Online Methods

Expression and Purification of DnaA

Residues 76-399 of *A. aeolicus* DnaA (containing the AAA+ and Duplex-DNA binding regions) were expressed as a TEV-protease cleavable His_6-MBP fusion and purified as previously described[25]. As a final purification step, untagged DnaA proteins (from TEV cleavage) were run over an S-200 size-exclusion column (GE) in gel-filtration buffer (50 mM HEPES pH 7.5, 500 mM KCl, 10% (v/v) glycerol, 5 mM $MgCl_2$, 100 μM ADP). Monomeric species were pooled, concentrated and flash-frozen for storage at -80°C. For mutagenesis studies, changes were introduced into the His_6-MBP-DnaA construct using QuickChange (Stratagene).

Crystallization and DNA Soaking

Following gel filtration of DnaA in crystallization buffer (20 mM HEPES pH 7.5, 250 mM KCl, 250 mM KBr, 10% (v/v) glycerol, 10 mM $MgCl_2$, 100 μM AMPPCP), monomeric species were pooled, concentrated to 10 mg/ml at 4°C, and flash-frozen for storage at -80°C. Crystallization by hanging-drop vapor diffusion was performed by mixing 1.3 μL of freshly-thawed DnaA in crystallization buffer and 1 μL of well solution (15-35 mM Sodium Cacodylate pH 6.5, 26% 1,2-propandiol and 1-2% PEG 2000 MME) at 18°C. Large rod-like crystals appeared within one to two weeks and reached maximal size around three weeks. Crystals were transferred by looping to a low-salt soaking solution (20 mM HEPES pH 7.5, 30 mM Sodium Cacodylate pH 6.5, 10% (v/v) glycerol, 10 mM $MgCl_2$, 26% 1,2-propandiol, 2.5% PEG 2000 MME and 200 μM AMPPCP) containing 5 mM ssDNA. After 6 h, crystals were looped and transferred to a second drop of soaking solution containing 5 mM ssDNA, and left overnight to ensure both complete removal of remaining salt and allow time for binding. The crystals were then looped and flash frozen in liquid nitrogen in preparation for data collection.

Previous biochemical studies revealed no apparent sequence preference for ssDNA by *A. aeolicus* DnaA[25], so DNAs of various sequences and lengths were all individually tested (dT_n (n = 3 to 12) and dA_n (n = 3 to 12), Elim Biopharmaceuticals). Data collection and structure determination revealed that dA_{12} generated the strongest electron density, although similar, albeit weaker and less connected density, was observed for dT oligos and smaller dA substrates.

Data Collection and Structure Determination

Data were collected at Beamline 8.3.1 at the Advanced Light Source (ALS)[44] and processed using HKL-2000[45]. Crystals belong to the space group $P2_12_12_1$, with dA_{12} soaked crystals having unit cell dimensions *a* = 99.8 Å, *b* = 114.2 Å and *c* = 201.3 Å (**Table S1**). Data were phased using molecular replacement (MR) as implemented in PHENIX[46], using a DNA-free DnaA tetramer as a search model (PDB ID 2HCB)[14]. Initial F_o-F_c electron density maps containing clear density for DNA were generated using rigid body and grouped B-factor refinement with PHENIX[46]. Further refinement was conducted using multidomain, NCS-restrained, simulated annealing in PHENIX[46], 4-fold multidomain NCS averaging with a custom solvent mask (including the region of DNA binding), density modification using resolve[47], and manual model building in COOT[48]. During the final stages of refinement, 4-fold multidomain NCS and secondary structure restraints were retained for AMPPCP and the entire protein except residues 255 to 265, which differed between chains as a result of crystal packing interactions. Composite, simulated-annealing omit maps generated with CNS[49], were used as a guide for building with COOT. DNA and waters were manually added to the model, and final rounds of refinement with PHENIX were conducted with grouped B-factor modeling, as well as NCS restraints and TLS modeling of individual protein domains (comprising three TLS groups total: the AAA+-core (aa 76-241) plus AMPPCP; the AAA+ α-helical "lid" (aa 242-254 and 266-241); and the duplex-DNA binding domain (aa 291-399)). All panels of figures with renderings of structures and electron density were prepared with PyMol[50].

The final model contains one DnaA tetramer bound to one dA_{12} per asymmetric unit. A clear 5' or 3' break between successive dA_{12} substrates was not present in the electron density, indicating that during the soaking procedure, different DNA molecules bound in multiple registers to consecutive DnaA protomers throughout the crystal. Accordingly, a terminal 5' phosphate, which was not present in the substrate used for soaking, was added to the modeled dA_{12} DNA.

Polarity of DNA Binding

During refinement, DNA was initially modeled independently into the DnaA pore with one of two possible polarities. Compared to the 5→3' polarity presented in the paper, refinement of the model with the dA_{12} substrate running 3'→5' (from the arginine finger side to the nucleotide-binding face of a DnaA protomer) resulted in only marginally higher R_{work} and R_{free} values (~0.1%), but also the appearance of off-model positive difference density and on-model negative difference density in F_o-F_c maps (the model as presented in the paper did not display such features). Simultaneous refinement with two DNAs, each at half occupancy, with opposing polarities of the dA_{12} substrate resulted in ~0.3% higher R_{work} and R_{free} values, and again showed unfavorable difference density in F_o-F_c maps.

Recognizing that these differences, while consistent with our build, were subtle and did not definitively resolve the ssDNA binding polarity to DnaA, we set out to further test our assignment. To this end, we designed and had synthesized (by Trilink BioTechnologies) two specialized, di-adenosyl nucleotide substrates that would give rise to a clear distinction in binding orientation: 5'-p(Br-A)pAp and 5'-p(ε-A)pAp, where "Br-A" indicates a bromo-deoxyadenosine label, "ε-A" indicates an etheno-deoxyadenosine label, and "p" indicates a phosphate moiety. Soaking of crystals with these dinucleotide substrates was performed as described for ssDNA substrates (we note that in our soaking trials with oligos as short as dA_3, we observed density associated with DnaA protomers consistent with that seen

for the trinucleotide repeats when using dA_{12}). Unfortunately, data collected with the ε-A substituted oligo yielded maps with density for dinucleotides bound to each monomer but additional density for the EthenoA was not clearly visible, likely due to the low ~3.4 Å resolution limit of the DnaA crystals. At the same time, SAD datasets collected with the Br-A substituted oligo did not yield useful maps, due either to the weak diffraction of the crystals (and accompanying radiation damage as we attempted to maximize data signal-to-noise at the bromine absorption maximum[51]), to incomplete bromine labeling, or both. We note that we carried out soaks with longer Br-dA labeled (and Br-dU labeled) oligos, but these efforts were not successful again because of weak diffraction. Additional experiments to test the orientation (e.g., using labeled oligo/protein pairs and FRET) were considered, but ruled out due to the small binding site size for substrate DNA, and an inability to find a suitable pair of labeling sites that could report on differing binding orientations.

As a consequence, although our data are supportive of the polarity presented in our model, we cannot definitively rule out the possibility that ssDNA might also be binding to DnaA in the crystal in an opposing direction. Nonetheless, several findings support the idea that DnaA binds single-stranded DNA in a defined orientation that is consistent with the direction suggested here. For example, following nucleoprotein complex formation on *oriC*, DnaA melts AT-rich regions in the DUE[12]; two independent reports have found that *E. coli* DnaA binds specifically to only one strand (the so-called "top" strand) of the DUE during this process[15, 16]. The importance of DnaA binding polarity becomes clear during the next stage of initiation, when the DnaB helicase is loaded. Modeling studies based on the known DnaB translocation polarity (5'→3') and known pairwise interactions between DnaB, DnaC and DnaA, have suggested that top-strand loading involves a direct interaction between DnaA and DnaC that has been observed biochemically and depends on the AAA+ domains of the two proteins[19]. Since AAA+ domains assemble with a defined orientation, in which the arginine finger face of one protomer points into the nucleotide binding face of a second

subunit, it follows that DnaA molecules likely position themselves on the top strand with only one of their two AAA+ domain surfaces presented to DnaC. While the polarity of the DnaA-DnaC interaction has not been established, a mutation on the arginine finger face of the *E. coli* DnaA AAA+ domain, R281A, is reported to disrupt helicase loading, but not *oriC* melting[52]; this finding suggests that DnaA interacts with DnaC using its arginine-finger face. In our structures, the 5' end of the modeled DNA resides near the arginine finger face of DnaA, a configuration consistent with these data.

ssDNA Extension Assay

Extension of dT_{21} oligonucleotides labeled with Cy3 and Cy5 (FR-dT_{21}) by DnaA was monitored by Förester Resonance Energy Transfer (FRET) using a FluoroMax-4 (Horiba Jobin Yvon) spectrofluorimeter. Measurements were carried out at 25°C in 20 μL with 25 nM of FR-dT_{21} and either 10 μM of DnaA in DnaA extension buffer (50 mM HEPES pH 7.5, 125 mM KCl, 2% (v/v) glycerol, 10 mM $MgCl_2$ and 2 mM ADP or ADP•BeF_3) or 10 μM of RecA in RecA extension buffer (25 mM Tris-acetate pH 7.5, 100 mM Na-acetate, 10 mM Mg-acetate, 1 mM DTT and 2 mM ADP or ATPγS). Emission scans from 545 to 700 nm were collected following the excitation of Cy3 at 530 nm, divided by the excitation intensity, and then corrected for the wavelength-dependent sensitivity of the detector. FRET efficiencies and distances were determined by comparing the Cy3 fluorescence from the doubly-labeled substrate (FR-dT_{21}) with the Cy3 fluorescence from a substrate only having a Cy3 label (C3-dT_{21}) under the same conditions.

Influence of Proteins on Dye Behavior

To ensure that all influences on dye behavior were properly considered when processing the FRET data from the DNA extension assay (**Figure 3**), the fluorescence and absorbance of each dye was monitored independently for each experimental condition. Emission and absorbance scans of substrates labeled only with the Cy3 donor (C3-dT_{21}) were collected in buffer alone, and with protein

in the presence of ATP mimics or ADP (**Figure S8c*ii*, S8c*iii*, S8d*ii* and S8d*iii***). Emission scans revealed pronounced protein- and nucleotide-dependent enhancement of donor fluorescence, but negligible differences in donor absorbance. Similar, but less significant effects have been observed previously for RecA at a concentration of 1 μM (comparable to our working concentration of 10 μM)[37]. A similar enhancement in acceptor fluorescence (and lack of effect on acceptor absorbance) also was observed in the doubly-labeled substrate, FR-dT_{21} (**Figure S8c*iv*, S8c*v*, S8d*iv* and S8d*v***). These effects are not surprising, as the spectral properties of fluorescent dyes are known to undergo dramatic variation depending on chemical environment[53, 54]. However, these controls also indicated that we needed to take into account additional corrections to obtain accurate distance measurements. In particular, the changes in donor fluorescence, but not donor absorbance, were indicative of changes in the donor quantum yield (Φ_D), which is used to calculate R_0 (Å), the distance corresponding to a FRET efficiency of 50%:

$$R_0 = 8.79 * 10^{-5} (J\kappa^2 n^{-4} \Phi_D)^{1/6}$$

where J = the spectral overlap between the donor emission and acceptor absorption; κ^2 = a geometric factor that depends on the orientation of donor and acceptor; n = the refractive index of the medium between donor and acceptor[54].

To determine the donor quantum yield under different experimental conditions, we used Rhodamine 6G as a standard for calibration, with an assumed quantum yield of 0.95 in EtOH[55]. To calculate the quantum yields seen in **Table S5**, we collected the fluorescence and absorbance of both the standard and the donor-only labeled substrate (C3-dT_{21}) in the corresponding RecA and DnaA buffers. We then used these data to determine ratios between the integrated fluorescence and absorbance, while correcting for the fractional absorbance at the excitation wavelengths used. Fluorescein in 0.1 M NaOH (known to have a quantum yield of 0.95[56]) was also measured as a control. To ensure reliable readings, all absorbance measurements were conducted with 1 μM dye/substrate, either alone or in the presence of 10 μM of the indicated protein.

All emission measurements were conducted with 25 nM dye/substrate, either alone or in the presence of 10 μM of the indicated protein. Since the presence of protein had no influence on dye absorbance (**Figure S8c*iii* and S8c*v***), the quantum yield of the donor in the presence of different proteins was determined simply by using its value in buffer, and multiplying by the observed changes in fluorescence. R_0 values were then calculated for each sample using the corresponding values for quantum yield.

Determination of FRET Efficiencies and DNA Length

To determine the efficiency of transfer (E) from the FRET data collected using the DNA extension assay (**Figure 3, S6, S8 and S9**), the emission of the donor from the donor-only labeled substrate (C3-dT_{21}, F_D) was compared to the emission of the donor from the doubly-labeled substrate FR-dT_{21} (F_{DA}) under equivalent experimental conditions as follows:

$$E = 1 - \frac{F_{DA}}{F_D}$$

[57]. The efficiencies for different samples can be found in **Table S5**. Solution distances were subsequently obtained using the relation:

$$R = R_0 \left[\frac{1-E}{E} \right]^{1/6}$$

[54], the values of which also can be found in **Table S5**.

DNA Strand Displacement Assay

The DnaA-dependent displacement of single strands from duplex-DNA was monitored using a Cy3 label on one of two strands (the "bottom" strand, **Table S3**). All measurements were carried out at 25°C in 80 μL of binding buffer containing 50 mM HEPES pH 7.5, 125 mM KCl, 2% (v/v) glycerol, 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 1 mM DTT and 2 mM ADP or ADP•BeF_3 (a non-hydrolyzable ATP analog that mimics the properties of ATP[25, 58]). After a short 2 min incubation of 25 nM duplex-DNA with various DnaA concentrations (**Figure 4**), 50 nM of unlabeled bottom strand was added for an additional 30 min

to capture displaced top strands. Following quenching with 10x stop buffer containing 200 mM EDTA, 10 mg/mL Proteinase K and 4%(v/w) SDS, displaced strands were separated on native polyacrylamide gels in Tris/boric acid/EDTA (TBE) buffer and visualized using a Molecular Dynamics Typhoon. The time dependence of DNA strand displacement by DnaA can be found in **Figure S11**. Sequences of substrates used can be found in **Table S3**.

ssDNA Binding Assay

Binding of 5' fluorescein-labeled dT_{25} oligonucleotides (F-dT_{25}, **Table S3**) to DnaA was monitored by fluorescence polarization using a Victor 3V (Perkin Elmer) multi-label plate reader (**Figure S4**). Measurements were carried out at 25°C in 20 μL of binding buffer containing 50 mM HEPES pH 7.5, 125 mM KCl, 2% (v/v) glycerol, 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 1 mM DTT and 2 mM ADP•BeF_3. The concentration of F-dT_{25} was held constant at 10 nM while the concentration of DnaA was varied. All data points represent the average of three independent measurements, with error bars representing the standard deviation between measurements. Binding curves were fit to the Hill equation to obtain $K_{d,app}$ values (**Table S4**) as described previously[25].

Oligomerization Characteristics of ssDNA-Binding Mutants

To confirm that ssDNA binding mutations did not affect the ATP-dependent oligomerization properties of DnaA, we employed a previously established glutaraldyhyde-crosslinking assay[25]. Crosslinking was performed by incubating 50 μg/ml of various AaDnaA proteins in 80 μL of a reaction buffer (50 mM HEPES pH 7.5, 10% (v/v) glycerol, 125 mM KCl, 5 mM $MgCl_2$, 2 mM DTT) containing 2 mM ADP•BeF_3 at 25°C for 5 minutes. Glutaraldehyde (Polysciences Inc.) was then added to 1 mM final concentration using 8.8 μL of a 10 mM stock. Reactions were incubated at 25°C for an additional 1 minute before quenching with 8 μL of 200 mM glycine followed by the addition of 30 μL of gel loading buffer (100 mM Tris pH 6.8, 24% (v/v) glycerol, 8% (w/v) SDS, 200 mM DTT, 0.02% (w/v) bromophenol blue). Crosslinked proteins were loaded in a

volume of 15 µL and separated on denaturing 4.5% polyacrylamide gels (80:1 acrylamide:bisacrylamide) in 0.1 M sodium phosphate, 0.1% SDS buffer (pH 7.2)[59, 60], and visualized by silver staining (**Figure S4b**).

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