DNA replication is tightly controlled to ensure accurate inheritance of genetic information. In all organisms, initiator proteins possessing AAA+ (ATPases associated with various cellular activities) domains bind replication origins to license new rounds of DNA synthesis. In bacteria the master initiator protein, DnaA, is highly conserved and has two crucial DNA binding activities. DnaA monomers recognize the replication origin (oriC) by binding double-stranded DNA sequences (DnaA-boxes); subsequently, DnaA filaments assemble and promote duplex unwinding by engaging and stretching a single DNA strand. While the specificity for duplex DnaA-boxes by DnaA has been appreciated for over 30 years, the sequence specificity for single-strand DNA binding has remained unknown. Here we identify a new indispensable bacterial replication origin element composed of a repeating trinucleotide motif that we term the DnaA-trio. We show that the function of the DnaA-trio is to stabilize DnaA filaments on a single DNA strand, thus providing essential precision to this binding mechanism. Bioinformatic analysis detects DnaA-trios in replication origins throughout the bacterial kingdom, indicating that this element is part of the core oriC structure. The discovery and characterization of the novel DnaA-trio extends our fundamental understanding of bacterial DNA replication initiation, and because of the conserved structure of AAA+ initiator proteins these findings raise the possibility of specific recognition motifs within replication origins of higher organisms.

The master bacterial DNA replication initiator, DnaA, is a highly conserved multifunctional protein that utilizes distinct domains to achieve its two key DNA binding activities. DnaA recognizes double-stranded (ds)DNA using a helix–turn–helix motif (domain IV), whereas an ATP-dependent DnaA filament interacts with a single DNA strand using residues within the initiator specific motif (ISM; an α-helical insertion that distinguishes the family of replication initiators) of the AAA+ domain (domain III) (Extended Data Fig. 1a–d). In contrast to DnaA, bacterial replication origins are diverse; they contain variable numbers of DnaA-boxes and seemingly lack a common architecture. Therefore, the sequence information within oriC that directs DnaA filament assembly onto a single DNA strand is unknown.

To investigate how DnaA filament formation could be localized to the DNA replication origin of Bacillus subtilis, we began by characterizing site-directed mutants of the DNA unwinding region in vivo (Fig. 1a and Extended Data Fig. 1e). To enable identification of essential sequences without selecting for suppressor mutations, we generated a strain in which DNA replication could initiate from the replication origin (oriN) integrated into the chromosome (Fig. 1b and Supplementary Information). Activity of oriN requires its cognate initiator protein, RepN; both of these factors act independently of oriC/DnaA. Expression of repN was placed under the control of a tightly regulated inducible promoter, thus permitting both the introduction of mutations into oriC and their subsequent analysis after removal of the inducer to shut off oriN activity (Fig. 1c and Extended Data Fig. 2).

At the B. subtilis replication origin, DNA unwinding by DnaA is detected downstream of DnaA-box elements and includes a sequence of 27 continuous A:T base pairs that is thought to facilitate DNA duplex opening (Fig. 1a). Surprisingly, we were able to delete the entire AT-rich sequence (∆27) without abolishing origin activity, although the mutant strain did display a slow growth phenotype indicating that the AT-cluster is required for efficient origin function (Fig. 1d). Interestingly, further deletions extending three or six base pairs (Δ30, Δ33) severely impaired oriC-dependent initiation (Fig. 1d), and a deletion series targeting the sequence between the GC-rich and AT-rich clusters confirmed that this region alone was essential for origin function (Fig. 1e). Scrambling this entire region also inhibited cell growth (t1–t6Scr), demonstrating that the specific sequence is required, rather than the spacing between the flanking elements (Fig. 1f). To explore this region in more detail, sequences were scrambled three base pairs at a time by exchanging each triplet for its complement. Phenotypic and marker frequency analyses revealed that disruption of sequences closest to the GC-cluster (t1Scr and t2Scr) caused the greatest defect in DNA replication initiation, indicating that the region proximal to the DnaA-boxes is most important for origin activity (Fig. 1f). Although mutagenesis of neither t4 nor t5 alone produced a detectable effect on DNA replication initiation under the conditions tested, they may become important when origin firing is suboptimal as was observed in the AT-cluster deletion mutant (Fig. 1d).

To determine whether this essential DNA sequence between the GC- and AT-clusters has a role in DNA melting per se, an open complex formation assay was performed. DnaA was incubated with oriC plasmids containing either the wild-type or scrambled sequence (t1–t6Scr), potassium permanganate was added to oxidize distorted bases within the DNA, and base modification was detected by primer extension. Scrambling the sequence inhibited open complex formation, indicating that this region is necessary for DnaA-dependent unwinding (Fig. 1g).

DnaA monomers are thought to bind DnaA-boxes before ATP-dependent filament formation. Using the strain capable of oriC-independent initiation, the seven DnaA-box sequences were individually scrambled to abolish DnaA binding. Culturing these strains in the absence of oriN activity revealed that mutation of DnaA-box6 severely inhibited growth, and mutation of DnaA-box7 resulted in a marked growth defect, while mutation of the remaining DnaA-boxes had no observable effect (Fig. 2a). Marker frequency analysis confirmed that mutation of DnaA-box7 markedly impaired origin activity, whereas mutation of the remaining DnaA-boxes resulted in only modest decreases in initiation frequency (Fig. 2a). These results indicate that DnaA-boxes proximal to the essential unwinding region are most critical for origin activity.

To directly test whether these DnaA-boxes promote DnaA filament assembly at the essential unwinding region we used a previously described DnaA filament formation assay. Here two cysteine residues are introduced within the AAA+ domain such that the protein remains functional and when the DnaA filament assemblies

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the cysteine residues from interacting protomers come into close proximity. DNA scaffolds were assembled using oligonucleotides, and the cysteine-specific crosslinker bis(maleimido)ethane (BMOE; 8 Å spacer arm) was used to capture the oligomeric species formed on each substrate.

Incubation of DnaA with duplex substrates containing DnaA-box6, DnaA-box7 and the GC-rich region produced a set of larger oligomers (Fig. 2c). Taken together, these results indicate that the assay was capturing DnaA filament formation on ssDNA (Fig. 2c, d and Extended Data Fig. 1d).

Critically, DnaA oligomer formation on the 5'-tailed substrate was specific. DnaA filament assembly was abolished when the DnaA-box sequences within the duplex region were scrambled and it was notably reduced when the single-stranded region was replaced with its complementary sequence (Fig. 2c). Taken together, these results suggest that DnaA filaments are loaded from duplex DnaA-boxes onto ssDNA bearing a 5'-tail. This model is consistent both with biochemical experiments showing that *Escherichia coli* DnaA preferentially interacts with the corresponding single-strand of its DNA unwinding element and with single molecule studies showing that *Aquifex aeolicus* DnaA filaments form with 3'→5' polarity.

DnaA oligomer size was proportional to the length of the 5'-tailed tail up to the formation of a heptamer, after which further DNA extension
did not promote longer filaments (Fig. 2e, f). We noted that this limit corresponded to a poly(A) tract in the DNA sequence and wondered whether this sequence inhibited DnaA filament formation. When the poly(A) tract was replaced by sequences from the beginning of the DNA unwinding region, DnaA oligomer length increased beyond a heptamer (Fig. 2e, f). This result suggests that the origin unwinding region is designed to limit DnaA filament formation to a precise position within oriC.

To identify a possible single-strand binding motif recognized by DnaA, individual base pairs within the essential unwinding region were inverted and origin activity was analysed in vivo. Marker frequency analysis revealed that altering either of two A:T base pairs, which were spaced three nucleotides apart from each other, resulted in the most significant loss of origin activity; in contrast the surrounding mutations had only modest effects (Fig. 3a). Re-examination of the unwinding region shows that A:T base pairs are spaced at three nucleotide intervals throughout this sequence (Fig. 1a). This observation is strikingly congruent with the mechanism proposed for binding of the DnaA filament to ssDNA, where each protomer engages a set of three nucleotides (Fig. 3b).

We hypothesized that an array of triplet nucleotide motifs recognized by DnaA are present within the unwinding region and that the motifs proximal to the DnaA-boxes are most important for origin activity. To test this model in vivo we created a set of nested deletions that removed either one or three base pairs (Extended Data Fig. 4). All of the single base-pair deletions significantly lowered the replication initiation frequency and several considerably inhibited cell growth (especially the same A:T base pairs noted above), whereas triplet deletions encompassing the single deletions had little or no effect (Fig. 3c, d). These results are consistent with the model that single base-pair deletions act both by disrupting a specific trinucleotide motif and by shifting the register of downstream trinucleotide motifs relative to the DnaA filament start point at the DnaA-boxes.

To test the model that the ssDNA binding motif is indeed a repeating trinucleotide, DnaA filament formation was analysed in vitro using tailed substrates that contained either single or triplet base deletions (Fig. 3e). Whereas deletion of one base produced shorter oligomers, deletion of three bases restored formation of full-length complexes.
Taken together with the in vivo deletions, these results indicate that DnaA filaments bind to ssDNA by recognizing a specific trinucleotide motif found within the unwinding region. We have termed this trinucleotide motif the ‘DnaA-trio’.

To define the precise sequence of the DnaA-trio, DnaA filament formation was observed using a series of DNA scaffolds in which the 5'-tails were extended by increments of one nucleotide. We observed that additional oligomeric species appeared after the following sequences were added: 3'-GAT-5', 3'-AAT-5' and 3'-GAA-5', suggesting that these triplets represent individual DnaA-trio motifs (Fig. 4a).

However, it was surprising that a longer oligomer was not formed after addition of the first 3'-GAT-5' motif proximal to the GC-cluster, since mutagenesis of this sequence in vivo resulted in strong phenotypes (Fig. 3a, c, d). In structures of the archaeaDna initiator OrcI bound to a replication origin the protein was observed to make two contacts with the DNA, one through its carboxy (C)-terminal DNA binding domain (analogous to DnaA domain IV) and another through its AAA+ motif5,16. We wondered whether DnaA might similarly be capable of contacting both a DnaA-box and the first DnaA-trio, thereby accounting for the absence of a DnaA trimer. Importantly, BMOE crosslinking of cysteines in the AAA+ domain would not detect this activity as the assay captures DnaA oligomers formed on either dsDNA or ssDNA12.

To test this hypothesis, we used the amine-specific crosslinker bis(sulfosuccinimidyl)suberate (BS3) which, in contrast to BMOE, only captures DnaA oligomers formed on a single DNA strand (Extended Data Figs 3 and 5). Crosslinking by BS3 reveals a DnaA dimer forming in the presence of the first 3'-GAT-5', indicating that DnaA does recognize this sequence (Extended Data Fig. 5). Taken together with the BMOE crosslinking showing that a DnaA dimer is formed on the dsDNA scaffold containing just DnaA-boxes 6 and 7 and the GC-cluster, the data suggest that the DnaA protein initially bound at DnaA-box7 undergoes a conformational change (detected by BS3) to engage the first DnaA-trio motif following the GC-cluster. Several lines of evidence support the notion that DnaA adopts distinct conformations when it engages either dsDNA or ssDNA17,18.

To support the assignment of the DnaA-trio, we performed a targeted mutagenesis of the proposed sequence. The results indicate that each of the positions (3'-GAT-5') appears important for DnaA filament formation, specifically the nucleotides at positions 1 and 2, and the deoxyribose group at position 3 (Fig. 4b and Extended Data Fig. 6). Interestingly, in the crystal structure of DnaA bound to a ssDNA substrate, the protein makes no base-specific contacts1. These observations suggest either that the sequence of the DnaA-trios is important for an intermediate step in DNA duplex recognition and melting before full engagement of the product single-strand, or that the specific base sequence promotes the DNA backbone to adopt a favourable geometry for DnaA binding.

Using this information, we first searched for DnaA-trios within other well-characterized origin unwinding elements (Fig. 4c, underlined)19–21. In these cases a set of at least three DnaA-trios could be identified. These DnaA-trios were located proximal to a DnaA-box that shared the same orientation as B. subtilis DnaA-box7, and the regions between the DnaA-box and the DnaA-trios were GC-rich. Using these additional criteria we next interrogated predicted bacterial DNA replication origins (DoriC22) for similar patterns. Figure 4c shows that similar elements can be identified within putative oriC regions throughout the bacterial kingdom. A sequence logo of the DnaA-trios indicates that the preferred motif is 3'-5'/5'-AT-5' (Fig. 4d), with the central adenine being most highly conserved. We also observed that in most cases a pair of tandem DnaA-boxes preceded the GC-cluster (Extended Data Table 1).

We propose that the DnaA-trio constitutes a new element within bacterial replication origins. Our findings indicate that DnaA-trios play an essential role during DNA replication initiation by providing specificity for DnaA filament formation on a single DNA strand, thereby promoting DNA duplex unwinding. Our analysis also indicates that the arrangement of tandem DnaA-boxes in close proximity to DnaA-trios is a widespread strategy used to direct DnaA filament growth onto the unwinding region, with a single DnaA protein probably binding dsDNA via domain IV before engaging a DnaA-trio via its AAA+ motif. Bioinformatic analysis identifies DnaA-trio motifs adjacent to a DnaA-box throughout the bacterial kingdom. Underlined sequences indicate experimentally determined DnaA-dependent unwinding sites. DnaA-trios sequence logo (WebLogo23), schematic of DnaA filament formation from double-stranded DnaA-boxes (triangles) onto a single strand containing the DnaA-trios.

Figure 4 | Identification of the DnaA-trio motif. a, Varying the length of 5'-tailed substrates identifies the likely DnaA-trio sequence. Lane 2 shows DnaA filament formation on a duplex DNA scaffold (DnaA-box6, DnaA-box7, GC-rich cluster). Letters indicate the nucleotide sequentially added to the 5'-tail. b, Targeted mutagenesis of the proposed DnaA-trio motif. c, Bioinformatic analysis identifies DnaA-trio motifs adjacent to a DnaA-box throughout the bacterial kingdom. Underlined sequences indicate experimentally determined DnaA-dependent unwinding sites. d, DnaA-trios sequence logo (WebLogo23). e, Schematic of DnaA filament formation from double-stranded DnaA-boxes (triangles) onto a single strand containing the DnaA-trios.
Analysis of replication initiator proteins from both bacteria and archaea shows that the ISM within AAA+ domains is used for DNA binding, and the recent structure of the Drosophila origin recognition complex (ORC) suggests that this is also probably the case for eukaryotes, supporting the model that DNA binding by the ISM is a universal feature of replication initiators. 1,4,15,16,27 We find here, for the first time, that the interaction of the B. subtilis replication initiator ISM with the origin involves recognition of a specific DNA sequence. We speculate that motifs analogous to the DnaA-trio might be present in replication origins of higher organisms and recognized by the ISM of ORC proteins. These sites need not be trinucleotides, nor would they necessarily share the same spacing observed for the DnaA-trios as they would need to accommodate the arrangement of AAA + interactions within the respective heterohexameric ORC17,27. The discovery of ISM binding motifs in higher organisms would greatly facilitate origin identification, an elusive problem precluding the understanding of DNA replication control in eukaryotes.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** H.M. and T.T.R. conceived and designed experiments; H.M., T.T.R. and O.H. constructed plasmids and strains; H.M. and O.H. performed microscopy experiments; T.T.R. purified proteins, performed the growth and marker frequency analysis experiments; H.M. and T.T.R. interpreted results and wrote the paper.

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No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Nutrient agar (Oxoid) was used for routine selection and maintenance of both B. subtilis and E. coli strains. For experiments in B. subtilis cells were grown using Luria-Bertani medium. Supplements were added as required: chloramphenicol (5 μg ml⁻¹), erythromycin (1 μg ml⁻¹), kanamycin (5 μg ml⁻¹), spectinomycin (50 μg ml⁻¹). Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich.

Marker frequency analysis. Genomic DNA was harvested from cells during the exponential growth phase and the relative amount of DNA from the replication origin (ori) and terminus (ter) was determined by qPCR. Strains were grown in Luria-Bertani medium to an absorbance, A₆₀₀ nm, of 0.3–0.5 whereupon sodium azide (0.5%) was added to prevent further metabolism. Chromosomal DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen). The DNA replication origin (oriC) region was amplified using primers 5′-GAATTCCTCGAGCGACT-3′ and 5′-GATTTCGGCGGAATTCCAG-3′; the region adjacent to oriN was amplified using primers 5′-CTCCTTCGCGAAGGATGA-3′ and 5′-CCCTTCATCGGGTCCAG-3′; the DNA replication terminus (ter) region was amplified using primers 5′-TATCTCGATGTGGAGA-3′ and 5′-ATTGCTGATTTGCAAAGGT-3′. Either Rotor-Gene SYBR Green (Qiagen) or GoTaq (Promega) qPCR mix was used for PCR reactions. qPCR was performed in a Rotor-Gene Q Instrument (Qiagen). By use of crossing points (Cₚ) and PCR efficiency a relative quantification analysis (ΔΔCₚ) was performed using Rotor-Gene Software version 2.0.2 (Qiagen) to determine the origin:terminus (ori:ter) ratio of each sample. These results were normalized to the ori:ter ratio of a DNA sample from B. subtilis spores, which only contain one chromosome and thus have an ori:ter ratio of 1. Error bars indicate the standard deviation of three technical replicates. All experiments were independently performed at least twice and representative data are shown.

Protein expression. BL21 (DE3)-pLysS cells were transformed with the appropriate expression construct (Supplementary Table 2) and selected on nutrient agar plates containing 100 μg ml⁻¹ of ampicillin and 25 μg ml⁻¹ of chloramphenicol. A single transformant colony was used to inoculate an overnight starter culture grown at 37 °C, 180 rpm, in Luria-Bertani medium supplemented with 100 μg ml⁻¹ of ampicillin and 34 μg ml⁻¹ of chloramphenicol. A 5 ml sample from a 1 litre growth culture was used to inoculate 1,200 ml of Luria-Bertani medium to an absorbance, A₆₀₀ nm, of 0.5. Cells were induced with 1 mM IPTG and cultured for a further 3 h at 37 °C. Cells were pelleted by centrifugation at 31,000 × g for 10 min before resuspension in 45 ml of resuspension buffer (25 mM HEPES-KOH (pH 7.6); 50 mM potassium glutamate; 10 mM magnesium acetate; 20% sucrose; 30 mM imidazole). The cell suspension was then flash-frozen in liquid nitrogen.

Protein purification. DNA was flash-frozen in liquid nitrogen. The clarified lysate was diluted to a 1 ml HiTrap HP column (GE), which had previously been equilibrated with FactorXa cleavage buffer. The Factor Xa-cleaved fraction was eluted in 7.5 ml of Ni binding buffer. The eluted fraction was diluted into 42.5 ml of Q binding buffer and purified on a 1 ml HiTrap Q HP column as previously described. Peak fraction(s) were pooled and dialysed into 1 l of final dialysis buffer (25 mM HEPES-KOH (pH 7.6); 250 mM potassium glutamate; 1 mM EDTA; 20% sucrose; 20% PEG₈₀₀₀) using 3.5k MWCO SnakeSkin dialysis tubing (Life Technologies) at 4 °C overnight before aliquoting, flash-freezing in liquid nitrogen and storage at –80 °C. Removal of the amino (N)-terminal His-tag, after incubation with FactorXa, was confirmed by anti-pentaHis (Qiagen) western blotting.

C-terminally His-tagged DnaA (WT-CC and ΔdomainI-II)-CC purification was performed as for the tag-free variants, except that the protein was dialysed into final dialysis buffer after the first HiTrap Q HP column purification before aliquoting, flash-freezing and storing.

HBSu purification was performed exactly as for DnaA, except that the HiTrap Q HP column was substituted for a 1 ml HiTrap Heparin HP column (GE) and the composition of buffers was modified accordingly. Ni binding buffer (25 mM Tris-HCl (pH 8.0); 400 mM NaCl; 30 mM imidazole). Ni elution buffer (25 mM Tris-HCl (pH 8.0); 400 mM NaCl; 500 mM imidazole). Heparin binding buffer (25 mM Tris-HCl (pH 8.0); 100 mM NaCl; 1 mM EDTA). Heparin elution buffer (25 mM Tris-HCl (pH 8.0); 2 M NaCl; 1 mM EDTA). Factor Xa cleavage buffer (25 mM Tris-HCl (pH 8.0); 100 mM NaCl; 2 mM CaCl₂; 20% sucrose; 20% PEG₈₀₀₀). Peak fractions were determined by SDS–PAGE and Coomassie staining owing to the absence of tryptophan, tyrosine and cysteine residues.

Open complex formation assays. KMnO₄ footprinting essays were assayed as described in ref. 9, except for the following changes. DnaA was not pre-incubated with ATP. The unwinding buffer contained 2 mM ATP, rather than 5 mM, and 500 mg of plasmid pTR541 (wild type) or pTR542 (t16-8°) was used per 75 μl-scale reaction. DnaA was added to final concentrations of 0, 100, 250 and 1,000 nM. Assembled reactions were incubated at 37 °C for 10 min. KMnO₄ treatment was then performed at 37 °C for 10 min. Six micromoles of 3-mercaptoethanol was used to quench reactions; however, EDTA was omitted. KmO₄-treated DNA was immediately purified using a Qiagen PCR clean-up kit, eluting in 20 μl of EB buffer. KmO₄-treated templates were not linearized before primer extension. Primer extensions were performed on a 20 μl scale using 0.1 μl of Vent exo- DNA polymerase (NEB) in 1× manufacturer’s reagent buffer supplemented with 4 mM MgSO₄, 200 μM each dNTP, 200 nM Cy-5 labelled oligonucleotide (5′-Cy-5-AGCTTCGAGCAGATTAAAG-3′) and 4 μl of PCR-purified template DNA per reaction. Reactions were subjected to thermocycling using a 3° Prime thermal cycler (Techne) with 1 min initial denaturation at 98 °C, followed by 35 cycles of (10 s at 98 °C; 30 s at 65 °C; 30 s at 72 °C). Reactions were quenched by addition of an equal volume of stop buffer (95% formamide; 10 mM EDTA; 10 mM NaOH; 0.01% OTA and 0.01% Orange G) and products subjected to denaturing PAGE (6% acrylamide: bisacrylamide (19:1); 8 μm urea in 1× TBE). Resolved products were visualized using a Typhoon Trio Variable Mode Imager (GE Healthcare). The DnaA-trio marker was generated by primer extension performed under the same conditions as described for KmO₄-treated substrates, but using a PCR product as template generated with a primer corresponding to the end of the first DnaA-trio (5′-TAGGGCGTGTGTGGATTTGTG-3′). All experiments were independently performed at least twice and representative data are shown.

FIlament assembly assays (BMOE). DNA scaffolds were prepared by mixing each oligonucleotide (50 nM final concentrations) in 10 mM HEPES-KOH (pH 7.6); 250 mM NaCl; 10 μM DTT; 20% sucrose; 0.01% Tween-20 and 2 mM nucleotide (ADP or ATP). Reactions were incubated at 37 °C for 5–90 min in a heat-block and slowly cooled to room-temperature in the heat-block before use. Filament formation was promoted by mixing DNA-A/C/D/ proteins (WT, I190A, ΔdomainI-II) (200 nM final concentration) with DNA scaffold (15 nM) on a 20 μl scale in 30 mM HEPES-KOH (pH 7.0), 100 mM potassium glutamate, 100 mM NaCl, 10 mM magnesium acetate, 25% glycerol, 0.01%Tween-20 and 2 mM nucleotide (ADP or ATP). Reactions were incubated at 37 °C for 5–12 min before addition of 4 mM BMOE (ThermoFisher Scientific). Reactions were incubated at 37 °C for 5–12 min before quenching by addition of 60 mM cysteine. Reactions were incubated once more at 37 °C for 10–12 min before fixing by the addition of 10% glycerol and 0.1% ascorbic acid. Reactions were visualized on a 2% agarose gel stained with ethidium bromide.

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Oligonucleotides used for plasmid construction are listed in Extended Data from J. Errington). Second, pHM396 was generated by digestion of pJS1 with PvuII′ steps. First, pJS1 was generated by ligation with a HindIII-BamHI PCR product (gift from H. Strahl).

Mutated regions, sequences were subcloned using BglII/FspAI. hsdR−(F α). Data Table 3.

Chroma and details are available upon request. Digital images were acquired and device) camera (Photometrics). All filters were Modified Magnetron ET Sets from Chroma.

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Extended Data Figure 1 | Structure of DnaA proteins. a, Primary domain structure of DnaA. Key functions are listed below the relevant domain. b, Structure of *Thermatoga maritima* DnaA domain III, highlighting the single-strand binding residue Val176 (Ile190 *B. subtilis*) within the ISM (PDB accession number 2Z4S). c, Structure of *E. coli* DnaA domain IV bound to a DnaA-box (PDB accession number 1J1V). d, Structure of *A. aeolicus* DnaA domain III (blue shades) and domain IV (cyan shades) bound to a single DNA strand (orange), highlighting the single-strand binding residue Val156 (Ile190 *B. subtilis*) (PDB accession number 3R8F). e, Scheme used to construct mutants within the *B. subtilis* DNA replication origin. The green arrow highlights the location of a DnaA-box mutation.
Extended Data Figure 2 | Characterization of the inducible repN/oriN replication initiation system. Repression of repN expression inhibits DNA replication in a ΔoriC mutant. A large deletion was introduced into the B. subtilis replication origin using a strain harbouring the inducible oriN/repN construct. Strain growth was found to be dependent upon addition of the inducer IPTG. a, Strains streaked to resolve single colonies. b, A GFP-DnaN reporter was used to detect DNA replication after removal of IPTG from inducible oriN/repN strains. Scale bar, 5μm. c, Genetic map indicating the location of oriN at the aprE locus in strain HM1108. d, Analysis of DNA replication initiation at oriC and oriN. Marker frequency analysis was used to measure the rate of DNA replication initiation in the presence and absence of IPTG (0.1 mM). Genomic DNA was harvested from cells during the exponential growth phase and the relative amount of DNA from either the endogenous replication origin (oriC) or the aprE locus (oriN) compared with the terminus (ter) was determined using qPCR (mean and s.d. of three technical replicates). Cell doubling times (in minutes) are shown above each data set.
Extended Data Figure 3 | Wild-type DnaA assembles into filaments on 5′-tailed substrates. DnaA filament formation using amine-specific crosslinking (BS3) on DNA scaffolds (represented by symbols above each lane). Protein complexes were resolved by SDS–PAGE and DnaA was detected by western blot analysis.
| Strain | Wild-type sequence indicating deletions | Resulting sequence |
|--------|----------------------------------------|--------------------|
| Mononucleotide | | |
| Wild-type | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |
| TR141   | 3’-GATGATAATGAGAT-5’ | 3’-ATGATAATGAGAT-5’ |
| TR114   | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |
| TR143   | 3’-GATGATAATGAGAT-5’ | 3’-GATAATGAGAT-5’ |
| TR147   | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |
| TR137   | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |
| TR144   | 3’-GATGATAATGAGAT-5’ | 3’-GATGAAATGAGAT-5’ |
| TR145   | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |
| TR153   | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |
| Trinucleotide | | |
| Wild-type | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |
| TR116   | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |
| TR163   | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |
| TR139   | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |
| TR153   | 3’-GATGATAATGAGAT-5’ | 3’-GATGATAATGAGAT-5’ |

Extended Data Figure 4 | DNA sequence of unwinding regions after mononucleotide and trinucleotide deletions. Resulting sequences grouped in boxes are identical for more than one deletion.
Extended Data Figure 5 | Crosslinking with BS3 captures a distinct DnaA oligomer. DnaA was incubated with various DNA scaffolds and different crosslinking agents were added to capture distinct DnaA oligomers. a, Crosslinking with BMOE detects DnaA oligomers forming on both duplex and tailed substrates. b, Crosslinking with BS3 only detects DnaA oligomers forming on tailed substrates, revealing an interaction between DnaA and the first DnaA-trio motif located downstream of the GC-cluster.
Extended Data Figure 6 | The nucleotide at the third position of the DnaA-trio is required to stabilize DnaA. DNA scaffolds containing the first two nucleotides of a DnaA-trio either with or without a 5′-phosphate are unable to stabilize binding of an additional DnaA protomer, indicating that the nucleotide at the third position is required. Combined with the data shown in Fig. 4b where the position is abasic, the results suggest that the sugar at the third position plays a critical role in DnaA binding.
Extended Data Figure 7 | Relationship between the DnaA-box and the DnaA-trios. (a) Sequence of the origin region used for constructing DNA scaffolds. Symbols below represent duplex DnaA-boxes (triangles), the GC-rich region (green rectangles), the two strands of the unwinding region (red or pink rectangles) and the AT-rich region (blue rectangle). Loading of the DnaA filament onto a single-stranded 5′-tail requires a DnaA-box and DnaA domains III–IV, but the DnaA-box position and orientation are flexible.
## Extended Data Table 1 | Bacterial replication origin regions in Fig. 4c

| Organism                                | Genome accession # | Reference or DoriC accession # (ORI) | Genome position shown in Figure 4 | Tandem DnaA-boxes (spacing) |
|------------------------------------------|--------------------|-------------------------------------|----------------------------------|-----------------------------|
| *Aquifex aeolicus* VF5                   | NC_000918          | 17                                  | 166853 to 166897                 | N                           |
| *Bacillus subtilis* 168                 | NC_000964          | 34                                  | 1860 to 1902                     | Y (-1)                      |
| *Bdellovibrio bacteriovorus* HD100       | NC_005363          | ORI10040030                         | 1569 to 1597                     | N                           |
| *Bifidobacterium bifidum* PRL2010        | NC_014638          | ORI94010761                         | 2048 to 2071                     | Y (0)                       |
| *Bordetella pertussis* Tohama I         | NC_002929          | ORI10030012                         | 4084583 to 4084611               | Y (+3)                      |
| *Borrelia afzelii* HLJ01                | NC_018887          | ORI96010684                         | 460118 to 460149                 | Y? (+1)(+9)                 |
| *Clostridium botulinum* A str. Hall      | NC_009698          | ORI92010335                         | 1517 to 1552                     | Y (-1)                      |
| *Corynebacterium glutamicum* ATCC 13032  | NC_003450          | ORI10010055                         | 1984 to 2010                     | Y (0)                       |
| *Enterococcus faecalis* V583             | NC_004668          | ORI10010096                         | 1498 to 1533                     | Y (0)                       |
| *Escherichia coli* MG1655                | NC_000913          | 35                                  | 3925780 to 3925809               | N                           |
| *Helicobacter pylori* 26695              | NC_000915          | 20                                  | 1607488 to 1607525               | Y (0)                       |
| *Leuconostoc citreum* KM20               | NC_010471          | ORI92310382                         | 1790 to 1819                     | Y (0)                       |
| *Listeria monocytogenes* EGD-e           | NC_003210          | ORI10010047                         | 1773 to 1802                     | Y (-1)                      |
| *Oceanobacillus iheyensis* HTE831        | NC_004193          | ORI10010074                         | 1746 to 1778                     | Y (-1)                      |
| *Staphylococcus aureus* NCTC 8325        | NC_007795          | ORI10010183                         | 2075 to 2104                     | Y (-1)                      |
| *Streptococcus pneumoniae* R6            | NC_003098          | ORI10010044                         | 1458 to 1487                     | Y (0)                       |
| *Streptomyces coelicolor* A3(2)          | NC_003888          | 36                                  | 4270070 to 4270096               | Y (0)                       |
| *Synechococcus elongatus* PCC 7942       | NC_007604          | 37                                  | 2695870 to 2695899               | Y (0)                       |
| *Thermotoga maritima* MSB8               | NC_000853          | 21                                  | 157010 to 157040                 | N                           |
| *Treponema pallidum* Nichols             | NC_000919          | ORI10010003                         | 1568 to 1588                     | Y (-1)                      |

References 17, 20, 21 and 34-37 are cited in the table.
## Extended Data Table 2 | Oligonucleotides used for plasmid construction

| Product | Template | Primer #1 | Sequence (5′→3′) | Primer #2 | Sequence (5′→3′) |
|---------|----------|-----------|------------------|-----------|------------------|
| pTR346  | pHM327   | oTR490    | GGCCCACTATTACTTCTACTATTTTTTATAAATATATATATTAATAC | oTR491    | GAAGTAATAGTGGGCCTGTGGATTTGTGGATAAGTTG |
| pTR83   | pHM327   | oTR539    | GGCCCACTATTACTTCTACTATTTTTTATAAATATATATATTAATACATTATC | oTR540    | AGAAGTAATAGGGCCTGTGGATTTGTGGATAAGTTG |
| pTR153  | pHM327   | oTR384    | TCAAGGTCGCATGAACAAAACAGAACTTATCAATG | oTR385    | CGGATCTTATTTTCCGGCAACTGCGTCTTTAAGC |
| pTR208  | pTR74    | oTR386    | TCAAGGTCGCATGAACAAAACAGAACTTATCAATG | oTR387    | CGGAAAATAAGATCCGGCTGCTAACAAAGCCCGAAAG |
| pTR350  | pTR54    | oTR498    | ACTATTACCATTATCCGTTAGGAGGATAAAAATG | oTR499    | GGATAATGGTAATAGTAGGGCCTGTGGATTTGTG |
| pTR377  | pHM327   | oTR539    | GGCCCACTATTACTTCTACTATTTTTTATAAATATATATATTAATACATTATC | oTR540    | AGAAGTAATAGGGCCTGTGGATTTGTGGATAAGTTG |
| pTR284  | pHM327   | oTR449    | GGCCCAACTATTACTTCTACTATTTTTTATAAATATATATATTAATACATTATC | oTR450    | AGAAGTAATAGGGCCTGTGGATTTGTGGATAAGTTG |
| pTR285  | pHM327   | oTR451    | GGCCCACTTTATTACTTCTACTATTTTTTATAAATATATATATTAATACATTATC | oTR452    | AGAAGTAATAGGGCCTGTGGATTTGTGGATAAGTTG |
| pTR286  | pHM327   | oTR452    | GGCCCACTTTATTACTTCTACTATTTTTTATAAATATATATATTAATACATTATC | oTR453    | AGAAGTAATAGGGCCTGTGGATTTGTGGATAAGTTG |
| pTR301  | pHM327   | oTR469    | CTTCTACTTTTTTTTTAAAATATATATATATATATATATATATATAT | oTR470    | TATAAAAAAGTAGAATAGATAGGGCCTGTGGATTTGTG |
| pTR302  | pHM327   | oTR471    | TTTTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATTAT...
Extended Data Table 3 | Oligonucleotides used to assemble DNA scaffolds

| Oligo 1 | Sequence (5’–3’) | Oligo 2 | Sequence (5’–3’) | Figure |
|---------|------------------|---------|------------------|--------|
| GGGCCGCTTGTTGGTTTGGTTGGAAGT | GGGCCGCTTGTTGGTTTGGTTGGAAGT | 2a, 2f, E4a, E6b | |
| ATATTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | ATATTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 2b | |
| AGGGCCGCTTGTTGGTTTGGTTGGAAGT | AGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a, 4b, E6a, E6b, Ed | |
| AGGGCCGCTTGTTGGTTTGGTTGGAAGT | AGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AGGGCCGCTTGTTGGTTTGGTTGGAAGT | AGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AGGGCCGCTTGTTGGTTTGGTTGGAAGT | AGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a, 5d | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AGGGCCGCTTGTTGGTTTGGTTGGAAGT | AGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AGGGCCGCTTGTTGGTTTGGTTGGAAGT | AGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AGGGCCGCTTGTTGGTTTGGTTGGAAGT | AGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AGGGCCGCTTGTTGGTTTGGTTGGAAGT | AGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AGGGCCGCTTGTTGGTTTGGTTGGAAGT | AGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AGGGCCGCTTGTTGGTTTGGTTGGAAGT | AGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | AAGATTTTTTATTAGAATAGGTTGAAAAAGTGTTTTTGTTTTTGGTTTGGTTGGAAGT | 4a, 5d | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
| TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | TAGGGCCGCTTGTTGGTTTGGTTGGAAGT | 4a | |
ERRATUM

doi:10.1038/nature18932

Erratum: The bacterial DnaA-trio replication origin element specifies single-stranded DNA initiator binding
Tomas T. Richardson, Omar Harran & Heath Murray

_Nature_ **534**, 412–416 (2016); doi:10.1038/nature17962

In Fig. 2c of this Letter, the rectangle at the bottom right corner was erroneously labelled 'ADP' rather than 'ATP'; this has now been corrected online.