigation.

The levels of bacterial Csps increased from 3 to 50 times at low temperature[38,54] and decreased after the cells adapted to the low temperature.[35,57] In *S. islandicus* REY15A, the SisSSB was upregulated only about 1.8 times at 55°C. In addition, the overexpression of SisSSB causes serious growth retardance even lethal at normal growth temperature. Thirdly, the basic level of SisSSB in REY15A is high (Figure 7B). These properties of SisSSB differ apparently from those of bacterial Csps which suggest that SisSSB participates in other nucleic acid processing in addition to melting RNA secondary structure. In bacteria, a common way, 5′ UTR of Csps mRNA works as RNA thermoswitches and adopts alternative RNA conformation at normal/lower temperature to regulate the expression of bacterial Csps at the post-transcriptional level.[54,57] While how SisSSB was regulated at lower temperature needs further investigation.

OB-fold-containing SSBs play vital roles in DNA replication, HRR, DDR, DNA damage repair, and genomic stability in almost all living organism.[50,58–61] Our genetic analysis reveals that SisSSB plays minor role if any in these processes. A recent study showed that deletion of the gene encoding SacSSB caused strain DP-5 sensitive to DNA damage agents, e.g., cisplatin, metronidazole, and 4-NQO. In addition, the HRR frequency of DP-5 decreased and the loss of SacSSB resulted in a 29-fold higher mutation rate at low growth temperatures.[62] This is different from our study in *S. islandicus* REY15A. After the deletion of *Sisssb*, we could easily knockout *Sisdbp* coding gene by CRISPR-based genome editing. In this method, the target site is identified and cleaved by the CRISPR-Cas complex in *S. islandicus* REY15A, causing the DNA double-strand break which is repaired through homologous recombination.[25,30] The easiness in getting double-knockout strain Δ*dbp*Δ*ssb* from Δ*ssb* strain suggests that homologous recombination is independent of SisSSB. The reason behind the differences between ours and the recent

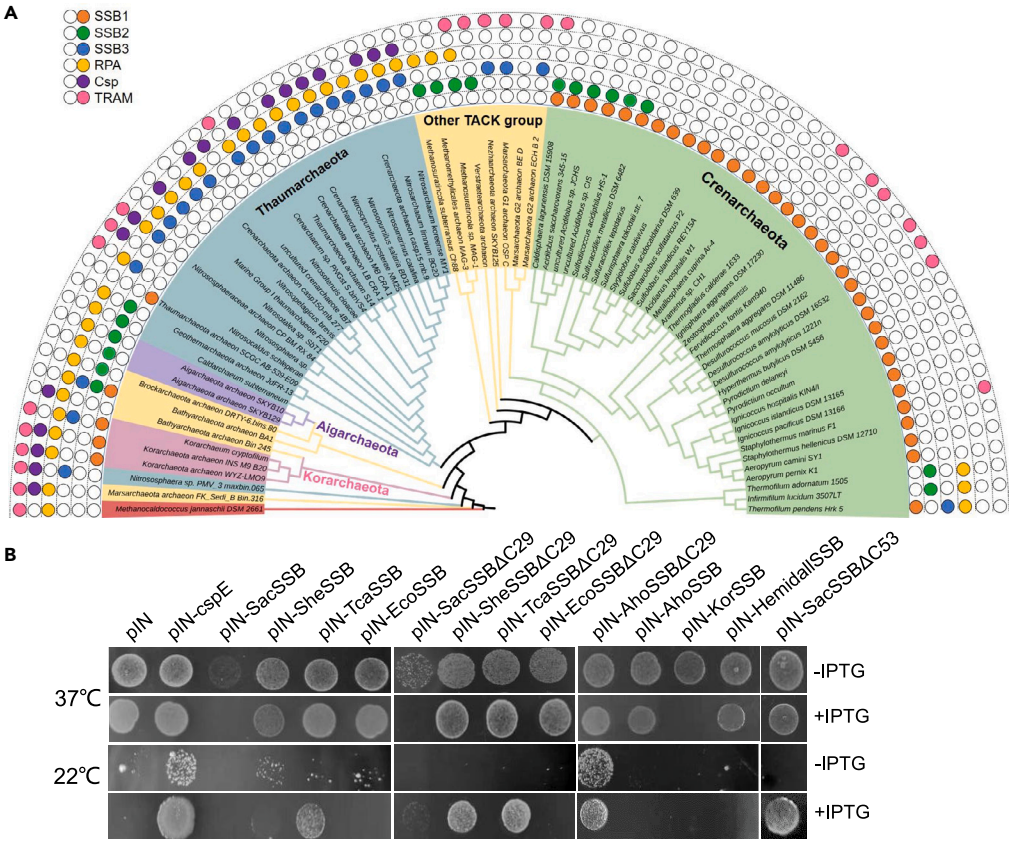

Figure 8. The RNA chaperon activity is conserved among crenarchaeal SSBs

(A) Distribution of SSB, RPA, bacterial CSP homolog, and TRAM in the TACK superphyla. The phylogenetic tree was constructed using the maximum likelihood method based on 16S rDNA sequences with bootstrap of 1000. Totally 77 archaeal species were selected in which 76 species were from TACK and *Methanococcus jannaschii* was selected as an outgroup. The colored balls indicate species having the indicated coding proteins while the empty cycles indicate species with no identified homolog.

(B) Dot assay showing that the N-terminal OB-fold domains of SheSSB, TcaSSB, SacSSB, and AhoSSB were able to complement the cold sensitivity of *E. coli* BX04. The procedure was the same as that in Figure 5B. See also Figures S6 and S7.

report on *S. acidocaldarius* SSB is not clear. It could be due to difference between two microbes. For example, *S. acidocaldarius* differs from *S. islandicus* in cell division; deletion of CdvB3 led to retardant growth in *S. acidocaldarius*,[63] while this deletion had no effect on the growth of *S. islandicus*[64]; CdvB2 is essential for *S. islandicus*,[64] while it could be deleted in *S. acidocaldarius*[65]

SSBs have been considered as an essential component of DNA replisome.[66,67] In addition, the interactomes of bacterial SSB and eukaryotic RPA contain numerous DNA replication proteins.[8–10,68] Until now, mini chromosome maintenance protein in the replisome of *S. solfataricus* is the only interacting protein identified of Crenarchaeota SSB.[69] In this study, we found that SisSSB is not required for the DNA replication in *S. islandicus* REY15A. One possibility is that there is un-identified SSB function in the replisome. Alternatively, the crenarchaeal cell may not require an SSB for DNA replication. Based on the comparison of DNA polymerases, the DNA replication machinery of Crenarchaeota archaea is reported unique.[70] Our study may raise an intriguing question about how the crenarchaeal cells maintain faithful replication of the genome in the absence of SSB. In addition, since SisSSB exhibits the strongest unwinding activity on RNA/DNA and approximately 2-fold overexpression causes global cell cycle elongation, we assume that SisSSB also functions in transcription process. Study on the structure and reconstruction of the replisome from Crenarchaea as well as the cellular nucleic acid targets will be promising subjects for further investigation.

Limitations of the study

The cellular nucleic acid targets have not been identified. The activities of other type archaeal SSBs have not been investigated. These issues need further studies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108389.

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AUTHOR CONTRIBUTIONS

Y.X., Q.H., and Y.S. conceived and designed the research and prepared this manuscript; Y.X. and Z.J. carried out most of the experiments; M.Z. and P.W. helped in bioinformatic analysis; X.Z. helped in protein purification and EMSA assay; Q.G. and Y.Y. helped in cell cycle synchronization and data analysis; Q.S. gave critical suggestions on experiments and manuscript preparation; X.F. helped in genome sequence analysis and RNA-binding assay; J.N. helped in transcriptomic analysis; and X.D. helped in cold-sensitive assays. All authors edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-SisSSB Rabbit polyclonal antibody	This paper	N/A
Anti-SisDBP Mouse polyclonal antibody	This study	N/A
Anti-SisTBP Rabbit polyclonal antibody	This study	N/A
Anti-His Mouse Monoclonal Antibody	Transgen	M20105
Goat Anti-Rabbit IgG (H + L)	Transgen	R10327
Goat Anti-Mouse IgG (H + L)	Transgen	R10818
Bacterial and virus strains		
S. islandicus REY15A(E233S)(Δ*pyrEF*Δ*lacS*)	Laboratory self-storage	*Liu* et al.[26]
Δ*ssb*	This paper	N/A
Δ*dbp*	This paper	N/A
Δ*dbp*Δ*ssb*	This paper	N/A
Para::ssb	This paper	N/A
E.coli RL211	Prof. Li Huang Laboratory	*Zhang* et al.[40]
E.coli BX04	Prof. Xiuzhu Dong Laboratory	*Zhang* et al.[39]
Chemicals, peptides, and recombinant proteins		
C-6×his-EcoSSB	This paper	N/A
C-6×his-KorSSB	This paper	N/A
C-6×his-HemidallSSB	This paper	N/A
Deposited data		
Genome sequencing data	This paper	Genebank: PRJNA982601(https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA982601%20)
RNA sequencing data	This paper	Genebank: GSE234768(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE234768)
Oligonucleotides		
See Table S2		

RESOURCE AVAILABILITY

Lead contact

Further information should be directed to and will be fulfilled by the Lead Contact, Yulong Shen (yulgshen@sdu.edu.cn).

Materials availability

All materials reported in this paper will be shared by Yulong Shen (yulgshen@sdu.edu.cn) upon request.

Data and code availability

All data supporting the findings of this study are available within the article and its Supplementary Information, or from the corresponding author upon reasonable request. The genome DNA sequences and RNA-seq data were deposited at the National Center for Biotechnology Information with the accession numbers SRA: PRJNA982601 and GEO: GSE234768, respectively. All the datasets are publicly accessible.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Growth conditions for *Saccharolobus islandicus* REY15A

S. islandicus REY15A(Δ*pyrEF*Δ*lacS*) (named E233S for simplicity) was grown in STVU medium containing mineral salt, 0.2% (w/v) sucrose (S), 0.2% (w/v) tryptone (T), 0.01% (w/v) uracil and a mixed vitamin solution (V). The sucrose (S) is replaced with D-arabinose (A) to make ATVU medium if needed. The medium was adjusted to pH 3.3 with sulfuric acid, as described previously. Normally, cells were cultured aerobically at 75°C with shaking (145 rpm) to OD_{600} = 0.6–0.8, then transferred to fresh medium with an initial estimated OD_{600} of 0.05 for follow-up growth curve determination.

METHOD DETAILS

Genetic manipulation

The genes encoding SisSSB(SiRe_0161) and SisDBP(SiRe_1003) were knocked out using the endogenous CRISPR-based genome editing system in *S. islandicus* REY15A.[25] Firstly, the genomic DNA of E233S was extracted using a bacterial genome rapid extraction kit (SparkJade Co., Shandong, China) and used as the template for high fidelity and SOE (splicing overlap extension) PCR by ApexHF HS DNA polymerase (Accurate Biotechnology Co., Hunan, China). The spacer consisting of a 5′-CCN-3′ downstream of a 40-nt DNA sequence was selected on the target gene for making of the artificial CRISPR array in the pGE plasmid. The donor DNA and spacer fragments were ligated to the pGE plasmid digested with SphI/XhoI and BspQI, respectively. The recombinant pGE plasmid was introduced into the wild type E233S by electroporation. The genotype of single colonies obtained were determined by PCR amplification with the flanking/gene-specific primer pairs. The strains and plasmid constructed and used in this study are listed in Table S1 and the oligonucleotide sequences for PCR and the *in vitro* assay are listed in Table S2.

Western blotting

Antibodies against SisSSB and SisTBP were produced in rabbit using synthetic specific peptides (amino acids 135–148 RGGRRQENEEGEEE for SSB[SiRe_0161]; amino acids 18–31, SIPNIEYDPDQFPG for TBP[SiRe_1138]). Antibodies against DBP were produced in mouse using the full length DBP expressed in and purified from *E. coli*. To quantify the protein in the cell, the cells (estimated per 5×10^8) were harvested by centrifugation at 5,000 *g* for 10 min and suspended in 100 μL PBS buffer (pH7.4). Then the cells were disrupted by sonication. Total protein was obtained without any centrifugation and the soluble fraction was obtained by collecting the supernatant after centrifugation at 10, 000 *g* for 10 min and filtration with a 0.45 μM cut-off filter. Western blotting was carried out following the standard procedure. The antibodies against SisTBP (loading control) and SisSSB were incubated at the same time.

Transcriptomic analysis

The transcriptomic analysis was performed as previously described.[71,72] E233S, Δ*ssb*, and Δ*dbp* strains were grown at 75°C with an initial OD_{600} of 0.05 in STVU medium and the cells were harvested when the OD_{600} reached 0.4–0.5. Cells were pelleted at 6,000 *g* for 10 min. The pellet was washed in PBS, frozen with liquid nitrogen and transported on dry ice. The samples were analyzed by Beijing Novogene Bioinformatics Technology Co., Ltd (Beijing, China). Total RNA was extracted using the Trizol reagent (Ambion, Austin, TX, USA) and assessed by Aagilent 2100 bioanalyzer. The libraries were prepared using purified mRNA from total RNA in which the rRNA was removed with probes. The libraries were then sequenced using the Illumina NovaSeq 6000. Filtered high quality clean reads were mapped to the reference genome sequence of *S. islandicus* REY15A.[18] Fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) was used to estimate the gene expression levels, and differential expression analysis (Δ*ssb* versus E233S and Δ*dbp* versus E233S) was performed using the DESeq R package (1.18.0). p values were adjusted using the Benjamini & Hochberg method. Corrected P-value of 0.005 and |log2(fold-change)| > 1 were set as the threshold as significantly differential expression.

Detection of the ssDNA interactome

The method to detect the ssDNA interactome in *S. islandicus* was according to a previous study.[17] Cells were cultured at 75°C to OD_{600} = 0.5–0.8 in STVU medium and collected by centrifugation at 5,000 *g* for 10 min. The pellet was washed with PBS, then resuspended in TEDG buffer (50 mM Tri-HCl pH8.0, 5 mM EDTA Na_2, 1 mM DTT, 5% glycerol) containing 150 mM NaCl. The cells were disrupted by sonication. After centrifugation (10, 000 *g* for 20 min), the supernatant was filtered with a 0.45 μm cut-off filter. Streptavidin-coated agarose beads 6FF (Smart-Lifesciences, Changzhou, China) were washed three times with NN buffer (20 mM NaH_2PO_4, 0.15 M NaCl , pH7.4), then 2.2 nmol (~31 μg) of 5′ biotined-45nt ssDNA was incubated with the beads with gently shaking at room temperature for 10 min. The beads were washed three times with NN buffer and mixed with 30 mg of the cell lysate. After the mixture was incubated at 55°C for 120 min, the beads were washed 3 times with the TEDG buffer containing 150 mM NaCl. Bound proteins were eluted with TEDG buffer containing 1500 mM NaCl. The eluate was TCA/acetone precipitated, resuspended in TEDG buffer, and run on a 15% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue G250 or silver. SisSSB and SisDBP were identified by Western blotting. Distinct protein bands were excised. The excised proteins as well as the whole interactomes were identified by mass spectrometry.

Flow cytometry

Cells were cultured in liquid STVU medium and estimated 3.0–5.0×10^7 cells were harvested and fixed with 70% cold ethanol overnight. The fixed cells were pelleted at 4°C, 800 *g* for 20 min. The cells were re-suspended and washed with 1 mL of PBS buffer. The cells were pelleted again, re-suspended in 100 μL of PBS buffer containing 50 μg/mL propidium iodide (PI), and incubated on ice for 30–60 min. The DNA content of the cells was analyzed using the ImageStreamX MarkII Quantitative imaging analysis for flow cytometry system (Merck Millipore, Germany). About 20,000 cells were collected for each sample and the data of the single cells were analyzed with the Flowjo software.

Cell cycle synchronization

Cells of different strains were synchronized as previously described.[26,72] Cells were grown at 75°C with shaking (145 rpm) in 30 mL STVU medium with an initial estimated OD_{600} of 0.045. Acetic acid (6 mM, final concentration) was added when the OD_{600} reached 0.15–0.2 and treated for 6 h. If needed, 0.2% D-arabinose was added after the addition of acetic acid. Then cells were centrifuged at 3,000 *g* for 10 min at room temperature and washed twice with 0.7% (w/v) sucrose to remove the acetic acid. Cells were suspended with 30 mL pre-warmed STVU medium and cultured as above.

Genome sequencing

Δssb*ssb*, Δ*ssb* and Δ*ssb*Δ*dbp* strains were first grown at 75°C and then transferred to 55°C with an initial OD_{600} of 0.05 for 45 days in liquid STVU medium, during which the culture medium was replaced with fresh medium for three times. The cells were pelleted by centrifugation at 6,000 *g* for 10 min and washed in PBS, frozen with liquid nitrogen and transported under cold conditions with dry ice. Genome sequencing analysis was performed by Novogene (Beijing, China). Briefly, the genomic DNA was extracted and then quantified by Qubit 2.0 Fluorometer (Thermo Scientific). Sequencing libraries were generated using NEBNext μLtra DNA Library Prep Kit for Illumina (NEB, USA) using 1.0 μg DNA per sample. The whole genome was sequenced using Illumina NovaSeq PE150. The sequenced data were filtered and the clean data were mapped to the reference genome sequence of *S. islandicus* REY15A[18] using BWA software with parameters are as follows: mem -t 4 -k 32 -M -R. SAMTOOLS software was used to count the coverage of the reference sequence to the reads and to explain the alignment results following the parameters: depth -d 200000. Single nucleotide polymorphism (SNP) and small fragments (<50bp) variation were e-detected by SAMTOOLS. The parameters were as follows: mpileup -m 2 -F 0.002 -d 10000 -u -L10000. Large segments fragments (≥50bp) variation was found by the BreakDancer software. The parameters were as follows: -q 20-day prefix. The obtained variations of Δ*ssb* and Δ*ssb*Δ*dbp* were compared with that of Δ*ssb*::*ssb* respectively, common mutations were removed and the specific variations in Δ*ssb* and Δ*ssb*Δ*dbp* genome were used to show the variations caused at lower temperature.

Anti-transcription termination assay in *E. coli*

To determine the RNA hairpin melting activity of SisSSB in a heterologous host, a recombinant plasmid pINIII carrying IPTG-inducible SSBs was transformed into *E. coli* RL211. The cells were grown to an OD_{600} of ~1.0 in LB medium with 100 μg/mL ampicillin. Aliquots (6 μL) of the culture were spotted onto LB plates containing 100 μg/mL ampicillin and 0.2 mM IPTG with or without 30 μg/mL chloramphenicol. The plates were incubated at 37°C for 2–3 days. *E. coli* RL211 carrying IPTG-inducible CspA, CspE or Sis10b or with an empty pINIII were used as positive and negative controls, respectively.

Protein purification

Gene fragments of SisSSB, SacSSB, SheSSB, TcaSSB, and EcoSSB and their mutants were obtained by PCR. Gene fragments of AhoSSB, KorSSB, HemidallSSB, and KorSSB were obtained by chemical synthesis after codon optimization. The SSB genes were then cloned into pET15bm plasmids with *Nde*I and *Sal*I restriction sites and transformed into *E. coli* BL21(DE3) CondonPlus-RIL. Cells were cultured at 37°C with 180 rpm shaking to OD_{600} = 0.4–0.6 in LB medium with 100 μg/mL ampicillin sodium and 34 μg/mL chloramphenicol. IPTG (0.3 mM) was added and the cells were cultured at 37°C for 5 h. The cells were harvested by centrifugation and resuspended in Buffer A (50 mM Tris-HCl, pH7.4, 200 mM NaCl, 5% glycerol) and lysed by sonication. The cells carrying no tag SisSSB, SacSSB, SheSSB, TcaSSB, and AhoSSB were disrupted by sonication. Cell extracts were heated for 20 min at 65°C and then centrifugation at 4°C, 10, 000 rpm for 20 min. The cell debris of strains carrying plasmid expressing C-His-tagged KorSSB, HemidallSSB, and Eco SSB was removed by centrifugation (without heat treatment). Polyethyleneimine (PEI) (0.5%, W/V) was added into the supernatant to remove the nucleic acids. The mixture was centrifuged at 10,000 *g* for 30 min at 4°C. The proteins were then precipitated with 0.6 g/mL $(NH_4)_2SO_4$ and collected by centrifugation under the conditions as above. The protein samples were then dissolved and dialyzed overnight in buffer A. The dialyzed mixture was centrifuged, and the supernatant was then filtered through a membrane filter (0.45 μm). The non-tagged proteins were purified by a cation exchange column (SP) and a Heparin column sequentially. The proteins were eluted in buffer B (50 mM Tris-HCl pH7.4, 1.5 M NaCl, 5% glycerol) with 0–100% salt gradience. The fractions were collected at 50%–70% salt gradience. The C-his SSBs samples were load into a Ni column pre-equilibrated with buffer A and the proteins were eluted in buffer A with 400 mM imidazole. For further purification, proteins were loaded into a size exclusion chromatography column Superdex 200 increase 10/300 (GE Healthcare). The protein concentration was determined by the Bradford method.

Unwinding assay

A 40 nt ssRNA (or ssDNA) FAM-labelled at the 5′ end was annealed to a 40 nt partial complementary ssRNA (of ssDNA) labeled at the 3′ end with a BHQ1 quenching at a molecular ratio of 1:1. Annealed nucleic acid products were 22 bp paired fragments (*Tm*: 59°C) at one end and 18 bp unpaired bases at another end. The wild-type SisSSB (20 μM) was incubated for 30 min at 37°C with 100 nM annealed substates in 200 mM Tris–HCl, pH 7.4, and 10 mM $MgCl_2$. Changes in fluorescence values were measured using a fluorescence spectrophotometer (ENSPIE-2300, PE, America) with a 460 nm excitation wavelength and a 515 nm emission wavelength.

Complementation of the cold-sensitive strain *E. coli* BX04

SSBs and their mutants were cloned into pINIII plasmids with *Nde*I and *Bam*HI restriction sites and transformed into *E. coli* BX04. Colonies were picked and cultured overnight in LB medium with 100 μg/mL ampicillin under 180 rpm shaking at 37°C. The overnight culture was transferred to fresh LB medium containing 100 μg/mL ampicillin with 1% inoculum and incubated for 3–5 h. Cultures were adjusted to OD_{600} 1.0 with fresh medium, diluted and spot on the LB plates with 100 μg/mL ampicillin, with or without 0.2 mM IPTG then incubated for 2–5 days at 37°C and 22°C, respectively.

Electrophoretic mobility shift assay (EMSA)

The 40 nt 5′-FAM-labelled ssDNA (ssDNA-FAM) and ssRNA (ssDNA-FAM) were used as the substrates to determine the binding capacity of SSBs. The binding assay was performed in a 20 μL reaction mixture containing 20 nM ssDNA/ssRNA, 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 20 mM NaCl, 50 μg/mL BSA, 1 mM DTT, 5% glycerol, and different concentrations of purified proteins (for ssRNA, 0.5 U/μL RNase inhibitor was added). The reaction mixture was incubated at 37°C for 30 min before loaded onto a 12% native polyacrylamide gel. After running in 0.5×TBE, the gel was visualized using an Amersham ImageQuant 800 biomolecular imager (Cytiva).

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of EMSA and western blotting was performed by using online ImageJ.JS (https://ij.imjoy.io/), statistical data analysis was performed by T-test. All the error bars represent the standard deviation.