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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.

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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 competiveness 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

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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 (DiaA in *E. coli* and HobA in *Helicobacter pylori*) or repressed (SirA in *B. subtilis*) by the binding of regulatory proteins to domain I. 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: Soj^{K16A} is unable to bind ATP, while Soj^{G12V} can bind ATP but cannot dimerize due to a steric clash in the dimerization interface; both of these proteins inhibit DnaA activity. The Soj^{R189A} 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

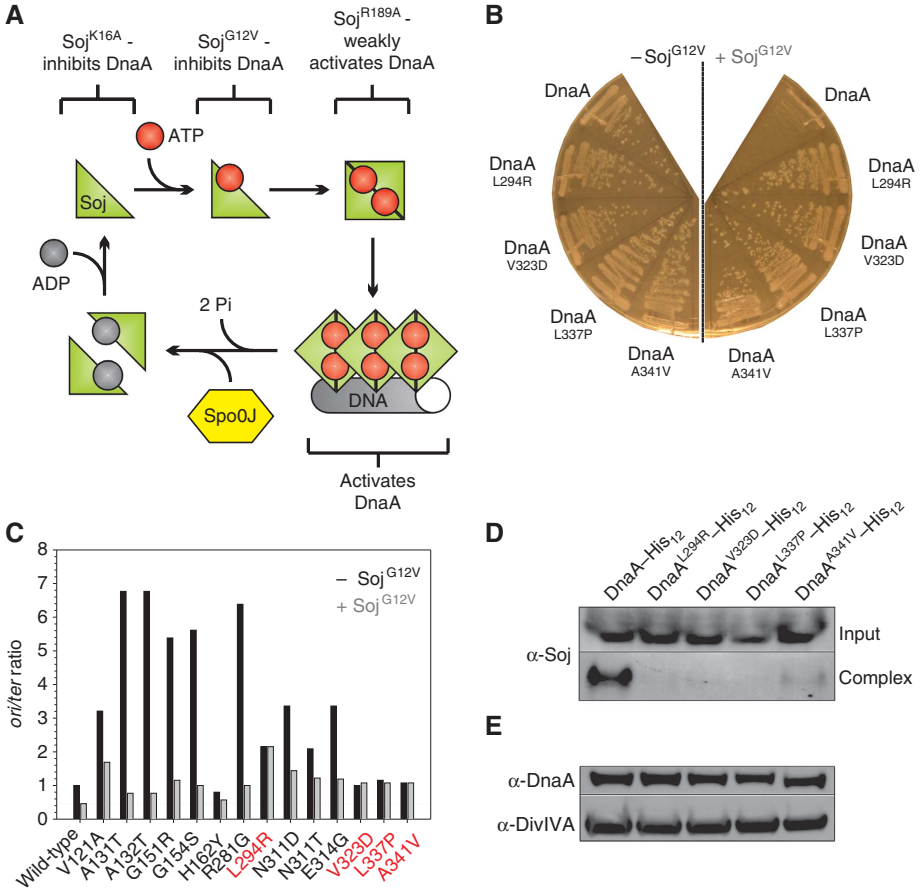

Figure 1 Specific mutations in *dnaA* either bypass or suppress the inhibition of DNA replication initiation by Soj^{G12V}. (**A**) Pathway of the Soj activity cycle. (**B**) Point mutations in DnaA introduced by error-prone PCR were found to overcome the small colony phenotype characteristic of Soj^{G12V} overexpression. Strains were grown on NA plates in the presence or absence of 1% xylose to induce *soj^{G12V}* expression. Wild-type (HM524), DnaA^{L294R} (HM527), DnaA^{V323D} (HM528), DnaA^{L337P} (HM529), DnaA^{A341V} (HM530). (**C**) The *oriC*-to-terminus ratios of *dnaA* point mutations generated using PCR mutagenesis were determined using MFA in the presence and absence of Soj^{G12V} overexpression (1% xylose). Suppressor mutations (red) were found to be recalcitrant to Soj^{G12V} activity. Cells were grown in LB medium at 30°C. Values were normalized to the *ori:ter* ratio of the wild-type strain grown in the absence of xylose. DnaA^{V121A} (HM713), DnaA^{A131T} (HM714), DnaA^{A132T} (HM710), DnaA^{G151R} (HM705), DnaA^{G154S} (HM706), DnaA^{H162Y} (HM707), DnaA^{R281G} (HM708), DnaA^{N311D} (HM712), DnaA^{N311T} (HM709), DnaA^{E314G} (HM711). (**D**) The Soj^{G12V} suppressor mutations in *dnaA* perturb the formation of a Soj:DnaA–His$_{12}$ complex *in vivo*. Cells were grown in LB medium at 30°C, crosslinked with formaldehyde, and the DnaA–His$_{12}$ complexes were purified before the crosslinks were reversed and proteins were separated by SDS–PAGE. Soj and DnaA–His$_{12}$ 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 Soj found in a complex with DnaA–His$_{12}$ following purification (Complex). DnaA–His$_{12}$ (HM657), DnaA^{L294R}–His$_{12}$ (HM716), DnaA^{V323D}–His$_{12}$ (HM555), DnaA^{L337P}–His$_{12}$ (HM658), DnaA^{A341V}–His$_{12}$ (HM725). (**E**) The amount of each DnaA^{Sup}–His$_{12}$ 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 Soj^{G12V} (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 *soj*^{G12V} allele and plated under Soj^{G12V} 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 mutation 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 Soj^{G12V} inhibition ($DnaA^{Hyp}$ proteins) by having a high basal rate of initiation, and suppressors that had an approximately wild-type rate of initiation but were resistant to Soj^{G12V} inhibition ($DnaA^{Sup}$ proteins: $DnaA^{L294R}$, $DnaA^{V323D}$, $DnaA^{L337P}$, $DnaA^{A341V}$). The suppressor mutations were each independently cloned into *dnaA* and transformed into the Soj^{G12V} 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 Soj^{G12V} overexpression (Supplementary Figure S2).

The ability of Soj to form a complex with DnaA–His_{12} and $DnaA^{Sup}$–His_{12} proteins *in vivo* was investigated using nickel affinity purification following formaldehyde crosslinking. Compared with wild-type DnaA–His_{12}, all four $DnaA^{Sup}$–His_{12} proteins were defective in their ability to form a complex with Soj (Figure 1D). Western blot analysis confirmed that all DnaA–His_{12} 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 Soj^{G12V} 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_5-tag. Conjugation of these proteins to the sensor chip using a ligand thiol coupling technique produced a homo-orientated DnaA surface. Wild-type and mutant Soj proteins were then systematically injected over the wild-type and $DnaA^{Sup}$ 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 $DnaA^{L294R}$ and $DnaA^{V323D}$ proteins were severely defective in their interaction with Soj:ADP, and the $DnaA^{A341V}$ protein displayed an intermediate interaction profile (Figure 2B). Furthermore, all the $DnaA^{Sup}$ proteins failed to support complex formation with monomeric Soj^{G12V} (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 Soj^{G12V}. 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 Soj^{G12V}. By contrast, complex formation was dramatically reduced when the $DnaA^{Sup}$ proteins were tested. In addition, the DnaA:DnaA complex (108 kDa) was reduced in the presence of Soj^{G12V}.

Cytological analysis of GFP–Soj^{G12V} localization in *B. subtilis* cells suggests that it associates with origin bound DnaA (Murray and Errington, 2008). To ascertain if Soj^{G12V} is capable of interacting with a DnaA:DNA complex *in vitro*, a pull-down experiment was performed. His-tagged Soj^{G12V} was incubated with pBsoriC4 in the presence and absence of native DnaA ($DnaA^{nat}$). Proteins and DNA were crosslinked 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 Soj^{G12V} 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 $DnaA^{Sup}$ proteins disrupt complex formation.

Mapping of the $DnaA^{Sup}$ and $DnaA^{Hyp}$ 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

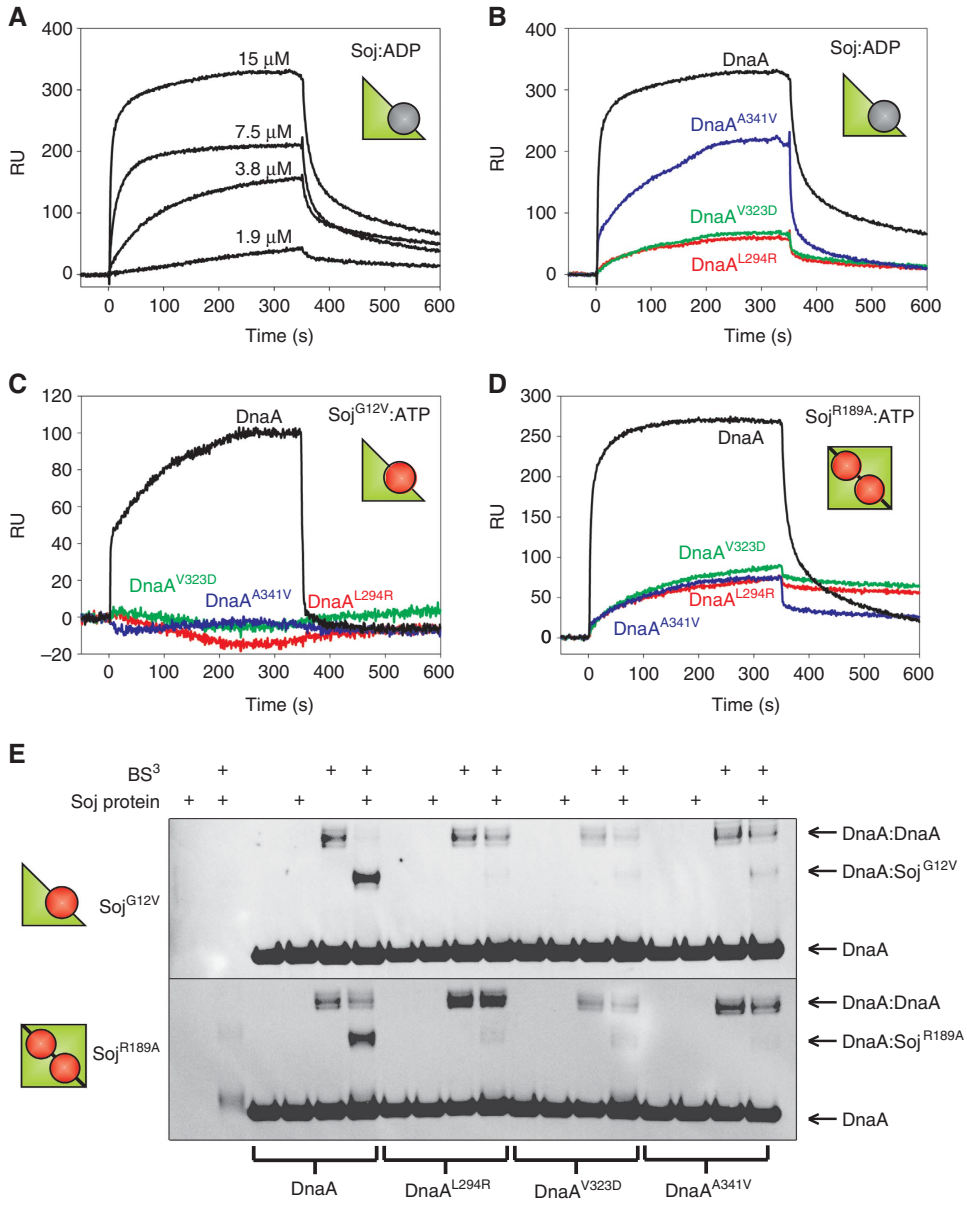

Figure 2 Soj directly interacts with DnaA. (**A**–**D**) SPR sensorgrams. DnaA proteins were immobilized onto the SPR chip surface via a unique C-terminal cysteine residue to create a homogenous surface. Cartoon representations of Soj are shown to indicate the quaternary state of various proteins. (**A**) Two-fold serial dilution of wild-type Soj:ADP injected over DnaA^{H485C}. (**B**–**D**) The indicated Soj proteins (15 μM) were injected over wild-type and mutant DnaA surfaces starting at time zero for 360 s. (**E**) *In vitro* crosslinking assay using the primary amine-specific crosslinker (BS^3) in the presence of DNA (pBSoriC4; 3 nM) and ATP (2 mM). Protein complexes were separated by SDS–PAGE and the DnaA protein was visualized by western blotting. Pluses located above each lane indicate the presence of BS^3 and/or Soj protein (32 μM). The identity of the DnaA proteins (3 μM) are indicated below. The identity of the Soj proteins is indicated to the left of each gel.

of *dnaA*, including the *dnaA* promoter and all of *oriC*), all of the isolated DnaA^{Hyp} substitutions were located within the AAA+ motif of DnaA, while all of the DnaA^{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^{Hyp} 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^{Hyp} 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^{Hyp} 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

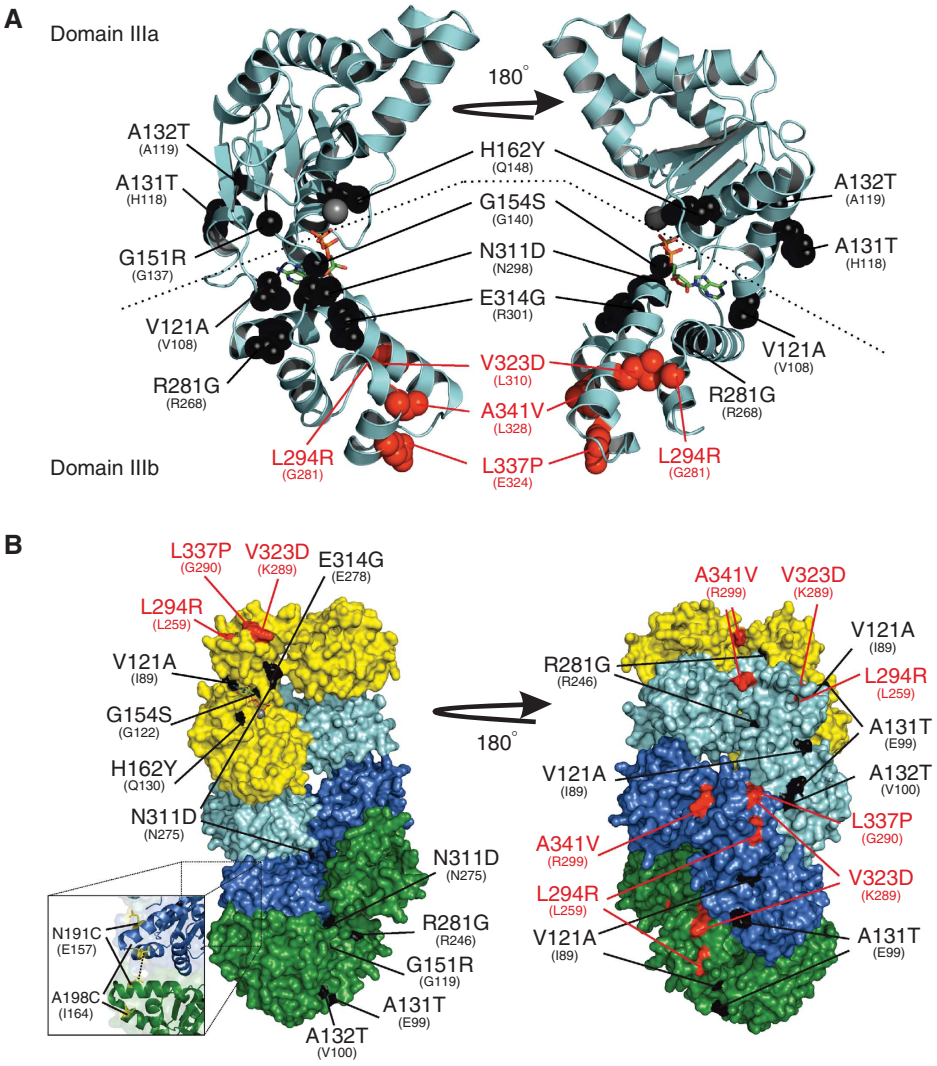

Figure 3 The DnaA hypermorph and suppressor substitutions are located in domain III. (**A**) A cartoon representation of monomeric DnaA from the *T. maritima* crystal structure (PDB ID: 2Z4S) bound to ADP (stick). Domains IIIa and IIIb are separated by a dashed line. The Soj^{G12V} hypermorph (black) 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.

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+ 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 (~9 Å) to the A198 residue of the adjacent monomer. DnaA^{N191C,A198C} (hereafter referred to as DnaA^{cc}) 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^{CC} 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. Critically, mutation of the arginine finger residue (R264A) that coordinates the γ-phosphate of the ATP from the neighbouring DnaA molecule (Erzberger *et al*, 2006) greatly

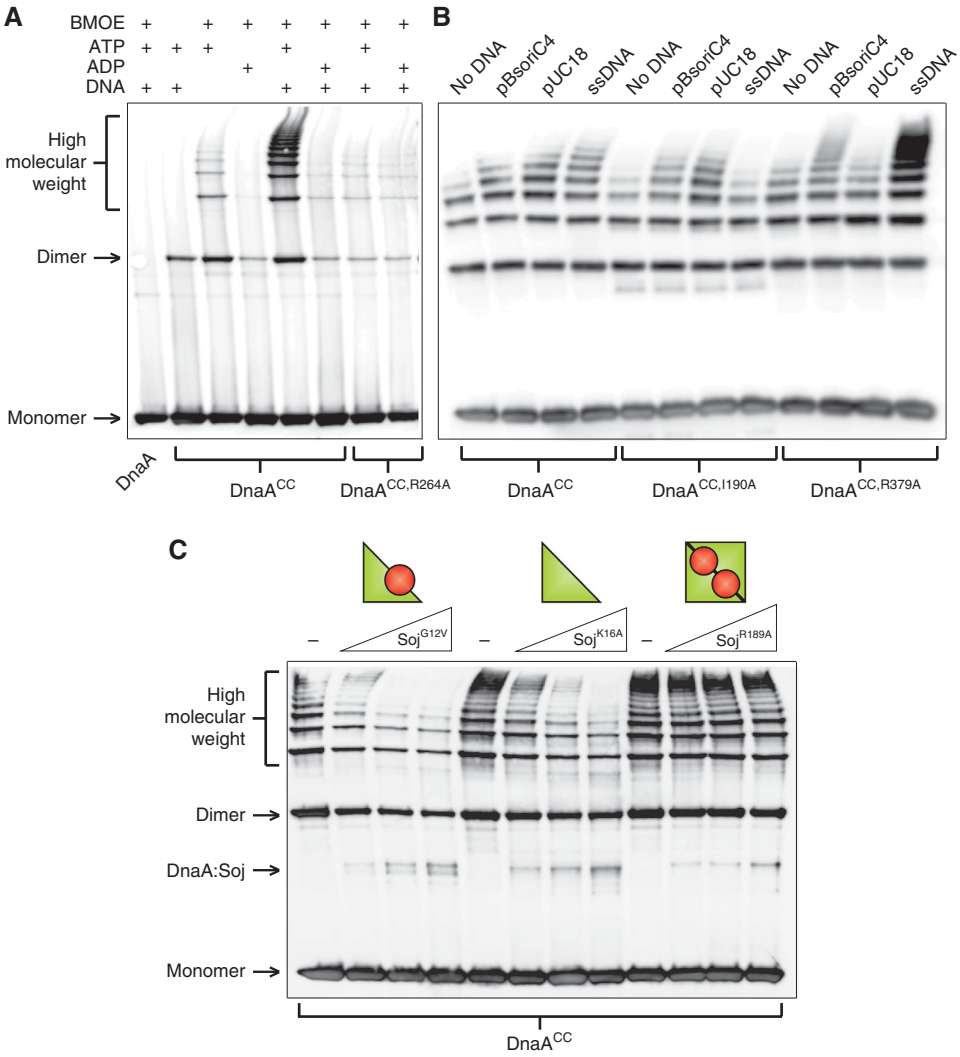

Figure 4 Monomeric Soj inhibits DnaA oligomerization *in vitro*. (**A**) *In vitro* oligomer formation assays using the cysteine-specific crosslinker BMOE. DnaA proteins were incubated in oligomer formation buffer for 15 min prior to the addition of BMOE. Pluses located above each lane indicate the presence 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.

diminished DnaA oligomerization (lanes 7 and 8). 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 pUC18 plasmid. Interestingly, oligomerization of $DnaA^{CC,R379A}$ was stimulated by a plasmid that harboured *oriC* (Figure 4B). These results correlated with the ability of $DnaA^{CC,R379A}$ 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 $DnaA^{CC,R379A}$ to a greater degree than $DnaA^{CC}$. 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^{G12V} 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^{K16A} (monomeric) and Soj^{R189A} (dimeric) proteins was also investigated. While Soj^{K16A} clearly disrupted DnaA oligomer formation, Soj^{R189A} 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 $DnaA^{L294R}$ and $DnaA^{V323D}$ were highly resistant to Soj^{G12V} activity (Figure 5A). In comparison, oligomers formed by $DnaA^{A341V}$ were partially susceptible to Soj^{G12V}, consistent with the intermediate response observed between the two proteins by SPR analysis (Figures 2B and 5A). Importantly, all of the $DnaA^{Sup}$ proteins were found to form oligomers under the same conditions as wild type (Supplementary Figure S6A). These results indicate that the effect of Soj^{G12V} 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^{G12V} addition are highly resistant to Soj^{G12V} inhibition (lane 3) when compared with oligomers formed after Soj^{G12V} 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^{G12V} only acted on one of these complexes. Soj^{G12V} was found to disrupt oligomerization of both $DnaA^{CC}$ in the presence of single-stranded DNA and $DnaA^{CC,I190A}$ 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^{G12V} inhibition of DnaA oligomerization was dependent on this domain I self-interaction, we created a truncated version of $DnaA^{CC}$ that lacked domains I and II ($DnaA^{III/IV,CC}$). As expected from the interaction analysis described above, the $DnaA^{III/IV}$ 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^{G12V} but were not affected by dimeric Soj^{R189A} (Supplementary Figure S7B). These results indicate that the oligomerization activity observed for the $DnaA^{CC}$ 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^{G12V} inhibition of DnaA is independent of DnaA ATP binding, ATP hydrolysis, and DNA-binding activities

Because oligomerization of $DnaA^{CC}$ 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 ($DnaA^{R313A}$) with α-P^{32} ATP in the presence and absence of Soj^{K16A}. The $DnaA^{R313A}$ protein was utilized to prevent hydrolysis of the bound ATP during the experiment (Supplementary Figure S8A) and Soj^{K16A} 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 $DnaA^{R313A}$ in the presence or absence of Soj^{K16A} (Figure 5C), indicating that monomeric Soj does not inhibit ATP binding. Furthermore, Soj^{G12V} did not stimulate the ATPase activity of wild-type DnaA (Supplementary Figure S8A) and oligomerization of the hydrolysis deficient $DnaA^{CC,R313A}$ protein remained sensitive to Soj^{G12V} 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^{G12V} was capable of inhibiting $DnaA^{CC}$ oligomer formation in the absence of DNA. As before, Soj^{G12V} inhibited $DnaA^{CC}$ oligomerization but had little effect on $DnaA^{CC,V323D}$ (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.

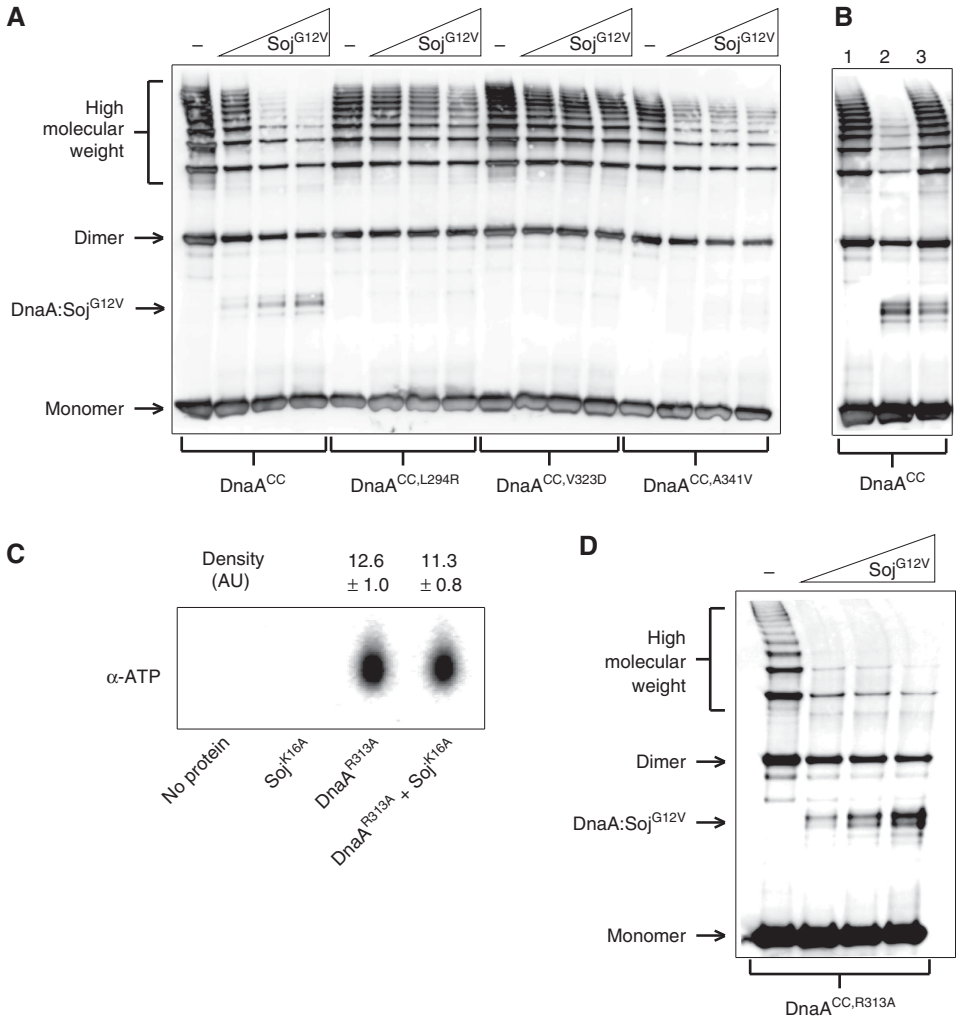

Figure 5 Monomeric Soj specifically prevents DnaA oligomerization *in vitro*. (**A**) *In vitro* oligomer formation assay with $DnaA^{Sup}$ proteins. DnaA proteins (3 μM) and Soj^{G12V} (12, 24, and 36 μM) were incubated in oligomer formation buffer in the presence of ATP (2 mM) and DNA (pBsoriC4; 3 nM) for 15 min prior to the addition of BMOE. DnaA proteins were separated by SDS–PAGE and visualized by western blotting. (**B**) Monomeric Soj is unable to disassemble pre-formed DnaA oligmers. 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^{G12V} was added at $T = 0$ and DnaA added at $T = 13$. Lane 3, DnaA was added at $T = 0$ and Soj^{G12V} added at $T = 13$. (**C**) Monomeric Soj does not affect DnaA ATP binding. $DnaA^{R313A}$ (3 μM) and/or Soj^{K16A} (36 μM) were incubated with α-P^{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. $DnaA^{R313A}$ (3 μM) and Soj^{G12V} (12, 24, and 36 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.

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 $dnaA^{N191C,A198C}$ ($dnaA^{CC}$) and BMOE was used to crosslink $DnaA^{CC}$ proteins in intact cells that were harvested during exponential growth. The strain harbouring $dnaA^{CC}$ mildly overinitated 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+ domains (Figure 6A–C). Thus, the site-specific crosslinking assay captures DnaA oligomers within *B. subtilis* cells that likely correspond to the complexes observed *in vitro*.

Figure 6A shows that overexpression of monomeric Soj^{G12V} resulted in an ~50% decrease in the percentage of $DnaA^{CC}$ 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

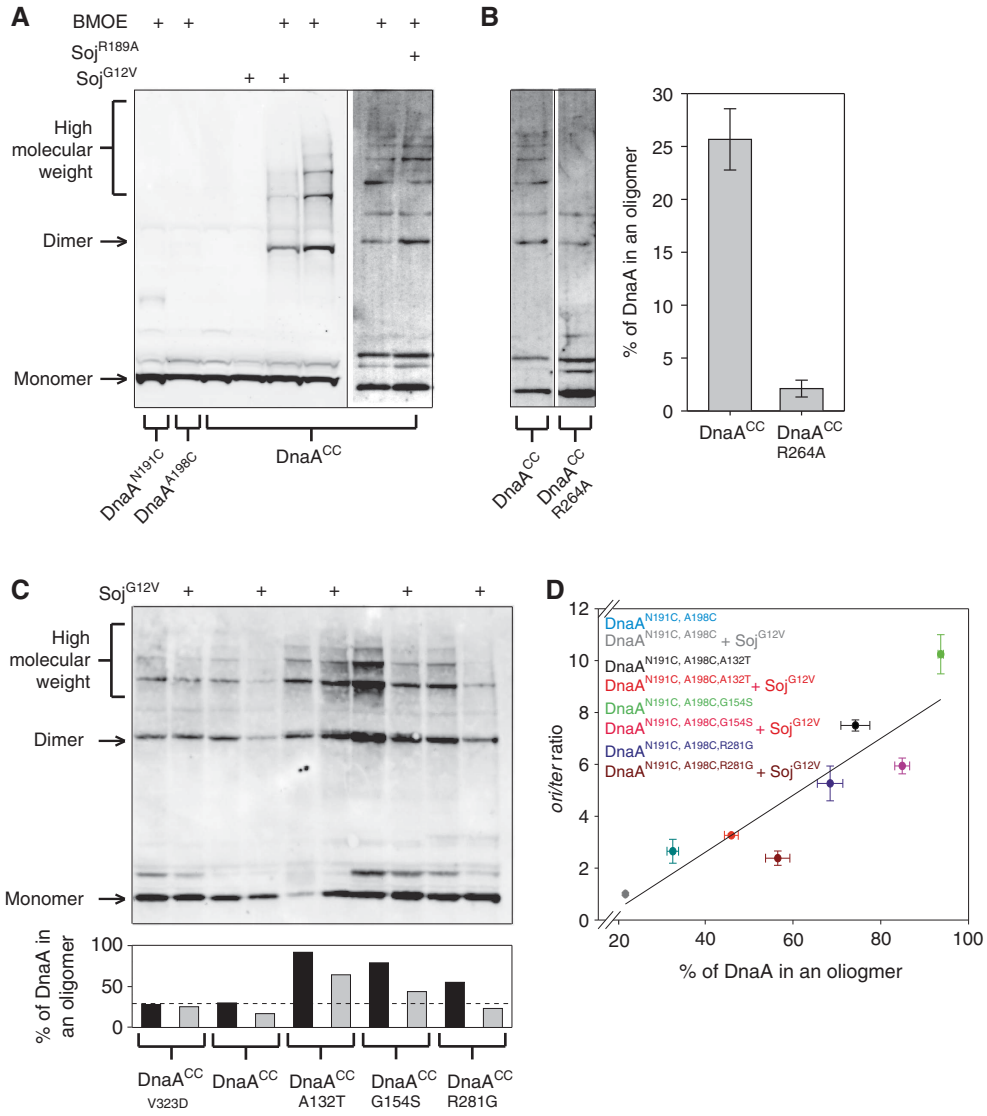

Figure 6 The DnaA oligomer forms *in vivo* and is inhibited by monomeric Soj. (**A**) A DnaA oligomer can be isolated *in vivo* and is specifically inhibited by monomeric Soj. Cells were grown in crosslinking medium at 30°C to an A_{600} of 0.5, washed, and resuspended in *in vivo* crosslinking buffer in the presence of BMOE. Where indicated, 1% xylose was added or removed (in the case of the $DnaA^{Hyp}$ strains) at an OD_{600} of 0.1 to modulate Soj protein expression. The identity of DnaA and/or Soj proteins are indicated below and above each lane, respectively. Proteins were separated by SDS–PAGE and DnaA was visualized by western blotting. $DnaA^{CC}$ Soj^{G12V} (sGJS006), $DnaA^{N198C}$ (sGJS021), $DnaA^{N191C}$ (sGJS022), $DnaA^{CC}$ Soj^{R189A} (sGJS033). (**B**) The DnaA oligomer is dependent on the arginine finger (R264) *in vivo*. Because the arginine finger mutation (*dnaA*^{R264A}) is lethal (Duderstadt *et al*, 2010), the allele was introduced into a strain harbouring a plasmid origin (*oriN*) that allows viability in the absence of DnaA and/or *oriC* (Hassan *et al*, 1997). The identity of the DnaA proteins expressed are indicated below the respective lanes. $DnaA^{CC}$ (sGJS051), $DnaA^{CC,R264A}$ (sGJS052). Quantification of three biological repeats is shown. Error bars represent the standard deviation of the data. (**C**) The hypermorphic DnaA mutants have an increased propensity to form oligomers *in vivo*. Reaction conditions and figure annotations are as described in (**A**). The bar chart below shows the quantification of the gel. DnaA was defined as being in a helix if found in a dimer or higher molecular weight complex. $DnaA^{CC}$ (sGJS006), $DnaA^{CC,V323D}$ (sGJS008), $DnaA^{CC,A132T}$ (sGJS036), $DnaA^{CC,G154S}$ (sGJS037), $DnaA^{CC,R281G}$ (sGJS038). (**D**) The percentage of DnaA found in an oligomer correlates with initiation frequency. The reactions in (**C**) (with the exception of $DnaA^{V323D}$) were repeated in triplicate, the lanes quantified using ImageJ, and the percentage of DnaA found in the helix calculated as described above. Error bars represent the s.e.m. of the data. Concurrently, the *oriC*-to-terminus ratios were determined by MFA. Errors bars represent the standard deviation of the data. Linear regression analysis yields an R^2 value of 0.82 while the Pearson product moment correlation coefficient is 0.903 (P-value = 0.00212).

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 *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 *in vivo*, 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 fulfil 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+ motifs 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^{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^{CC} oligomer formation assay captures a helical structure of *B. subtilis* DnaA.

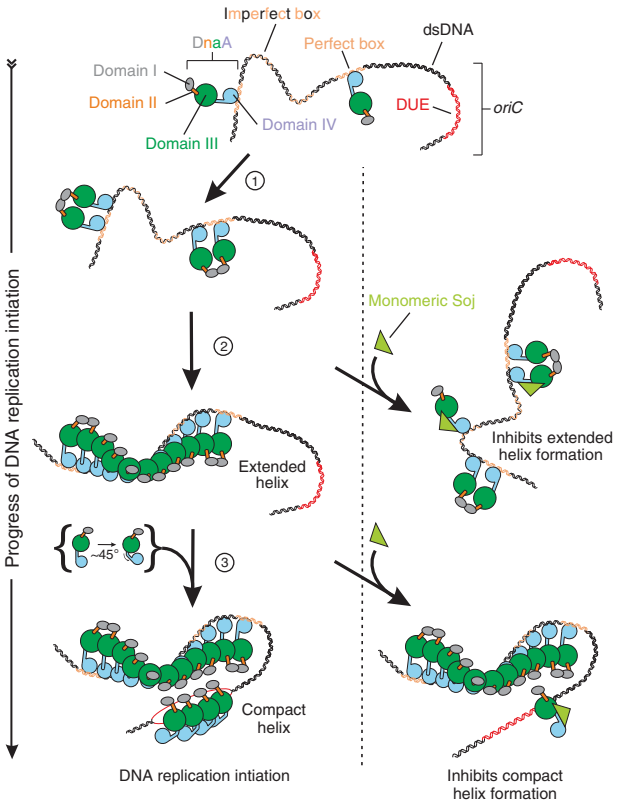

Figure 7 Model of monomeric Soj regulation of DnaA during DNA replication initiation. DnaA is stably bound to its high-affinity sites during most of the cell cycle. At initiation, additional DnaA molecules are recruited through a domain I interaction (step 1). At this point, Soj could interact with DnaA to prevent the structural transition necessary for it to form a helix on double-stranded DNA (step 2). This nucleoprotein complex facilitates the unwinding of the DUE and the loading of a compact helix onto ssDNA (step 3). Soj could interact with this newly recruited DnaA preventing the bending of the α-helix linking domains III and IV.

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 *et al*, 2006; Ozaki *et al*, 2008; Duderstadt *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 *in vitro* and replication origin function *in vivo* (Duderstadt *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 *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 *et al*, 2002, 2006; Ozaki *et al*, 2008; Duderstadt *et al*, 2010), that the α-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 *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 at 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 (Supplementry 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 IIIb 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^{G12V} 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 (Gorbatyuk 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 for the construction of all plasmids, and strain BL21 (DE3) pLysS (Stratagene) was used to express all proteins. The plasmid pET21-d (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 $MgCl_2$) 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 BS^3 (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 left 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–NH_4–citrate, 0.5% glucose, 6 mM $MgSO_4$, 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 grown at 30°C until the A_{600} 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 A_{600} 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 sonicated 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).

Acknowledgements

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Author contributions: Experimental design, data acquisition, and data analysis were performed by GS and HM. The manuscript was co-written by GS, JE, and HM.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abe Y, Jo T, Matsuda Y, Matsunaga C, Katayama T, Ueda T (2007) Structure and function of DnaA N-terminal domains: specific sites and mechanisms in inter-DnaA interaction and in DnaB helicase loading on *oriC*. *J Biol Chem* **282:** 17816–17827

Bowers JL, Randell JC, Chen S, Bell SP (2004) ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. *Mol Cell* **16:** 967–978

Carr KM, Kaguni JM (2001) Stoichiometry of DnaA and DnaB protein in initiation at the *Escherichia coli* chromosomal origin. *J Biol Chem* **276:** 44919–44925

Cassler MR, Grimwade JE, Leonard AC (1995) Cell cycle-specific changes in nucleoprotein complexes at a chromosomal replication origin. *EMBO J* **14:** 5833–5841

Chen LL, Rosa JJ, Turner S, Pepinsky RB (1991) Production of multimeric forms of CD4 through a sugar-based crosslinking strategy. *J Biol Chem* **266:** 18237–18243

Clarey MG, Erzberger JP, Grob P, Leschziner AE, Berger JM, Nogales E, Botchan M (2006) Nucleotide-dependent conformational changes in the DnaA-like core of the origin recognition complex. *Nat Struct Mol Biol* **13:** 684–690

Duderstadt KE, Chuang K, Berger JM (2011) DNA stretching by bacterial initiators promotes replication origin opening. *Nature* **478:** 209–213

Duderstadt KE, Mott ML, Crisona NJ, Chuang K, Yang H, Berger JM (2010) Origin remodeling and opening in bacteria relies on distinct assembly states of the DnaA initiator. *J Biol Chem* **285:** 28229–28239

Erzberger JP, Mott ML, Berger JM (2006) Structural basis for ATP-dependent DnaA assembly and replication-origin remodeling. *Nat Struct Mol Biol* **13:** 676–683

Erzberger JP, Pirruccello MM, Berger JM (2002) The structure of bacterial DnaA: implications for general mechanisms underlying DNA replication initiation. *EMBO J* **21:** 4763–4773

Felczak MM, Simmons LA, Kaguni JM (2005) An essential tryptophan of Escherichia coli DnaA protein functions in oligomerization at the *E. coli* replication origin. *J Biol Chem* **280:** 24627–24633

Fujikawa N, Kurumizaka H, Nureki O, Terada T, Shirouzu M, Katayama T, Yokoyama S (2003) Structural basis of replication origin recognition by the DnaA protein. *Nucleic Acids Res* **31:** 2077–2086

Funnell BE, Baker TA, Kornberg A (1987) *In vitro* assembly of a prepriming complex at the origin of the *Escherichia coli* chromosome. *J Biol Chem* **262:** 10327–10334

Gerdes K, Howard M, Szardenings F (2010) Pushing and pulling in prokaryotic DNA segregation. *Cell* **141:** 927–942

Gorbatyuk B, Marczynski GT (2005) Regulated degradation of chromosome replication proteins DnaA and CtrA in *Caulobacter crescentus*. *Mol Microbiol* **55:** 1233–1245

Gruber S, Errington J (2009) Recruitment of condensin to replication origin regions by ParB/SpoOJ promotes chromosome segregation in *B. subtilis*. *Cell* **137:** 685–696

Hassan AK, Moriya S, Ogura M, Tanaka T, Kawamura F, Ogasawara N (1997) Suppression of initiation defects of chromosome replication in *Bacillus subtilis* dnaA and *oriC*-deleted mutants by integration of a plasmid replicon into the chromosomes. *J Bacteriol* **179:** 2494–2502

Hester CM, Lutkenhaus J (2007) Soj (ParA) DNA binding is mediated by conserved arginines and is essential for plasmid segregation. *Proc Natl Acad Sci USA* **104:** 20326–20331

Ireton K, Gunther NWt, Grossman AD (1994) spo0J is required for normal chromosome segregation as well as the initiation of sporulation in *Bacillus subtilis*. *J Bacteriol* **176:** 5320–5329

Katayama T, Ozaki S, Keyamura K, Fujimitsu K (2010) Regulation of the replication cycle: conserved and diverse regulatory systems for DnaA and *oriC*. *Nat Rev Microbiol* **8:** 163–170

Kawakami H, Katayama T (2010) DnaA, ORC, and Cdc6: similarity beyond the domains of life and diversity. *Biochem Cell Biol* **88:** 49–62

Kurokawa K, Nishida S, Emoto A, Sekimizu K, Katayama T (1999) Replication cycle-coordinated change of the adenine nucleotide-bound forms of DnaA protein in Escherichia coli. *EMBO J* **18:** 6642–6652

Lee PS, Grossman AD (2006) The chromosome partitioning proteins Soj (ParA) and Spo0J (ParB) contribute to accurate chromosome partitioning, separation of replicated sister origins, and regulation of replication initiation in *Bacillus subtilis*. *Mol Microbiol* **60:** 853–869

Leonard AC, Grimwade JE (2005) Building a bacterial orisome: emergence of new regulatory features for replication origin unwinding. *Mol Microbiol* **55:** 978–985

Leonard AC, Grimwade JE (2010) Regulating DnaA complex assembly: it is time to fill the gaps. *Curr Opin Microbiol* **13:** 766–772

Leonard TA, Butler PJ, Lowe J (2005) Bacterial chromosome segregation: structure and DNA binding of the Soj dimer—a conserved biological switch. *EMBO J* **24:** 270–282

Livny J, Yamaichi Y, Waldor MK (2007) Distribution of centromere-like parS sites in bacteria: insights from comparative genomics. *J Bacteriol* **189:** 8693–8703

Miller DT, Grimwade JE, Betteridge T, Rozgaja T, Torgue JJ, Leonard AC (2009) Bacterial origin recognition complexes direct assembly of higher-order DnaA oligomeric structures. *Proc Natl Acad Sci USA* **106:** 18479–18484

Molt KL, Sutera Jr VA, Moore KK, Lovett ST (2009) A role for nonessential domain II of initiator protein, DnaA, in replication control. *Genetics* **183:** 39–49

Mott ML, Berger JM (2007) DNA replication initiation: mechanisms and regulation in bacteria. *Nat Rev Microbiol* **5:** 343–354

Murray H, Errington J (2008) Dynamic control of the DNA replication initiation protein DnaA by Soj/ParA. *Cell* **135:** 74–84

Ozaki S, Katayama T (2009) DnaA structure, function, and dynamics in the initiation at the chromosomal origin. *Plasmid* **62:** 71–82

Ozaki S, Katayama T (2011) Highly organized DnaA-oriC complexes recruit the single-stranded DNA for replication initiation. *Nucleic Acids Res* (advance online publication 3 November 2011; doi:10.1093/nar/gkr832)

Ozaki S, Kawakami H, Nakamura K, Fujikawa N, Kagawa W, Park SY, Yokoyama S, Kurumizaka H, Katayama T (2008) A common mechanism for the ATP-DnaA-dependent formation of open complexes at the replication origin. *J Biol Chem* **283:** 8351–8362

Ptacin JL, Lee SF, Garner EC, Toro E, Eckart M, Comolli LR, Moerner WE, Shapiro L (2010) A spindle-like apparatus guides bacterial chromosome segregation. *Nat Cell Biol* **12:** 791–798

Rozgaja TA, Grimwade JE, Iqbal M, Czerwonka C, Vora M, Leonard AC (2011) Two oppositely-oriented arrays of low affinity recognition sites in *oriC* guide progressive binding of DnaA during E. coli pre-RC assembly. *Mol Microbiol* **82:** 475–488

Schepers A, Diffley JF (2001) Mutational analysis of conserved sequence motifs in the budding yeast Cdc6 protein. *J Mol Biol* **308:** 597–608

Scholefield G, Veening JW, Murray H (2010) DnaA and ORC: more than DNA replication initiators. *Trends Cell Biol* **21:** 188–194

Scholefield G, Whiting R, Errington J, Murray H (2011) Spo0J regulates the oligomeric state of Soj to trigger its switch from an activator to an inhibitor of DNA replication initiation. *Mol Microbiol* **79:** 1089–1100

Simmons LA, Felczak M, Kaguni JM (2003) DnaA Protein of *Escherichia coli*: oligomerization at the *E. coli* chromosomal origin is required for initiation and involves specific N-terminal amino acids. *Mol Microbiol* **49:** 849–858

Speck C, Messer W (2001) Mechanism of origin unwinding: sequential binding of DnaA to double- and single-stranded DNA. *EMBO J* **20:** 1469–1476

Sullivan NL, Marquis KA, Rudner DZ (2009) Recruitment of SMC by ParB-parS organizes the origin region and promotes efficient chromosome segregation. *Cell* **137:** 697–707

Sutton MD, Carr KM, Vicente M, Kaguni JM (1998) Escherichia coli DnaA protein. The N-terminal domain and loading of DnaB helicase at the *E. coli* chromosomal origin. *J Biol Chem* **273:** 34255–34262

Tucker PA, Sallai L (2007) The AAA+ superfamily—a myriad of motions. *Curr Opin Struct Biol* **17:** 641–652

Weigel C, Schmidt A, Seitz H, Tungler D, Welzeck M, Messer W (1999) The N-terminus promotes oligomerization of the Escherichia coli initiator protein DnaA. *Mol Microbiol* **34:** 53–66

Wigley DB (2009) ORC proteins: marking the start. *Curr Opin Struct Biol* **19:** 72–78