Supporting information

Concerted actions of DnaA complexes with DNA unwinding sequences within and flanking replication origin *oriC* promote DnaB helicase loading

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Supporting Table S1. Oligonucleotides designed for this study

Name	5'-Sequence
delAT	TCTAAATAAATAGATCTAGAGGATCCCAGGTCTTTCTCAAGCCG
delATL	TAGAGGATCCCAGGTCTTTCTCAAGCCG
U-ATL	TATTAAAAAGAAGATCTATTTATTTA
L-ATL	TAAATAAATAGATCTTCTTTTTAATA
U-LM	GATCTATTTATTTAGAGATCTGTTCTATT
L-LM	AATAGAACAGATCTCTAAATAAATAGATC
M28	GATCTGTTCTATTGTGATCTCTTATTAG
M28rev	CTAATAAGAGATCACAATAGAACAGATC
DOR96R2R4-f	GAGGGGTTATACACAACTCAAAAAC
rightDOR102F	CTCAAAAACTGAACAACAGTTG
KWSmaIoriCFwd	CCCGGGCCGTGGATTCTAC
yy15	AGCCAGGAACCGGCCGTAACCATCTGGCGGC
yy16	GCCGCCAGATGGTTACGGCCGGTTCCTGGCT
yyc9	CAGACCGAAGCGAAATACGAAAAAGTG
yyc10	CACTTTTTCGTATTTCGCTTCGGTCTG

Supporting Table S2. Plasmids used in this study

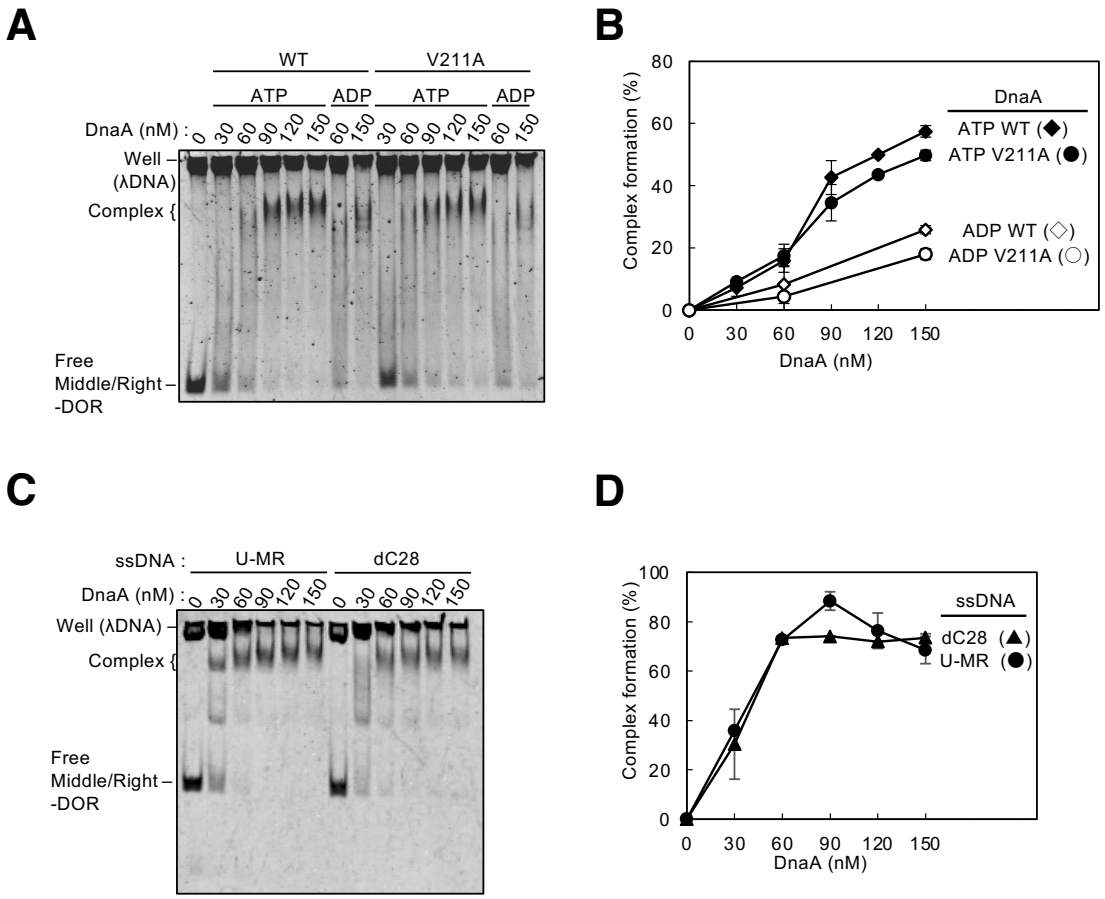
Name	Relevant structure	Reference or source
M13*oriC*MS9	M13mp18 bearing 431 bp chromosome-derived *oriC*	Shimizu *et al*., 2016
pRS*oriC*	pBR322 bearing chromosomal region (*gidA'-oriC-mioC-asnC*) and *asnA::frt-kan-frt*	Noguchi *et al*., 2015
pRSR5M*Tma*	pRSoriC R5M box::*Tma*DnaA box	Sakiyama *et al*., 2017
pRSR4*Tma*	pRS*oriC* R4 box::*Tma*DnaA box	Noguchi *et al.,* 2015
pECTM	pING-1 bearing arabinose-inducible promoter and Chi*dnaA*	Noguchi *et al*., 2015
pECTM-V211A	pECTM Chi*dnaA* V211A	Sakiyama *et al*., 2017
pECTM-R245A	pECTM Chi*dnaA* R245A	Sakiyama *et al*., 2017
pECTM-V211A/R245A	pECTM Chi*dnaA* V211A R245A	Sakiyama *et al*., 2017
pBS*oriC*	pBluescript II bearing *oriC*	Ozaki and Katayama, 2012
pBS-left*oriC*	pBS*oriC* Δ(R4-R2)	Ozaki and Katayama, 2012
pBS-left*oriC* ΔAT	pBS*oriC* Δ(R4-R2) ΔAT	This work
pBS-left*oriC* ΔATL	pBS*oriC* Δ(R4-R2) Δ(AT-L)	This work
pBS*oriC* R4*Tma*	pBS*oriC* R4*Tma*	This work
M13*oriC*MS9 R4*Tma*	M13*oriC*MS9 R4 box::*Tma*DnaA box	This work
pBAD/His-dnaC	pBAD/His-B bearing *dnaC*	This work
pBAD/His-dnaC K112R	pBAD/His-B bearing *dnaC K112R*	This work
pBAD/His-dnaC S177A	pBAD/His-B bearing *dnaC K177A*	This work

Supporting Table S3. Preparation of DOR used in this report

Name	Amplified region	Length (bp)	Primers	Template
Left-DOR	R1-I2	118	ori1 Dr2	pBS*oriC*
Middle/Right-DOR	R2-R4	118	DOR96R2R4-f ori-2	pBS*oriC*
Middle/Right-DOR-R4Tma	R2-R4 (R4Tma)	118	DOR96R2R4-f ori-2	pBS*oriC*R4*Tma*
Right-DOR	C3-R4	102	rightDOR102F ori-2	pBS*oriC*

Supporting Table S4. Strains used in this study

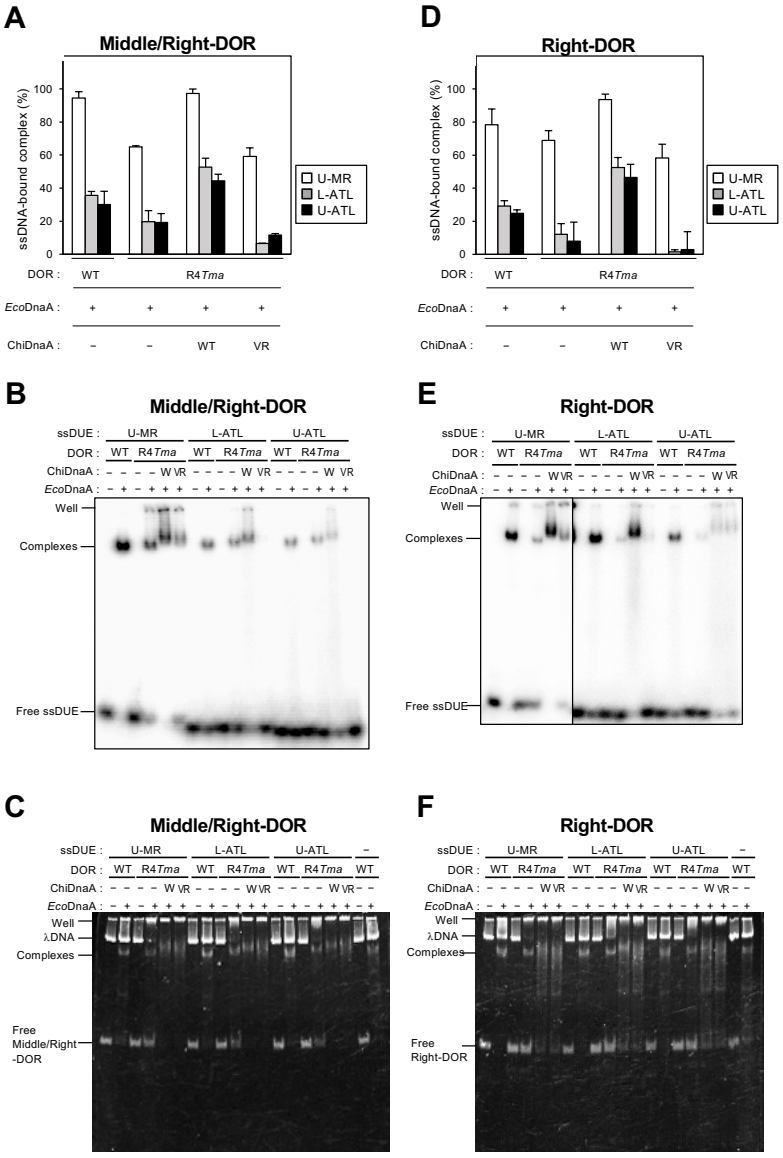
Strain	Relevant genotype	Sources
NY20	MG1655 *asnA*::*kan*	Noguchi e*t al*., 2015
NY21	MG1655 *asnA*::*kan oriC*ΔR4-box::TmaDnaA-box	Noguchi *et al*., 2015
SYM24	MG1655 *asnA*::*kan oriC*ΔR5M-box::TmaDnaA-box	Sakiyama *et al.*, 2018



Supporting Figure S1. DnaA complex formation with Middle/Right-DOR fragments (related to Figure 3).

(A and B) The indicated amounts of ATP–DnaA WT or V211A mutants were incubated for 5 min at 4°C with the Middle/Right-DOR fragments (35 nM). Two independent experiments were carried out, and representative gel images (in a black/white inverted mode) are shown. Migration positions of Middle/Right-DOR–DnaA complexes and protein-free DNAs are indicated (*A*). Band intensities of Middle/Right-DOR–DnaA complexes were quantified as "Complex formation (%)". Mean values with SDs (n = 2) are shown in the graphs (*B*).

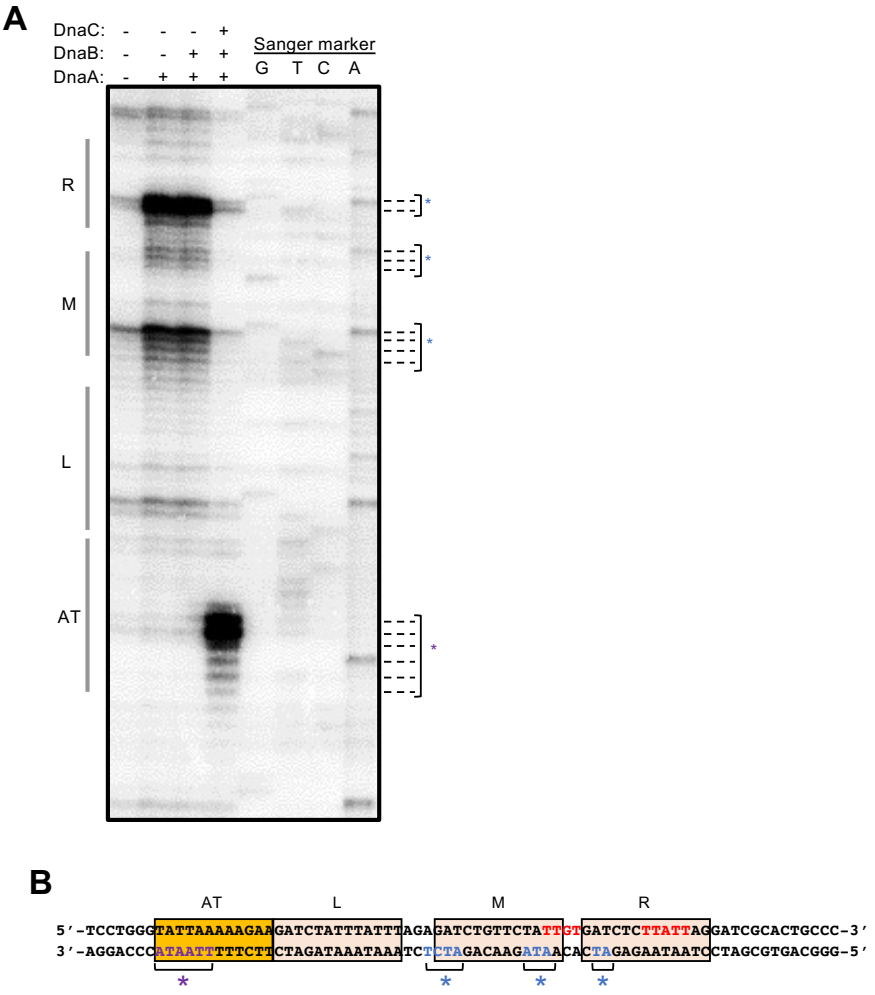
(C and D) The indicated amounts of ATP-DnaA were incubated for 5 min at 4°C with the Middle/Right-DOR fragments (35 nM), following incubation for 10 min at 30°C with 28-mer ssDUE U-MR or oligo-dC (dC28). Two independent experiments were carried out, and representative gel images and size makers are shown as above (*C*). Band intensities of Middle/Right-DOR − DnaA complexes were quantified as "Complex formation (%)". Two independent experiments were carried out and, mean values with SDs (n = 2) are shown in the graphs (*D*).



Supporting Figure S2. EMSA using the Middle/Right-DOR or Right-DOR DNA with or without substitution of the *Tma*DnaA box for the R4 box (related to Figure 4).

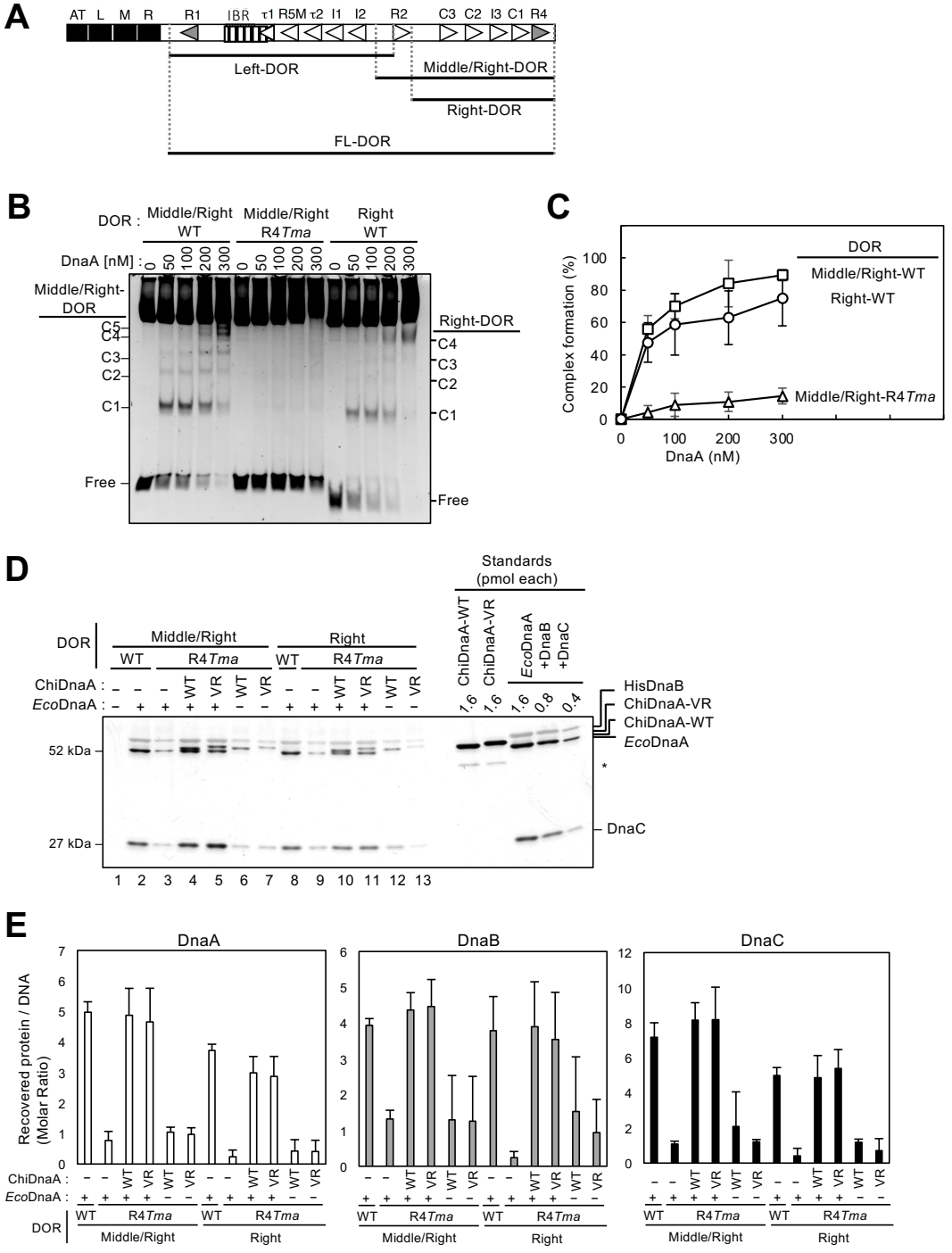
EMSA was performed with the Middle/Right-DOR (*A*, *C, and E*) or Right-DOR DNA (*B*, *D, and F*). DNAs with (R4*Tm*a) or without (WT) R4*Tma* substitution were incubated for 5 min at 4°C with ATP–*Eco*DnaA (150 nM) and λ phage DNA in the presence of ATP–ChiDnaA WT or VR (35 nM), followed by further incubation for 10 min at 30°C with ^{32}P-labeled-ssDNA U-MR, U-ATL, or L-ATL (16 nM) in buffer including 80 mM potassium chloride (see *Experimental procedures*). The resultant ssDNA–DnaA–DOR (*A*, *B, D, and E*) or DnaA–DOR complexes (*C and F*) were analyzed by radioactive imaging or GelStar staining, respectively. The amounts of ssDNA bound to the DnaA–DOR complexes, quantified as "ssDNA-bound complex (%)", are shown (*A* and *B*). Two independent experiments were carried out, and mean values with SDs (n = 2) are

shown in the graphs (*A* and *D*). When *Eco*DnaA and the DORs bearing the R4*Tma* substitution were co-incubated, abnormal complexes such as aggregates may have formed, including λ DNA, resulting in material remaining in the gel wells. However, the majority of the DORs constructed specific complexes including DnaA. +; presence. –, absence.



Supporting Figure S3. Annotation of primer extension products of $KMnO_4$-treated *oriC* plasmid **(related to Figure 4G)**.

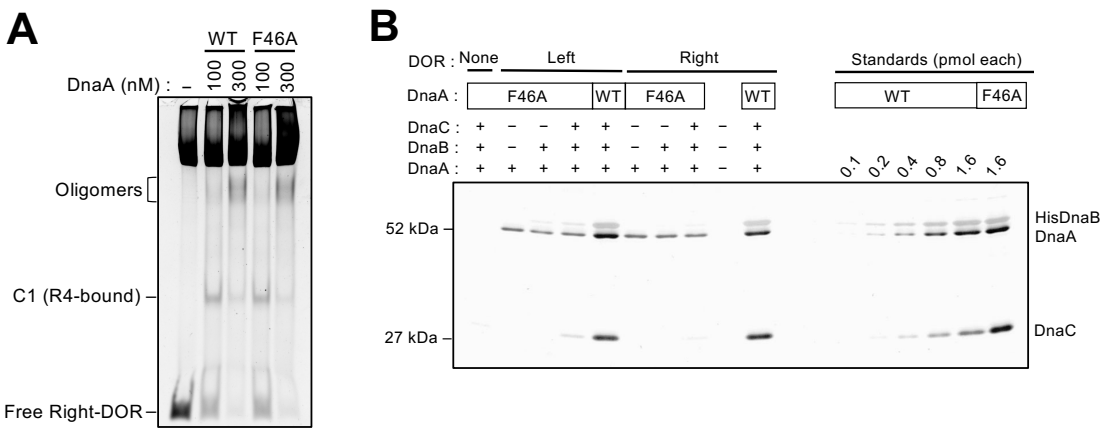
(A) Plasmid bearing *oriC* (5 nM) were incubated for 10 min at 37°C with IHF (100 nM) in the presence (+) or absence (-) of EcoDnaA (100 nM), DnaB K236A (300 nM), and DnaC (300 nM), followed by further incubation with 10 mM $KMnO_4$ and primer extension experiments. The products were analyzed using 7.5% denaturing sequencing gel with a series of Sanger sequence products obtained using the same primer and *oriC* plasmid (see *Experimental procedures*). Positions of sequence motifs (L, M, R, and AT) are shown on the left. Hypersensitive bands are indicated as asterisks (blue; DnaA-dependent hypersensitive sites, purple; hypersensitive sites in the presence of DnaA, DnaB, and DnaC). (B) Corresponding sequence to the primer extension products is shown. Red letters are the known DnaA-binding motifs. Hypersensitive sites shown in *panel A* are also indicated as purple and blue letters.



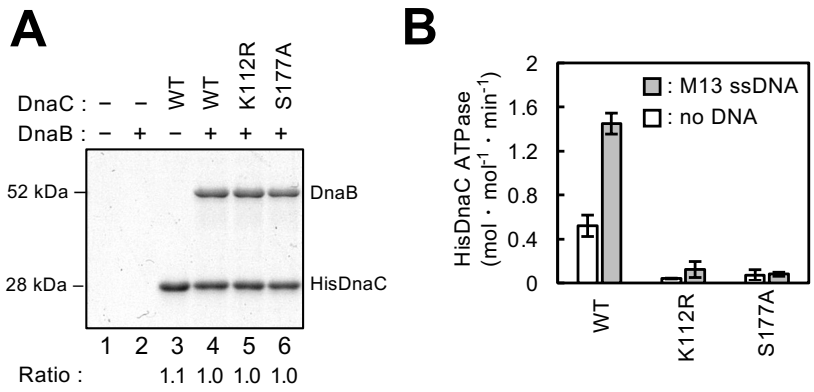
Supporting Figure S4. Role of DnaA H/B-motifs bound at the DnaA box R4 in DnaB binding, related to Figure 5.

(A) Schematic structures of DOR fragments are shown as bars. For symbols, see Figure 1A. (B) EMSA with the Middle/Right WT, Middle/Right R4*Tma* and Right-DOR fragments. Indicated concentrations of ATP–DnaA were incubated for 5 min on ice with the indicated DOR fragment (35 nM) and λ phage DNA, followed by further incubation for 10 min at 30°C. In the absence of

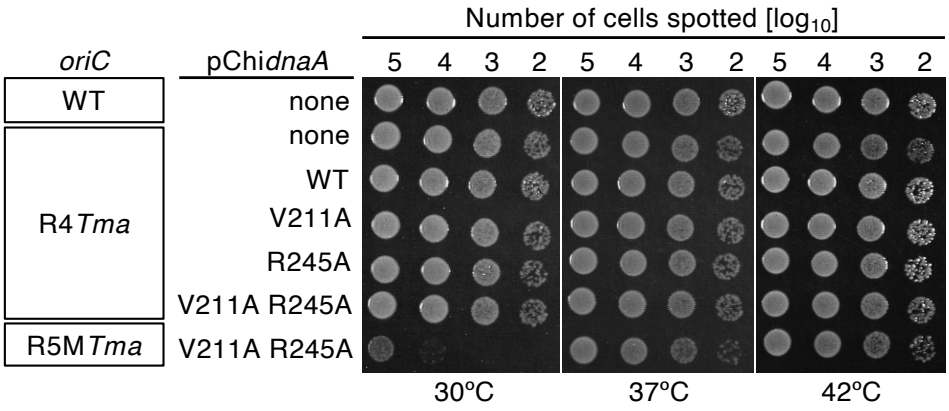
ssDUE or ChiDnaA, DnaA forms very unstable complexes on Middle/Right R4*Tma* (Noguchi *et al.*, 2015). (C) The levels of the DOR–DnaA subcomplexes, indicated as "Complex formation (%)", were quantified from the gel images. Two independent experiments were carried out, and mean values with SDs (n = 2) are shown in the graphs. (D) ATP–DnaA (10 pmol: 0.4 μM), His-DnaB (10 pmol as monomer), and DnaC (10 pmol) were incubated in the presence of biotinylated FL-, Middle/Right- or Right-DOR WT, or R4*Tma* (250 fmol, 10 nM), followed by pull-down assay. Proteins bound to DNA were analyzed by SDS–11% PAGE and silver staining. (E) The amounts of recovered proteins (DnaA, DnaB, and DnaC) were determined using standard curves, and the values from the negative control (No DOR) were subtracted. About 150 fmol DNA was recovered. The means and SDs (n = 2) of the numbers of DnaA, DnaB, and DnaC molecules recovered per DNA are indicated by bars.



Supporting Figure S5. DnaB binding to the Right-DOR–DnaA subcomplex requires DnaA Phe46 (related to Figure 5). (A) EMSA with the Right-DOR fragments using DnaA WT and F46A is shown. DNA fragments (35 nM) were incubated at 30°C for 10 min in 10 μL buffer G (20 mM HEPES-KOH at pH 7.6, 1 mM EDTA, 4 mM DTT, 5 mM magnesium acetate, and 10% [v/v] glycerol) containing the indicated ATP–DnaA, 0.25 mg/mL BSA, and 200 ng λ phage DNA as a competitor. The samples were subjected to 4% PAGE in Tris-borate at room temperature, followed by GelStar staining. The experiments were performed twice, and similar results were obtained. (B) Pull-down assay using DnaA F46A mutant protein. ATP–DnaA WT or F46A (10 pmol: 0.4 μM), His-DnaB (10 pmol as monomer), and DnaC (10 pmol) were incubated in the presence of biotinylated *oriC*, Left- or Right-DOR (250 fmol, 10 nM), followed by pull-down assay. Proteins bound to DNA were analyzed by SDS–11% PAGE and silver staining.



Supporting Figure S6. DnaC mutants sustained DnaB binding and ATPase activity (related to Figure 7). (A) DnaB WT (10 pmol as monomer) was incubated with His-DnaC WT or mutants (K112R or S177A) (10 pmol), followed by pull-down assay. The complexes of DnaB and His-DnaC were analyzed by SDS–11% PAGE and CBB staining. (B) ATPase activity of DnaC mutants. [γ-^{32}P]ATP-bound DnaC WT or mutants (K112R or S177A) (10 pmol: 0.4 μM) were incubated with His-DnaB (10 pmol: 0.4 μM as monomer) in the presence or absence of M13 ssDNA (1.3 nM), followed by incubation at 37°C for 60 min. [γ-^{32}P]ATP or [γ-^{32}P]Pi was analyzed by thin-layer chromatography and radioactive imaging. The mean values with SDs (n = 2) are shown in the graphs.



Supporting Figure S7. Roles for DnaA H/B-motifs in cell growth (related to Figure 8). Spot test of cells with chromosomal *oriC* R4*Tma* mutation harboring plasmids encoding ChiDnaA WT or mutants (V211A, R245A, or V211A R245A) or only vector plasmid (none). Serial dilutions of full-growth cultures (~10^9 cells/mL) at 30°C, 37°C, or 42°C were spotted onto LB-agar plates containing 100 μg/mL ampicillin and incubated at the indicated temperatures.

References

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