Soj/ParA stalls DNA replication by inhibiting helix formation of the initiator protein DnaA

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Control of DNA replication initiation is essential for normal cell growth. A unifying characteristic of DNA replication initiator proteins across the kingdoms of life is their distinctive AAA+ nucleotide-binding domains. The bacterial initiator DnaA assembles into a right-handed helical oligomer built upon interactions between neighbouring AAA+ domains, that in vitro stretches DNA to promote replication origin opening. The Bacillus subtilis protein Soj/ParA has previously been shown to regulate DnaA-dependent DNA replication initiation; however, the mechanism underlying this control was unknown. Here, we report that Soj directly interacts with the AAA+ domain of DnaA and specifically regulates DnaA helix assembly. We also provide critical biochemical evidence indicating that DnaA assembles into a helical oligomer in vivo and that the frequency of replication initiation correlates with the extent of DnaA oligomer formation. This work defines a significant new regulatory mechanism for the control of DNA replication initiation in bacteria.

Introduction

Successful replication and segregation of genetic information prior to cell division is essential for all living organisms. Loss of replication control can dramatically reduce an organism’s competitiveness in its environment, and in extreme cases can lead to unchecked cell proliferation or cell death. Throughout the kingdoms of life, chromosome duplication is instigated by DNA replication initiator protein complexes (Mott and Berger, 2007; Wigley, 2009; Kawakami and Katayama, 2010). A unifying characteristic of initiator proteins is their AAA+ nucleotide-binding domain, which is critical for their structure and function (Tucker and Sallai, 2007; Kawakami and Katayama, 2010).

Bacterial chromosomes are typically replicated bi-directionally from a single origin (oriC); an event orchestrated by the multi-domain initiator protein DnaA (Supplementary Figure S1; for review see Mott and Berger, 2007; Leonard and Grimwade, 2010). At the C-terminus, domain IV contains the helix-turn-helix and basic loop motifs required for specific double-stranded DNA-binding activity (Erzberger et al, 2002; Fujikawa et al, 2003). Domain III contains the AAA+ motif involved in ATP binding and ATP hydrolysis, as well as residues required for coordinating single-stranded DNA (Erzberger et al, 2002; Ozaki et al, 2008; Duderstadt et al, 2011). Domain II is a poorly conserved flexible linker ( Abe et al, 2007; Molt et al, 2009) connecting domains III–IV to domain I, which acts as a hub for additional protein interactions and directs loading of the replicative helicase (Sutton et al, 1998).

Initiation of DNA replication in bacteria requires stepwise structural transitions, resulting in the assembly of DnaA into an active initiation complex (for reviews see Ozaki and Katayama, 2009; Leonard and Grimwade, 2010). Through domain IV, DnaA is thought to stably bind conserved nine basepair sequences (DnaA-boxes) in the oriC region throughout the cell cycle (Cassler et al, 1995). These founding DnaA proteins recruit further DnaA molecules onto neighbouring low-affinity binding sites via dimerization of domain I (Simmons et al, 2003; Miller et al, 2009). Additional ATP-bound DnaA proteins then assemble onto this platform to form a large nucleoprotein complex observable by electron microscopy as a particle wrapped in DNA (Funnell et al, 1987). This oligomeric structure may correspond to the right-handed helix, built via interactions between neighbouring AAA+ domains, which has been observed by X-ray crystallography (Carr and Kaguni, 2001; Erzberger et al, 2006). Amino-acid substitutions in DnaA that perturb helix formation in vitro inhibit replication origin unwinding in vitro and functionality in vivo (Duderstadt et al, 2010), and it has recently been proposed that the DnaA helix destabilizes an AT-rich sequence within the origin (the DNA unwinding element; DUE) by stretching one strand of the DNA duplex to promote origin opening (Duderstadt et al, 2011). This activity appears to be accompanied by a transition in DNA-binding modes from double-stranded to single-stranded; a result of domain IV engaging the AAA+ motif of a neighbouring monomer within the helical oligomer (Erzberger et al, 2006; Duderstadt et al, 2010). This compact helix is thought to continue onto the upper strand of the now single-stranded DUE via residues in domain III, stabilizing the DUE in its unwound state (Speck and Messer, 2001; Ozaki et al, 2008). Following open complex formation, DnaA directly recruits the replicative helicase onto the single-stranded DNA via interactions with domains I and III (Sutton et al, 1998; Abe et al, 2007). The remaining replisomal components are then recruited in a stepwise manner, which culminates in an active DNA replication complex.

There are several steps during initiation at which regulatory systems have been found to control bacterial DNA replication (for review see Katayama et al, 2010). DnaA binding to oriC can be inhibited either by protein occlusion (SeqA in Escherichia coli, Spo0A in Bacillus subtilis, and CtrA...
in Caulobacter crescentus), by spatial sequestration (YabA in B. subtilis), or by titration (datA in E. coli and DBCs in B. subtilis). DnaA assembly at oriC can be either stimulated (DnaA in E. coli and HobA in Helicobacter pylori) or repressed (SirA in B. subtilis) by the binding of regulatory proteins to domain 1. Lastly, DnaA is inactivated following replisome formation through the stimulation of its ATP hydrolysis activity (Hda in E. coli and C. crescentus).

In a previous study we identified the highly conserved ParA protein (Soj) as a novel regulator of DNA replication in B. subtilis (Murray and Errington, 2008). Soj is a Walker-type ATPase that forms an ATP-dependent sandwich dimer that can bind DNA (Leonard et al., 2005). We have shown that the monomeric Soj protein inhibits DnaA, while dimerization of Soj switches the protein into an activator of DnaA (Scholefield et al., 2011). These results indicate that Soj acts as a molecular switch to control DnaA activity, with its opposing regulatory activities being dictated by its quaternary state. Detailed biochemical characterization of Soj proteins has identified amino-acid substitutions that arrest Soj quaternary changes at different steps (Figure 1A; Leonard and Grimwade, 2005; Hester and Lutkenhaus, 2007; Scholefield et al., 2011). Two separate substitutions inhibit Soj dimerization: SojR189A is unable to bind ATP, while SojG12V can bind ATP but cannot dimerize due to a steric clash in the dimerization interface; both of these proteins inhibit DnaA activity. The SojR189A substitution allows ATP-dependent dimerization but disrupts DNA-binding activity: this mutant protein is relatively inert, presumably because DNA-binding activity is required for Soj to efficiently activate DnaA (Scholefield et al., 2011).

Here, we have investigated the negative regulation of DnaA by monomeric Soj. We have identified amino-acid substitutions in DnaA that render the protein insensitive to

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**Figure 1** Specific mutations in dnaA either bypass or suppress the inhibition of DNA replication initiation by SojG12V. (A) Pathway of the Soj activity cycle. (B) Point mutations in DnaA introduced by error-prone PCR were found to overcome the small colony phenotype characteristic of SojG12V overexpression. Strains were grown on NA plates in the presence or absence of 1% xylose to induce SojG12V overexpression. Strains were grown on NA plates in the presence or absence of 1% xylose to induce SojG12V overexpression.

- Soj inhibit DnaA
- SojG12V weakly activates DnaA
- ATP
- ADP
- 2 Pi
- sojG12V
- DnaA
- V323D
- L294R
- A341V
- L337P
- DnaA
- L294R
- A341V
- L337P
- DnaA
- V323D
- L294R
- A341V
- L337P
- DnaA

(C) The oriC-to-terminus ratios of dnaA point mutations generated using PCR mutagenesis were determined using MFA in the presence and absence of SojG12V overexpression (1% xylose). Suppressor mutations (red) were found to be recalcitrant to SojG12V activity. Cells were grown in LB medium at 30°C. Values were normalized to the oriC/ter ratio of the wild-type strain grown in the absence of xylose.

(D) The SojG12V suppressor mutations in dnaA perturb the formation of a Soj:DnaA–His12 complex in vivo. Cells were grown in LB medium at 30°C, crosslinked with formaldehyde, and the DnaA–His12 complexes were purified before the crosslinks were reversed and proteins were separated by SDS–PAGE. Soj and DnaA–His12 were detected by western blot analysis. The top panel shows the amount of Soj protein in the cell lysate (Input) and the bottom panel shows the amount of DnaA found in a complex with DnaA–His12 following purification (Complex).

(E) The amount of each DnaA–His12 protein was determined by western blot analysis. DivIVA was used as a loading control.
inhibition by monomeric Soj and that do not form a complex with Soj in vivo. Using these proteins we show that Soj directly interacts with DnaA in vitro. Importantly, we have developed a site-specific crosslinking assay that detects DnaA oligomers assembling on single-stranded and double-stranded DNA substrates, both of which appear to represent a helical conformation built upon the AAA + domains. Using this assay we show that monomeric Soj specifically inhibits DnaA helix formation in vitro. Furthermore, we adapted our site-specific crosslinking assay to demonstrate that (i) DnaA forms oligomers in vivo, (ii) monomeric Soj inhibits DnaA oligomerization in vivo, and (iii) the extent of DnaA oligomerization in vivo correlates with the rate of DNA replication initiation. Together, these results establish the DnaA helix as an important target for regulation, as well as providing critical biochemical evidence supporting the physiological relevance of DnaA helix formation during DNA replication initiation.

Results

Specific point mutations in dnaA disrupt Soj inhibition in vivo

Previously, we have shown that monomeric Soj inhibits DnaA activity and forms a complex with DnaA in vivo (Murray and Errington, 2008). However, it remained unclear whether this regulation was mediated by a direct interaction between the proteins. To address this question, we screened for mutations in dnaA that suppress the growth inhibition caused by overexpressing monomeric SojG12V (Figure 1B). A chloramphenicol marker was integrated downstream of the dnaAN operon and genomic DNA from this strain was used as a substrate for error-prone PCR to generate point mutations in dnaA. PCR products were transformed into a strain harbouring an inducible sojG12V allele and plated under SojG12V overexpression conditions, resulting in slow growth of wild-type colonies. Genomic DNA from large colonies was backcrossed into the parent strain to confirm that the mutations conferring fast growth was linked to dnaA. DNA sequencing identified 14 distinct mutations that caused single amino-acid substitutions within DnaA.

To characterize the mutations in dnaA, marker frequency analysis (MFA) was used to measure the relative levels of origin and terminus DNA, thereby generating a measure of DNA replication initiation frequency (Figure 1C). The mutations within dnaA fell into two classes: hypermorphs that bypassed SojG12V inhibition (DnaASup proteins) by having a high basal rate of initiation, and suppressors that had an approximately wild-type rate of initiation but were resistant to SojG12V inhibition (DnaAHyp proteins) by having a low basal rate of initiation, and suppressors that had an approximately wild-type rate of initiation but were resistant to SojG12V inhibition (DnaASup proteins: DnaAL294R, DnaA323D, DnaA337P, DnaA341V). The suppressor mutations were each independently cloned into dnaA and transformed into the SojG12V overexpression strain to demonstrate that they were responsible for the large colony phenotype. The resulting strains displayed rates of DNA replication initiation and DnaA expression levels similar to wild type in the presence or absence of SojG12V overexpression (Supplementary Figure S2).

The ability of Soj to form a complex with DnaA–His12 and DnaA323D, His12 proteins in vivo was investigated using nickel affinity purification following formaldehyde crosslinking. Compared with wild-type DnaA–His12, all four DnaA323D–His12 proteins were defective in their ability to form a complex with Soj (Figure 1D). Western blot analysis confirmed that all DnaA–His12 proteins were expressed to a similar level as wild type (Figure 1E). Taken together, the data indicate that these amino-acid substitutions in DnaA suppress SojG12V inhibition by disrupting DnaA–Soj complex formation.

Soj interacts directly with DnaA in vitro

To test whether Soj and DnaA directly interact, we purified several DnaA and Soj proteins and measured binding in vitro using surface plasmon resonance (SPR). B. subtilis DnaA lacks cysteine residues, allowing for the introduction of a C-terminal cysteine following a His$_3$-tag. Conjugation of these proteins to the sensor chip using a ligand thiol coupling technique produced a homo-oriented DnaA surface. Wild-type and mutant Soj proteins were then systematically injected over the wild-type and DnaASup surfaces. SPR analysis showed that wild-type Soj in the monomeric, ADP-bound form (Soj:ADP) binds to DnaA with an $K_{d}$ of $\sim$30 μM (Figure 2A). In contrast, the DnaAL294R and DnaA323D proteins were severely defective in their interaction with Soj:ADP, and the DnaA341V protein displayed an intermediate interaction profile (Figure 2B). Furthermore, all the DnaASup proteins failed to support complex formation with monomeric SojG12V (Figure 2C).

To substantiate the results observed by SPR, DnaA proteins were subjected to primary amine-specific crosslinking (BS$_3$) in solution with and without SojG12V. Protein complexes were separated by SDS–PAGE and DnaA was detected by western blot analysis (Figure 2E). The appearance of a signal at a molecular weight expected for a Soj:DnaA complex (27 kDa + 54 kDa = 81 kDa) was observed in the presence of SojG12V. By contrast, complex formation was dramatically reduced when the DnaA323D proteins were tested. In addition, the DnaA:DnaA complex (108 kDa) was reduced in the presence of SojG12V.

Cytological analysis of GFP–SojG12V localization in B. subtilis cells suggests that it associates with origin bound DnaA (Murray and Errington, 2008). To ascertain if SojG12V is capable of interacting with a DnaA:DNA complex in vitro, a pull-down experiment was performed. His-tagged SojG12V was incubated with pBsoriC4 in the presence and absence of native DnaA (DnaA341V). Proteins and DNA were cross-linked using a concentration of formaldehyde that yielded a specific Soj:DnaA interaction (Supplementary Figure S3A). Complexes were then bound to nickel beads via the histidine tag on Soj, washed, and the crosslinks reversed. The amount of pBsoriC4 in these complexes was detected using qPCR. There was an $\sim$13-fold enrichment of pBsoriC4 bound to SojG12V in the presence of DnaA, indicating that Soj is capable of forming a complex with DnaA bound to DNA (Supplementary Figure S3B).

Taken together, the SPR and crosslinking assays indicate that monomeric Soj directly interacts with DnaA both in solution and bound to DNA, and that the substitutions in the DnaA323D proteins disrupt complex formation.

Mapping of the DnaA323D and DnaA341V substitutions onto DnaA structures suggests a mechanism for Soj regulation

We noted that although our PCR mutagenesis strategy targeted the entire dnaA region ($\sim$5 kb flanking either side
of dnaA, including the dnaA promoter and all of oriC), all of the isolated DnaA<sup>hyp</sup> substitutions were located within the AAA + motif of DnaA, while all of the DnaA<sup>sup</sup> substitutions were located in domain IIIb (or at the border between domains IIIb and IV, depending upon the assignment of domain boundaries; see Supplementary Figure S1). Strikingly, when these amino-acid substitutions were mapped onto the crystal structure of DnaA domain III from *Thermotoga maritima* (Figure 3A), all four of the suppressor substitutions were found to cluster in domain IIIb, strongly suggesting that this region is the binding site for Soj.

The DnaA<sup>hyp</sup> substitutions were found more widely distributed throughout the AAA + motif. Three of the amino acids (G151, G154, and V121) were located around the nucleotide-binding pocket, with the backbone of the latter two residues mediating direct contacts with the terminal phosphate(s) and sugar, respectively. However, the remaining six DnaA<sup>hyp</sup> substitutions appear to be surface exposed and distal from the nucleotide-binding pocket; thus, it was unclear what effect these substitutions were having. To gain insight into how the DnaA<sup>hyp</sup> proteins might affect DnaA activity, the positions of these amino-acid substitutions were mapped onto the helical crystal structure of *Aquifex aeolicus* DnaA bound to AMP-PCP (Figure 3B, note *A. aeolicus* DnaA lacks a 14 amino-acid stretch present in all classically studied bacteria including *B. subtilis*, which harbours two of the four
suppressor substitutions (V323D and L337P); see Supplementary Figures S1 and S4). Significantly, all of these hypermorphic substitutions were either buried inside (70%) or adjacent to the DnaA:DnaA interface and were generally solvent exposed only at each end of the helix. Since these substitutions lead to hyperactivity of DnaA, we speculate that they may promote AAA\(\text{ }^+\text{mediated oligomerization by increasing the affinity of the DnaA:DnaA interaction.}

Taken together with the observation that Soj G12V disrupts DnaA:DnaA complex formation (Figure 2E), we hypothesized that monomeric Soj regulates DnaA ATP-dependent oligomerization.

B. subtilis DnaA assembles into an ATP-dependent oligomer in vitro

To test this model we designed a crosslinking assay to specifically detect ATP-dependent oligomerization of DnaA (Chen, 1991). Guided by the A. aeolicus structure, a pair of cysteine residues were introduced into domain IIIa at N191 and A198 (Figure 3B, inset). Within the oligomer, the N191 residue from one DnaA monomer is in close proximity (\(\sim 9\) Å) to the A198 residue of the adjacent monomer. DnaA\(\text{N191C,A198C}\) (hereafter referred to as DnaA\(\text{C}^\text{C}\)) was incubated with the cysteine-specific crosslinker bis(maleimido)ethane (BMOE; spacer arm 8.0 Å), protein complexes were separated by SDS–PAGE, and DnaA was detected by western blot analysis.

Figure 4A shows that crosslinking of DnaA\(\text{C}^\text{C}\) captures multiple high molecular weight complexes that run as a ladder on the gel. Formation of these DnaA oligomers was dependent on ATP and dramatically stimulated by DNA. Critical, mutation of the arginine finger residue (R264A) that coordinates the \(\gamma\)-phosphate of the ATP from the neighbouring DnaA molecule (Erzberger et al., 2006) greatly

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**Figure 3** The DnaA hypermorph and suppressor substitutions are located in domain III. (A) A cartoon representation of monomeric DnaA from the T. maritima crystal structure (PDB ID: 2Z4S) bound to ADP (stick). Domains IIIa and IIIb are separated by a dashed line. The Soj G12V hypermorph (black) and suppressor (red) substitutions are shown as spacefill representations. The identity and positions of the B. subtilis amino-acid substitutions are indicated above the corresponding residue of the T. maritima protein. (B) The majority of the hypermorphic substitutions are located either adjacent to, or buried within, the DnaA:DnaA interface. A surface representation of the helical DnaA structure from A. aeolicus (PDB ID: 2HCB) bound to AMP-PCP. DnaA monomers are coloured independently (yellow, cyan, blue, and green). The amino-acid substitutions are coloured and annotated as in (A) above. The inset shows the location of the two residues changed to cysteines for the crosslinking assays, with the black dashed line indicating where BMOE acts.

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**Supplementary Figures S1 and S4**

1. **Supplementary Figure S1**: Diagram showing the DnaA hypermorph and suppressor substitutions in domain III.
2. **Supplementary Figure S4**: Diagram illustrating the interaction between DnaA and Soj in the oligomeric state. The figure highlights the key residues involved in the DnaA:Soj interaction and how these residues are affected by hypermorphic and suppressor substitutions.
The stimulation by DNA appeared to be non-specific as the formation of DnaA oligomers was indistinguishable when comparing plasmids with or without the *B. subtilis* origin of replication (pBsoriC4 versus pUC18, respectively), comparing supercoiled DNA with linear DNA, and comparing double-stranded DNA with single-stranded DNA (Figure 4B and data not shown).

Previous work has suggested that the crystalized DnaA oligomer cannot accommodate binding to double-stranded DNA through the helix-turn-helix in domain IV due to substantial steric clashes (Duderstadt et al., 2010). Because we readily observed that double-stranded DNA stimulates DnaA oligomer formation, we wondered whether this stimulation was dependent upon residues required for the DNA-binding activities of domains III and IV. An amino-acid substitution was introduced into domain III (I190A) that previously was shown to disrupt single-stranded DNA-binding activity of DnaA in vitro and DNA replication in vivo (Ozaki et al., 2008). As expected, oligomerization of DnaA CC,I190A was not stimulated by single-stranded DNA, although it was stimulated by double-stranded plasmids (Figure 4B). Next, to investigate the role played by domain IV in oligomer formation, arginine 379 was mutated to an alanine. In *E. coli* DnaA, the equivalent residue has been shown to interact with both specific basepairs and backbone phosphates in the minor groove of double-stranded DNA (Fujikawa et al., 2003). In contrast to the results obtained with DnaA CC,I190A, oligomerization of DnaA CC,R379A was stimulated by single-stranded DNA, but not by the double-stranded DNA.

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**Figure 4** Monomeric Soj inhibits DnaA oligomerization in vitro. (A) In vitro oligomer formation assays using the cysteine-specific crosslinker BMOE. DnaA proteins were incubated in oligomer formation buffer for 15 min prior to the addition of BMOE. Pluses located above each lane indicate the presence of BMOE (2 mM), nucleotide (2 mM), and/or DNA (pBsoriC4; 3 nM). The identity of the DnaA proteins (3 μM) are indicated below. DnaA proteins were separated by SDS–PAGE and visualized by western blotting. (B) DnaA oligomers form on both single-stranded and double-stranded DNA. DnaA proteins (3 μM) were incubated in oligomer formation buffer with NaCl (400 mM) in the presence of ATP (2 mM), with or without DNA, for 15 min prior to the addition of BMOE. The identity of each DNA substrate is indicated above the respective lanes, and the amount of DNA is equal in each reaction (120 fmol). Both plasmids are supercoiled and the single-stranded DNA (ssDNA) is oligonucleotide oGJS159. DnaA proteins were separated by SDS–PAGE and visualized by western blotting. (C) Monomeric Soj disrupts DnaA oligomer formation in vitro. Triangles above the lanes represent an increasing concentration of various Soj proteins (12, 24, and 36 μM). DnaA proteins (3 μM) were incubated in oligomer formation buffer in the presence of ATP (2 mM) and DNA (pBsoriC4; 3 nM) for 15 min prior to the addition of BMOE. DnaA proteins were separated by SDS–PAGE and visualized by western blotting.
stranded pUC18 plasmid. Interestingly, oligomerization of DnaA<sup>CC,R379A</sup> was stimulated by a plasmid that harboured oriC (Figure 4B). These results correlated with the ability of DnaA<sup>CC,R379A</sup> to bind to the respective plasmids as judged by an electrophoretic mobility shift assay (Supplementary Figure S5). We note that single-stranded DNA stimulated helix formation of DnaA<sup>CC,R379A</sup> to a greater degree than DnaA<sup>CC</sup>. This observation suggests that the arginine residue may interact with the phosphate backbone of the single-stranded substrate and inhibit the docking of domain IV into the AAA+ domain, a transition proposed to be critical for single-stranded DNA-binding activity (Duderstadt et al., 2010). Taken together, these experiments indicate that DnaA is capable of forming ATP-dependent oligomers on both single-stranded and double-stranded DNA substrates, in contrast to the apparent constraints placed upon domain IV within the structure of the DnaA oligomer (see Discussion).

**Monomeric Soj specifically inhibits DnaA oligomer formation in vitro**

The DnaA oligomer formation assay was then used to investigate the effect of Soj on DnaA activity. The presence of Soj<sup>G12V</sup> significantly reduced both the length and abundance of the DnaA oligomers formed in the presence of plasmid DNA (Figure 4C). To determine whether the observed inhibition was specific to monomeric Soj, the effect of Soj<sup>K16A</sup> (monomeric) and Soj<sup>R189A</sup> (dimeric) proteins was also investigated. While Soj<sup>K16A</sup> clearly disrupted DnaA oligomer formation, Soj<sup>R189A</sup> had little or no effect (Figure 4C) even though it was capable of interacting with DnaA (Figure 2D and E). Thus, despite sharing apparent interaction determinants, disruption of DnaA oligomerization is a specific property of the monomeric Soj protein. These results are consistent with our previous findings that monomeric Soj specifically inhibits DnaA initiation activity in vivo (Scholefield et al., 2011).

Oligomers formed by DnaA<sup>L294R</sup> and DnaA<sup>V323D</sup> were highly resistant to Soj<sup>G12V</sup> activity (Figure 5A). In comparison, oligomers formed by DnaA<sup>A341V</sup> were partially susceptible to Soj<sup>G12V</sup>, consistent with the intermediate response observed between the two proteins by SPR analysis (Figures 2B and 5A). Importantly, all of the DnaA<sup>Sup</sup> proteins were found to form oligomers under the same conditions as wild type (Supplementary Figure S6A). These results indicate that the effect of Soj<sup>G12V</sup> on DnaA oligomerization is dependent on the same residues required for the formation of a Soj:DnaA complex.

To determine whether monomeric Soj prevents DnaA oligomer formation and/or disassembles pre-formed DnaA oligomers, an order of addition experiment was performed. Figure 5B shows that DnaA oligomers formed prior to Soj<sup>G12V</sup> addition are highly resistant to Soj<sup>G12V</sup> inhibition (lane 3) when compared with oligomers formed after Soj<sup>G12V</sup> addition (lane 2), indicating that monomeric Soj acts by preventing DnaA oligomer formation.

Since the DnaA oligomer can form on both single-stranded and double-stranded DNA, it was possible that Soj<sup>G12V</sup> only acted on one of these complexes. Soj<sup>G12V</sup> was found to disrupt oligomerization of both DnaA<sup>CC</sup> in the presence of single-stranded DNA and DnaA<sup>CC,R379A</sup> in the presence of double-stranded DNA (Supplementary Figure S6B). Thus, monomeric Soj can inhibit DnaA oligomers forming on either single-stranded or double-stranded substrates, although we note that the degree of inhibition appeared to be greater in the presence of single-stranded DNA.

Previous work has established that domain I plays an important role in DnaA oligomerization in *E. coli* (Felczak et al., 2005). To test whether Soj<sup>G12V</sup> inhibition of DnaA oligomerization was dependent on this domain I self-interaction, we created a truncated version of DnaA<sup>CC</sup> that lacked domains I and II (DnaA<sup>III/IV,CC</sup>). As expected from the interaction analysis described above, the DnaA<sup>III/IV,CC</sup> protein retained the ability to interact with Soj (Supplementary Figure S7A). The truncated DnaA was readily capable of forming oligomers and, like the full-length protein, these oligomers were inhibited by monomeric Soj<sup>G12V</sup> but were not affected by dimeric Soj<sup>R189A</sup> (Supplementary Figure S7B). These results indicate that the oligomerization activity observed for the DnaA<sup>CC</sup> protein is independent of domains I and II, and taken together with the interaction experiments, they support a model in which monomeric Soj inhibits DnaA oligomerization by specifically regulating an activity of the AAA+ domain.

**Soj<sup>G12V</sup> inhibition of DnaA is independent of DnaA ATP binding, ATP hydrolysis, and DNA-binding activities**

Because oligomerization of DnaA<sup>CC</sup> was stimulated by both ATP and DNA, we investigated whether monomeric Soj inhibits DnaA oligomerization by modulating the interaction of DnaA to either of these molecules. To test whether monomeric Soj affects ATP binding to DnaA, we incubated an ATP hydrolysis deficient DnaA protein (DnaA<sup>R313A</sup>) with γ<sup>-</sup>P<sub>32</sub>ATP in the presence and absence of Soj<sup>K16A</sup>. The DnaA<sup>R313A</sup> protein was utilized to prevent hydrolysis of the bound ATP during the experiment (Supplementary Figure S8A) and Soj<sup>K16A</sup> was used because it is ATP-binding deficient (Scholefield et al., 2011). Reactions were assembled and incubated in an identical manner to the oligomer formation assay before the proteins were separated from the reaction buffer using magnetic nickel beads and washed to remove unbound ATP. The bound ATP was extracted and then separated by thin layer chromatography. There was no significant difference between the amount of ATP bound to DnaA<sup>R313A</sup> in the presence or absence of Soj<sup>K16A</sup> (Figure 5C), indicating that monomeric Soj does not inhibit ATP binding. Furthermore, Soj<sup>G12V</sup> did not stimulate the ATPase activity of wild-type DnaA (Supplementary Figure S8A) and oligomerization of the hydrolysis deficient DnaA<sup>CC,R313A</sup> protein remained sensitive to Soj<sup>G12V</sup> inhibition (Figure 5D), indicating that monomeric Soj does not act by regulating the ATPase activity of DnaA.

To determine whether monomeric Soj inhibits DnaA oligomerization by inhibiting the DNA-binding activity of DnaA, we determined whether Soj<sup>G12V</sup> was capable of inhibiting DnaA<sup>CC</sup> oligomer formation in the absence of DNA. As before, Soj<sup>G12V</sup> inhibited DnaA<sup>CC</sup> oligomerization but had little effect on DnaA<sup>III/IV,CC,R323D</sup> (Supplementary Figure S8B). These results indicate that monomeric Soj can regulate DnaA helix formation in a manner that is independent of the ability of DnaA to bind to DNA. Taken together, the data support the model that monomeric Soj directly interacts with the AAA+ domain of DnaA to specifically prevent ATP-dependent oligomer formation.
Monomeric Soj inhibits DnaA oligomer formation in vivo

To determine if the inhibition of DnaA oligomer formation caused by monomeric Soj in vitro was physiologically relevant, the site-specific crosslinking assay was modified to detect DnaA oligomers in vivo. The endogenous dnaA gene was replaced with dnaA\textsuperscript{N191C,A198C} (dnaACC\textsuperscript{N191C,A198C}) and BMOE was used to crosslink DnaA CC proteins in intact cells that were harvested during exponential growth. The strain harbouring dnaA\textsuperscript{CC} mildly overinitiated DNA replication compared with wild type, although its growth and morphology appeared normal (Supplementary Figure S9A). Following incubation with BMOE, cells containing DnaA CC (but not cells with single cysteine substitutions) formed oligomers and this was dependent upon the arginine finger required for the interaction of neighbouring AAA\textsuperscript{+} domains (Figure 6A–C).

Thus, the site-specific crosslinking assay captures DnaA oligomers within \textit{B. subtilis} cells that likely correspond to the complexes observed in vitro.

Figure 6A shows that overexpression of monomeric SojG12V resulted in a \( \approx \) 50% decrease in the percentage of DnaACC found in the oligomer compared with wild type, while overexpression of dimeric Soj R189A had no inhibitory effect (both Soj proteins were overexpressed to the same extent; Supplementary Figure S9B). Moreover, the oligomers formed by one of the DnaA Sup proteins (DnaA CC,V323D) were resistant to Soj G12V overexpression (Figure 6C). These results suggest that monomeric Soj is capable of directly inhibiting DnaA oligomer formation in vivo as well as in vitro.

In addition to analysis of the DnaA Sup protein, the in vivo oligomer formation assay was used to investigate the activity

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**Figure 5** Monomeric Soj specifically prevents DnaA oligomerization in vitro. (A) In vitro oligomer formation assay with DnaA\textsuperscript{Sup} proteins. DnaA proteins (3 \( \mu \)M) and Soj\textsuperscript{G12V} (12, 24, and 36 \( \mu \)M) were incubated in oligomer formation buffer in the presence of ATP (2 mM) and DNA (pBsoriC4; 3 nM) for 15 min prior to the addition of BMOE. DnaA proteins were separated by SDS–PAGE and visualized by western blotting. (B) Monomeric Soj is unable to disassemble pre-formed DnaA oligomers. Reaction conditions are the same as in (A). DnaA was crosslinked at \( T = 15 \) min. Lane 1, DnaA was added to the reaction buffer at \( T = 13 \). Lane 2, Soj\textsuperscript{G12V} was added at \( T = 0 \) and DnaA added at \( T = 13 \). Lane 3, DnaA was added at \( T = 0 \) and Soj\textsuperscript{G12V} added at \( T = 13 \). (C) Monomeric Soj does not affect DnaA ATP binding. DnaA\textsuperscript{R313A} (3 \( \mu \)M) and/or Soj\textsuperscript{K16A} (36 \( \mu \)M) were incubated with \( \alpha\textsuperscript{-}P\textsuperscript{32} \) ATP before being isolated from the reaction using magnetic nickel beads and denatured with methanol. The released nucleotides were separated on a PEI cellulose TLC plate and visualized by autoradiography. Density values were measured using ImageJ (\( n = 3 \)). (D) Monomeric Soj disrupts DnaA oligomers independent of DnaA ATPase activity. In vitro oligomer formation assay with the ATP hydrolysis deficient DnaA protein. DnaA\textsuperscript{R313A} (3 \( \mu \)M) and Soj\textsuperscript{G12V} (12, 24, and 36 \( \mu \)M) were incubated in oligomer formation buffer in the presence of ATP (2 mM) and DNA (pBsoriC4; 3 nM) for 15 min prior to the addition of BMOE. DnaA proteins were separated by SDS–PAGE and visualized by western blotting.
of several DnaA hyp proteins. The three DnaA hyp proteins tested (A132T, G154S, and R281G) showed an ~2–3-fold increased propensity to form oligomers compared with the wild type (Figure 6C). The helices formed by the DnaA hyp proteins remained susceptible to inhibition by Soj G12V, consistent with the results from the MFA described above (Figure 1C). These observations are compatible with the model that the DnaA hyp proteins bypass Soj G12V inhibition by promoting interactions between adjacent DnaA monomers, thus leading to an increase in DnaA oligomer formation and in the rate of DNA replication initiation.

Interestingly, the DNA replication initiation frequency of wild-type DnaA and each DnaA hyp protein (in the presence or absence of Soj G12V) strongly correlated with the percentage of protein observed in oligomers (correlation coefficient = 0.903) (Figure 6D). This result further supports
the biological significance of DnaA oligomer formation, and suggests that this activity may be the rate-limiting step required for DNA replication initiation in \textit{B. subtilis}.

**Discussion**

**Monomeric Soj inhibits DnaA helix formation**

In this report we show that monomeric Soj stalls DNA replication initiation by directly interacting with the initiator protein DnaA and specifically inhibiting DnaA oligomer formation. Based on the arguments outlined below, we propose that monomeric Soj regulates DnaA assembly into a right-handed helical structure. If this is correct, we have uncovered a novel mode of DnaA regulation mediated via the Soj protein. We also provide critical biochemical evidence demonstrating that the DnaA helix observed by X-ray crystallography assembles and has functional relevance \textit{in vitro}, thereby supporting the physiological role of both this DnaA structure and the proposed Soj regulatory mechanism.

To study the negative regulation of DnaA by monomeric Soj, we established a cysteine crosslinking assay capable of detecting an ATP-dependent DnaA oligomer. To achieve crosslinking via BMOE, this oligomer has to fulfill two requirements: first, the arginine finger residue (R264) has to coordinate the ATP of the neighbouring monomer, and second, the two cysteines from adjacent AAA + domains have to be brought within 9 Å of each other. This means that there are two relatively fixed contacts between neighbouring AAA + domains necessary for an efficient reaction. Since these dual requirements would allow minimal flexibility of the AAA + domains relative to each other, the DnaA\textsubscript{CC} site-specific crosslinking assay is most likely capturing the AAA + domains in an orientation analogous to the published crystal structure. Therefore, we propose that our DnaA\textsubscript{CC} oligomer formation assay captures a helical structure of \textit{B. subtilis} DnaA.

**The DnaA helix can form on single-strand and double-stranded DNA**

It has been proposed that the compact DnaA helix observed in the crystal structure could only form on single-stranded DNA through interactions in domain III, since domain IV is packed tightly into the compact helix, effectively sequestering the double-stranded DNA-binding interface (Erzberger \textit{et al}, 2006; Ozaki \textit{et al}, 2008; Duderstadt \textit{et al}, 2010). Consistent with this model and supporting the importance of the compact DnaA helix, it has been shown that amino-acid substitutions in the domain III–IV interface that affect DnaA oligomerization inhibit both open complex formation \textit{in vitro} and replication origin function \textit{in vivo} (Duderstadt \textit{et al}, 2010). However, it should be noted that genetic analyses, electron microscopy analyses, and footprinting assays together strongly indicated that ATP-bound DnaA must first cooperatively assemble into a large nucleoprotein complex built upon defined interactions on double-stranded DNA within oriC prior to open complex formation (Mott and Berger, 2007; Rozgaja \textit{et al}, 2011). Thus, it was not clear how the compact DnaA helix would be assembled at the replication origin.

We observed that an ATP-dependent DnaA helix can be built on either single-stranded or double-stranded DNA. The DnaA helix formed on single-stranded DNA was found to depend on I190 in domain III, located within the central pore of the helix. By contrast, the DnaA helix formed on non-specific double-stranded DNA was found to depend on R379 in domain IV and was independent of I190. These observations imply that during DnaA helix formation on double-stranded DNA, domain IV must be extended away from the AAA + helical core. This also leads to the conclusion, supported by the structures of DnaA bound to ADP and AMP-PCP (Erzberger \textit{et al}, 2002, 2006; Ozaki \textit{et al}, 2008; Duderstadt \textit{et al}, 2010), that the z-helix linking domains III and IV is a semi-flexible linker, allowing domain IV to adopt multiple conformations relative to the helical AAA + core.

These results are consistent with the model, originally proposed by Erzberger \textit{et al} (2006), whereby ATP-bound DnaA initially assembles into an extended helical structure that is engaged with specific double-stranded DNA-binding sequences through domain IV, followed by the transition to a compact helix which point domain IV dissociates from double-stranded DNA and folds into domain III, thereby promoting the single-stranded DNA-binding activity of residues located in the central channel formed by the AAA + motifs (Figure 7). Although the precise architecture of the DNA replication initiation complex has not yet been determined (see Ozaki and Katayama, 2011), our data help to reconcile how multiple distinct helical DnaA oligomers could be assembled specifically at bacterial replication origins.
In addition to the helical DnaA oligomer built upon the AAA+ core, it is important to note that domain I plays critical roles in DnaA assembly and activity. Many bacterial DnaA proteins can directly form homo-oligomers that are dependent upon domain I (Weigel et al., 1999; Simmons et al., 2003). This domain I self-interaction is required for DnaA both to recruit additional DnaA proteins to the replication origin and to load the replicative helicase (Felczak et al., 2005; Miller et al., 2009). Although this domain I self-interaction is not necessary for either assembly or ssDNA stretching activity of the DnaA helical oligomer (Supplementary Figure S7B; Erzberger et al., 2006; Duderstadt et al., 2010, 2011), it could stimulate DnaA helix formation indirectly either by increasing the local concentration of DnaA at oriC or by stabilizing DnaA oligomers at oriC. Future studies will be needed to determine how these distinct DnaA interfaces, along with the information encoded by the DNA sequence of oriC, act in concert to construct an active DnaA initiation complex.

**Potential mechanisms for Soj activity**

Our genetic analysis suggests that Soj directly interacts with domain IIIB (or the region linking domains IIb and IV) of DnaA. Comparison of the ADP-bound monomeric and the AMP-PCP-bound helical DnaA structures indicates that domain IIIB must shift and domain IV must bend to generate the space required for the arginine finger from the neighbouring monomer to engage the γ-phosphate of ATP (Erzberger et al., 2006). Furthermore, our biochemical analyses show that monomeric Soj neither inhibits ATP binding, nor stimulates ATP hydrolysis, nor disassembles pre-formed DnaA helices.

Based on these considerations, we envisage two potential mechanisms by which Soj could act to prevent helix formation (Figure 7). First, Soj could bind to domain IIIB and sterically inhibit helix assembly. Second, Soj could bind to domain IIIB and prevent DnaA undergoing the conformational changes required for it to assemble into a helix. Taking into account the observations that both monomeric and dimeric Soj proteins bind to DnaA, that the DnaA\(^{sup}\) substitutions impair the binding of both Soj conformations, and that prevention of DnaA helix formation is specific to monomeric Soj, we currently favour the latter allosteric model because binding of the larger Soj dimer would presumably create an even greater steric wedge between neighbouring DnaA molecules.

Our preliminary data suggest that dimeric Soj stimulates DnaA helix formation in vivo, consistent with its ability to increase the frequency of DNA replication initiation (GJS and HM, unpublished data). In addition, previous data indicate that Soj can positively regulate DnaA activity as a transcriptional regulator (Murray and Errington, 2008). Thus, we wonder whether Soj could allosterically regulate DnaA helix formation both negatively and positively, with the direction of regulation depending upon the quaternary state of the Soj protein.

Although the interaction studies indicate that both monomeric and dimeric Soj proteins bind to a similar region of DnaA (Figure 2C–E), we note that the dimeric Soj protein remained capable of interacting with all of the DnaA\(^{sup}\) substitutions, albeit to a lesser extent than with the wild-type protein (Figure 2D). This observation suggests that dimeric Soj may be able to interact with a second site on DnaA, and this additional contact could influence the outcome of the interaction between the two proteins. These questions regarding dichotomies between the activities and the interactions of monomeric and dimeric Soj proteins are currently under investigation.

**Biological implications of regulating DnaA helix formation**

The signal that switches Soj from a monomer to a dimer, and thus from an inhibitor to an activator of DnaA, is unknown. In all growth conditions tested so far, it appears that Soj dimerization is efficiently inhibited by the regulatory protein Spo0J (ParB). Therefore, Soj probably spends most of its time in the inhibitory monomeric state. Previous cytological studies have shown that GFP–Soj\(^{G125V}\) localizes as a focus at oriC in a DnaA-dependent manner (Murray and Errington, 2008), suggesting that Soj prevents DnaA helix formation at oriC. By stalling DNA replication initiation at the stage of DnaA helix formation, it would allow DnaA to remain primed for rapid activation (i.e., DnaA would already be ATP-bound and localized at the origin). We hypothesize that a cellular signal, such as an increase in nutritional availability, entry into a developmental programme, or a cell-cycle event, stimulates the conversion of Soj from monomer to dimer (perhaps locally around oriC), thereby swiftly promoting the initiation of DNA replication.

Chromosomally encoded par genes are found throughout all branches of the bacterial kingdom (Livny et al., 2007). Nonetheless, many species do not harbour par genes and we hypothesize that regulation of DnaA helix formation, representing the active initiation complex, is achieved through different mechanisms in various bacteria. In E. coli, which does not have par genes, the rate of ATP binding appears to limit DnaA oligomerization potential (Kurokawa et al., 1999). In C. crescentus DnaA is degraded at the end of every cell cycle (Corbatyuk and Marczynski, 2005), suggesting that helix formation could be limited by protein synthesis. Interestingly, although C. crescentus contains par genes, it appears that in this organism ParA is involved in segregation of the chromosome origin region (Ptacin et al., 2010; Scholefield et al., 2010). Whether this variation in ParA activity reflects the fact that DnaA oligomerization in C. crescentus is limited at a different step in the assembly pathway compared with B. subtilis is not known, but it will be informative to determine the rate-limiting step in DnaA oligomerization for a range of bacteria and correlate this with the activity of their respective ParA proteins.

**The B. subtilis Par system: roles in DNA replication and chromosome segregation**

par operons were originally identified on low-copy number plasmids where they ensure faithful segregation of plasmids into daughter cells (Gerdes et al., 2010). In these systems, the ParB protein binds to a centromere-like site on the plasmid (parS), followed by segregation of this nucleoprotein complex by the ATPase ParA. The chromosomal orthologues of Par proteins are found throughout the bacterial kingdom and have been shown to influence chromosome organization and segregation in a number of diverse species (for review see Gerdes et al., 2010). These results led to the hypothesis that Par proteins act in an analogous manner to their plasmid orthologues by forming a segregation machine that actively separates replicated chromosomes prior to cell division.

Despite the appeal of this model, rigorous examination of Soj/ParA activity during vegetative growth in B. subtilis has
so far provided little support for a role in chromosome segregation or origin localization (Lee and Grossman, 2006). However, it has been shown that Spo0J/ParB is required for proper DNA segregation in *B. subtilis* (Ireton et al, 1994), and recent work indicates that Spo0J affects chromosome organization by recruiting Condensin (the SMC complex) to the origin region of the chromosome (Gruber and Errington, 2009; Sullivan et al, 2009). Therefore, the *B. subtilis* Par system is involved in accurate chromosome segregation, although apparently not in an analogous manner to related plasmid systems. Moreover, it appears that the activity of the *B. subtilis* Par system is more complex than initially imagined, with the Spo0J-parS complex acting as a regulator of, and perhaps coordinating the activities of, factors involved in DNA replication (Soj) and DNA organization/segregation (SMC) leading to possible coordination between the two systems.

**AAA + inter-protein interactions as a regulatory target in ORC**

In eukaryotic organisms, DNA replication is initiated by the origin recognition complex (ORC; Orc1–6) in combination with Cdc6. Orc1, Orc4, Orc5, and Cdc6 contain AAA + domains, while Orc2 and Orc3 are predicted to have AAA +–like folds. Orc1 and Orc5 have been shown to bind ATP while Orc1 and Cdc6 have intrinsic ATPase activity (Kawakami and Katayama, 2010). Strikingly, ORC has been shown to undergo an ATP-dependent conformational change, and the structure of the DnaA helix can be docked into a low-resolution structure of ORC (Clarey et al, 2006). These observations suggest that the ATP-dependent conformational changes in ORC result from a similar reorientation of the AAA + domains observed in DnaA. Consistent with this model, the arginine finger residue in Orc4 and the ATPase activity of Cdc6 are both critical for reiterative helicase loading (Schepers and Diffley, 2001; Bowers et al, 2004). We suggest that detailed examination of DnaA conformational changes required for the assembly of an active initiation complex will likely underpin and inform the mechanistic understanding of related initiator complexes from higher organisms. Furthermore, our finding that DnaA helix formation is targeted for regulation by Soj opens up the possibility that the conformational changes observed for ORC will also be subject to regulation.

**Materials and methods**

**Strains and plasmids**

Strains and plasmids used in this study are described in the Supplementary data and listed in Supplementary Tables SI and SII and the relevant oligonucleotides in Supplementary Table SIII. *E. coli* strain DH5α (Invitrogen) was used as the construction of all plasmids, and strain BL21 (DE3) pLYS (Stratagene) was used to express all proteins. The plasmid pET21d (Invitrogen) was used as the expression vector for all proteins.

**Media and chemicals**

Nutrient agar (NA; Oxoid) was used for routine selection and maintenance of both *B. subtilis* and *E. coli* strains. For experiments in *B. subtilis*, cells were grown in either Luria-Bertani (LB) medium or casein hydrolysate medium. Supplements were added as required: 20 µg/ml tryptophan, 5 µg/ml chloramphenicol, 2 µg/ml kanamycin, 50 µg/ml spectinomycin. For plasmid and protein expression in *E. coli*, cells were grown in LB medium or Nutrient Broth (Oxoid) and supplemented with 30 µg/ml (for single copy plasmids) or 75 µg/ml ampicillin and 10 µg/ml chloramphenicol. Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich.

**Marker frequency analysis**

MFA was essentially done as previously described (Murray and Errington, 2008). For details see Supplementary data.

**Purification of in vivo protein–protein complexes**

This was done as previously described, for details see Supplementary data (Murray and Errington, 2008).

**Primary amine crosslinking assay**

Soj proteins (36 µM) were diluted into oligomer formation buffer (25 mM HEPES pH 7.6, 200 mM NaCl, 100 mM potassium glutamate, and 10 mM MgCl2) supplemented with 2 mM ATP and 3 nM pBSoriC4 at room temperature. DnaA (3 µM) was then immediately added and the reaction was incubated at 37°C for 15 min. The primary amine-specific crosslinker BS3 (Thermo Scientific) was then added (0.1 mM final) and the reaction was left to proceed for 3 min at 37°C. The reaction was quenched by the addition of TRIS (100 mM final pH 8) for 10 min at 37°C. Samples were separated by SDS–PAGE and bands were visualized by western blot analysis using α-DnaA polyclonal antibodies.

**In vitro helix formation assay**

Soj proteins (12, 24, and 36 µM) were diluted into oligomer formation buffer (see above) supplemented with 2 mM ATP and 3 nM pBSoriC4. DnaA (3 µM) was then added and the reaction was incubated at 37°C for 15 min. BMOE cysteine-specific crosslinker (Thermo Scientific) was added to a final concentration of 2 mM and the reaction was let to proceed for 3 min at 37°C. The reaction was quenched by the addition of cysteine (50 mM final) for 5 min at 37°C. Samples were separated by SDS–PAGE and bands were visualized by western blot analysis using α-DnaA polyclonal antibodies.

**In vivo helix formation assay**

Crosslinking media (Spizizen minimal media supplemented with 0.01 mg/ml Fe–NH4–citrate, 0.5% glucose, 6 mM MgSO4, and 0.02 mg/ml of all the natural amino acids except cysteine) was inoculated directly from −80°C with the required strain and left to grow overnight at 37°C. Cells were diluted 1:100 into fresh crosslinking media in a flask allowing for high aeration and growth at 30°C until the A600 reached ~0.1 at which point xylose (1% final) was added or removed by washing to induce or to repress soj alleles, respectively. Cell growth was allowed to continue until the A600 reached ~0.6. 10 ml of culture was collected by centrifugation at 14K r.p.m. for 1 min and resuspended in 2 ml *in vivo* crosslinking buffer (50 mM HEPES pH 7 and 10% Sucrose). For MFA, another 250 µl of cells was added directly to 25 µl of 10% sodium azide and then treated and analysed by qPCR. Cells were collected as above and resuspended in 100 µl *in vivo* crosslinking buffer and flash frozen in liquid nitrogen (flash freezing and the subsequent addition of DMSO did not affect cell viability). Frozen cells were thawed and BMOE was added to a final concentration of 2 mM. The reaction was left to proceed for 30 min at 37°C with shaking at 800 r.p.m. before being quenched by the addition of cysteine (100 mM final) for 5 min at 37°C. Cells were lysed by the addition of a 0.5 × reaction volume of SDS–PAGE sample loading buffer and DTT followed by heating to 90°C for 15 min and then briefly incubated at 4°C. Cell debris was removed by centrifugation at 14K r.p.m. for 15 min at 4°C. Finally, samples were concentrated using a 10-kDa MWCO centrifugal unit (Millipore) and analysed by SDS–PAGE. Bands were visualized by western blot analysis using α-DnaA polyclonal antibodies.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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Soj inhibits DnaA helix formation
G Scholfeifeld et al

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Conflict of interest

The authors declare that they have no conflict of interest.

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Soj inhibits DnaA helix formation
G Scholefield et al