**Supplementary Data**

**Single-stranded DNA recruitment mechanism in replication origin unwinding by DnaA initiator protein and HU, an evolutionary ubiquitous nucleoid protein**

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**Supplementary Table S1. Oligonucleotide used in this study**

| Name | 5' sequence | Note |
|---|---|---|
| M28-R1f | GATCTGTTCTATTGTGATCTCTTATTAGGATCGCACTGCC CTGTG | Using for construction of ssMR-left DOR |
| Left half DORr | CCCCTCATTCTGATCCCAGCTTATACGGTCCAGGATCACC GATCATTCACAGTTAATGATCCTTTCCAGGTTGTTGATC TT AAAAGCCGGATCCTTGTTATCCACAGGGCAGTGCGATC | |
| ori2 | GTTATTCATATGTCTAGCACGC | A primer for primer extension |
| KWSmaIoriCFwd | CCCGGGCCGTGGATTCTAC | |
| TMA28 | GCTATTATTAGTAGTAGTAAATAATAGG | |
| 306_PBS2 | GTAATACGACTCACTATAGGGCGA | |

**Supplementary Table S2. *oriC* plasmids and plasmids bearing *dnaA* used in this study**

| Name | Relevant structure | Source |
|---|---|---|
| M13*oriC*MS9 | M13mp18 bearing 431 bp chromosome-derived *oriC* | Shimizu, *et al.,* 2016 |
| M13*oriC*MS9 R1-I2 | M13*oriC*MS9 ΔR4-R2 | Sakiyama, *et al.,* 2017 |
| M13*oriC*MS9 R1-I1 | M13*oriC*MS9 ΔR4-I2 | Sakiyama, *et al.,* 2017 |
| M13*oriC*MS9 R1-τ2 | M13*oriC*MS9 ΔR4-I1 | Sakiyama, *et al.,* 2017 |
| M13*oriC*MS9 R1-R5M | M13*oriC*MS9 ΔR4-τ2 | Sakiyama, *et al.,* 2017 |
| M13*oriC*MS9 R1non | M13*oriC*MS9 ΔR1 box::R1-nonsense box | Sakiyama, *et al.,* 2017 |
| M13*oriC*MS9 τ1non | M13*oriC*MS9 Δτ1 box::τ1-nonsense box | Sakiyama, *et al.,* 2017 |
| M13*oriC*MS9 R5Mnon | M13*oriC*MS9 ΔR5M box::R5M-nonsense box | Sakiyama, *et al.,* 2017 |
| M13*oriC*MS9 τ2non | M13*oriC*MS9 Δτ2 box::τ2l-nonsense box | Sakiyama, *et al.,* 2017 |
| M13*oriC*MS9 I1non | M13*oriC*MS9 ΔI1 box::I1-nonsense box | Sakiyama, *et al.,* 2017 |
| M13*oriC*MS9 R1*Tma* | M13*oriC*MS9 ΔR1 box::*Tma*DnaA box | Noguchi, *et al.,* 2015 |
| M13*oriC*MS9 R5M*Tma* | M13*oriC*MS9 ΔR5M box::*Tma*DnaA box | Sakiyama, *et al.,* 2017 |
| pChi*dnaA* | pING-1 bearing arabinose-inducible promoter and Chi*dnaA* | Sakiyama, *et al.,* 2017 |
| pChi*dnaA* R285A | pChi*dnaA* Chi*dnaA*R285A | Sakiyama, *et al.,* 2017 |
| pChi*dnaA* V211A R245A | pChi*dnaA* Chi*dnaA* RV211A R245A | Sakiyama, *et al.,* 2017 |
| pOZ14 | pBluescript II bearing 149 bp chromosome-derived minimal *Tma-oriC* | Ozaki, et al, 2006 |

| | | |
|---|---|---|
| pOZsub1 | A pOZ14 derivative in which the sequence of *Tma*DnaA box 1 is randomized. | Ozaki, *et al.* 2012 |
| pOZsub2 | A pOZ14 derivative in which the sequence of *Tma*DnaA box 2 is randomized. | Ozaki, *et al.* 2012 |
| pOZsub3 | A pOZ14 derivative in which the sequence of *Tma*DnaA box 3 is randomized. | Ozaki, *et al.* 2012 |
| pOZsub4 | A pOZ14 derivative in which the sequence of *Tma*DnaA box 4 is randomized. | Ozaki, *et al.* 2012 |
| pOZsub5 | A pOZ14 derivative in which the sequence of *Tma*DnaA box 5 is randomized. | Ozaki, *et al.* 2012 |

**Supplementary Table 3. Strains used in this study**

| Strain | Relevant phenotype | Reference |
|---|---|---|
| MG1655 | Wild type | Laboratory stock |
| NY20 | MG1655 *asnA*::*frt-kan* | Noguchi, *et al.,* 2015 |
| SYM25 | NY20 *oriC*ΔR1-box::R1-nonsense | Sakiyama, *et al.,* 2017 |
| SYM5 | NY20 *oriC*Δτ1-box::τ1-nonsense | Sakiyama, *et al.,* 2017 |
| SYM6 | NY20 *oriC*ΔR5M-box::R5M-nonsense box | Sakiyama, *et al.,* 2017 |
| SYM7 | NY20 *oriC*Δτ2-box::τ2-nonsense box | Sakiyama, *et al.,* 2017 |
| SYM9 | NY20 *oriC*ΔI1-box::I1-nonsense box | Sakiyama, *et al.,* 2017 |
| NY20-frt | MG1655 *asnA*::*frt* | This work |
| SYM25-frt | NY20-frt *oriC*ΔR1 box::R1-nonsense box | This work |
| SYM5-frt | NY20-frt *oriC*Δτ1-box::τ1-nonsense box | This work |
| SYM6-frt | NY20-frt *oriC*ΔR5M box::R5M-nonsense box | This work |
| SYM7-frt | NY20-frt *oriC*Δτ2-box::τ2-nonsense box | This work |
| SYM9-frt | NY20-frt *oriC*ΔI1 box::I1-nonsense box | This work |
| NY20-dihfA | NY20-frt Δ*ihfA*::*frt-kan* | This work |
| SYM25-dihfA | SYM25-frt Δ*ihfA*::*frt-kan* | This work |
| SYM5-dihfA | SYM5-frt Δ*ihfA*::*frt-kan* | This work |
| SYM6-dihfA | SYM6-frt Δ*ihfA*::*frt-kan* | This work |
| SYM7-dihfA | SYM7-frt Δ*ihfA*::*frt-kan* | This work |
| SYM9-dihfA | SYM9-frt Δ*ihfA*::*frt-kan* | This work |
| NY20-dihfB | NY20-frt Δ*ihfB*::*spec* | This work |
| SYM25-dihfB | SYM25-frt Δ*ihfB*::spec | This work |
| SYM6-dihfB | SYM6-frt Δ*ihfB*::spec | This work |
| NY20-dihfAB | NY20-frt Δ*ihfA*::*frt-kan,* Δ*ihfB*::spec | This work |
| NY24 | NY20 *oriC*ΔR1-box::*Tma*DnaA box | Noguchi, *et al.,* 2015 |
| SYM24 | NY20 *oriC*ΔR5M-box::*Tma*DnaA box | Sakiyama, *et al.,* 2017 |

*(continued)*

| | | |
|---|---|---|
| NY24-frt | NY20-frt *oriC*ΔR1-box::*Tma*DnaA box | This work |
| SYM24-frt | NY20-frt *oriC*ΔR5M-box::*Tma*DnaA box | This work |
| NY24-dihfA | NY24-frt Δ*ihfA*::*frt-kan* | This work |
| SYM24-dihfA | SYM24-frt Δ*ihfA*::*frt-kan* | This work |
| NY24-dihfB | NY24-frt Δ*ihfB*::*spec* | This work |
| SYM24-dihfB | SYM24-frt Δ*ihfB*::*spec* | This work |
| NY24-dihfAB | NY24-frt Δ*ihfA*::*frt-kan ihfB*::*spec* | This work |
| NY20-dqueG | NY20-frt Δ*queG*::*frt-kan* | This work |
| SYM25-dqueG | SYM25-frt Δ*queG*::*frt-kan* | This work |
| SYM6-dqueG | SYM6-frt Δ*queG*::*frt-kan* | This work |
| SYM9-dqueG | SYM9-frt Δ*queG*::*frt-kan* | This work |
| SR08 | MG1655 Δ*queG*:: *frt-kan* | This work |
| KX95 | MG1655 Δ*ihfB*::*spec* | Kasho, *et al.,* 2014 |
| KMG-5 | MG1655 Δ*ihfA*::*frt-kan* | Kasho, *et al.,* 2014 |

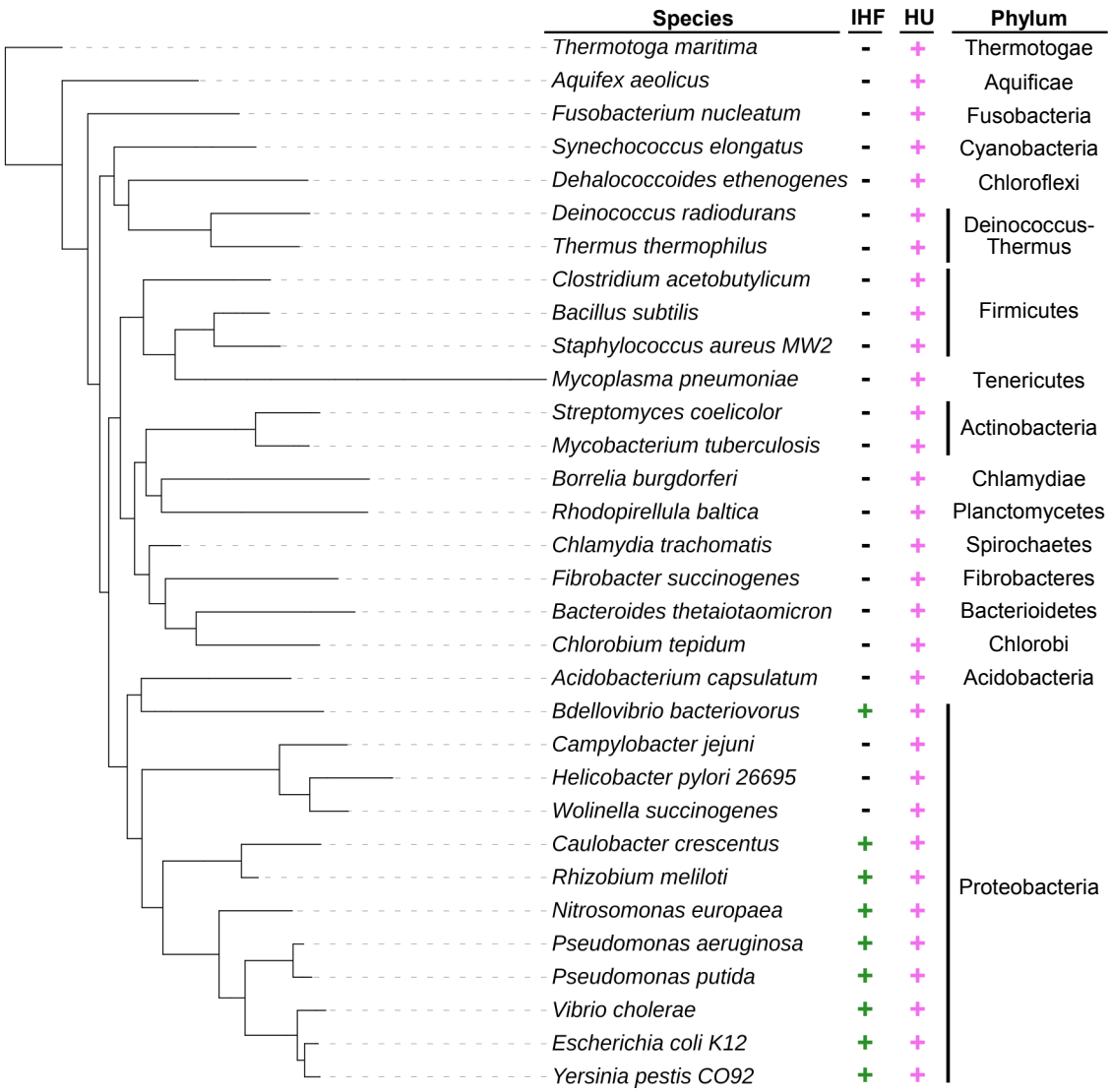
**Supplementary Methods**

**P1 phage transduction for strain construction**

For construction of Δ*ihfA*::*frt-kan* strains (NY20-dihfA, SYM25-dihfA, SYM5-dihfA, SYM6-dihfA, SYM7-dihfA, SYM9-dihfA, NY24-dihfA, and SYM24-dihfA), P1 phage lysates prepared from KMG-5 were used for transduction of strains NY20-frt, SYM25-frt, SYM5-dihfA, SYM6-frt, SYM7-frt, SYM9-frt, NY24-frt, and SYM24-frt. Transductants were screened on LB agar plates including 50 μg/mL kanamycin. For construction of Δ*ihfB*::*spec* strains (NY20-dihfB, SYM25-dihfB, SYM6-dihfB, NY24-dihfB, and SYM24-dihfB), P1 phage lysates prepared from strain KX95 were used for transduction of strains NY20-frt, SYM25-frt, SYM6-frt, NY24-frt, and SYM24-frt and transductants were screened on LB agar plates including 100 μg/mL spectinomycin. For construction of Δ*queG*::*frt-kan* strains (NY20-dqueG, SYM25-dqueG, SYM6-dqueG, and SYM9-dqueG), P1 phage lysates prepared from strain SR08 were used for transduction of strains NY20-frt, SYM25-frt, SYM6-frt, and SYM9-frt and kanamycin-resistant transductants were screened similarly. For construction of Δ*ihfA* and Δ*ihfB* double mutant strains (NY20-dihfAB and NY24-dihfAB), Δ*ihfA*::*frt-kan* was introduced into strains NY20-dihfB and NY24-dihfB using P1 phage lysates prepared form KMG-5 and kanamycin-resistant transductants were screened similarly, which was followed by conformation of spectinomycin-resistance as described above.

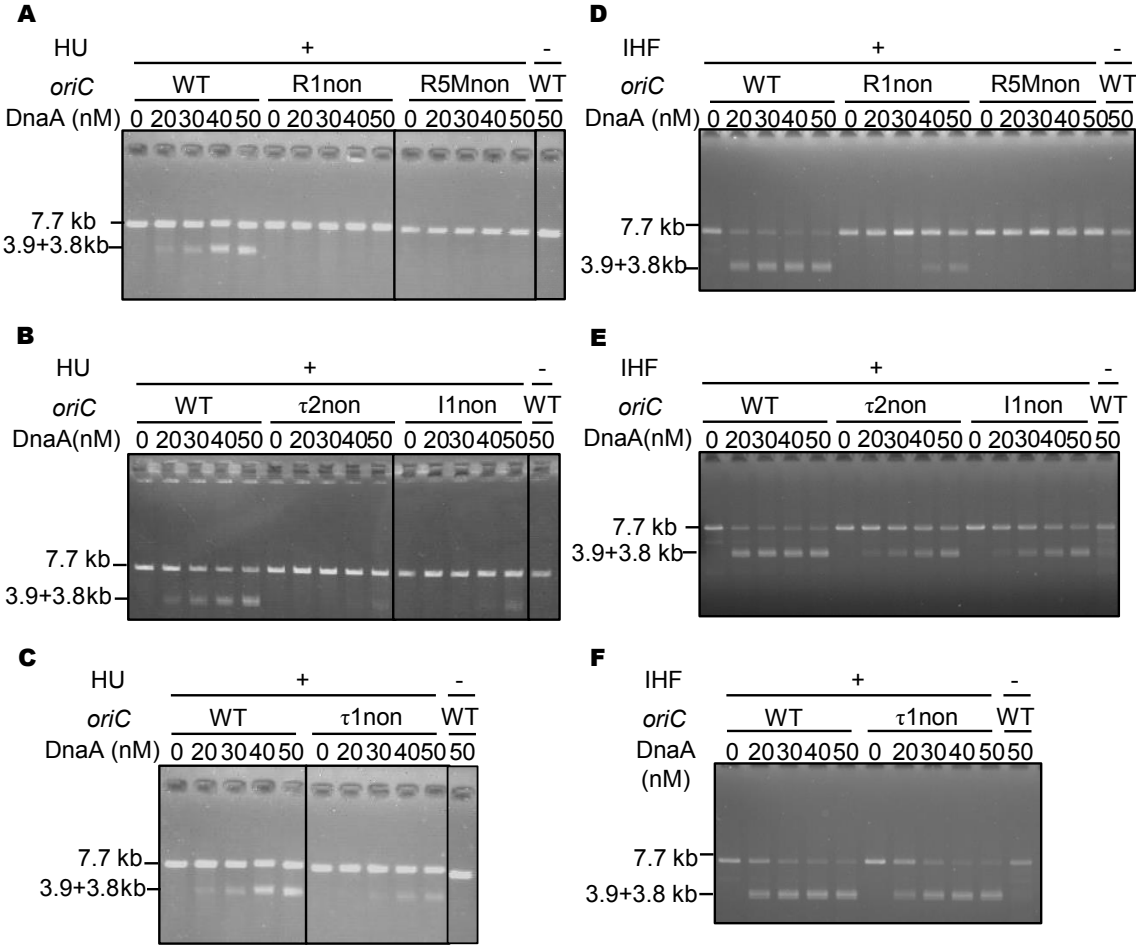
## Supplementary Figures

### Supplementary Figure S1



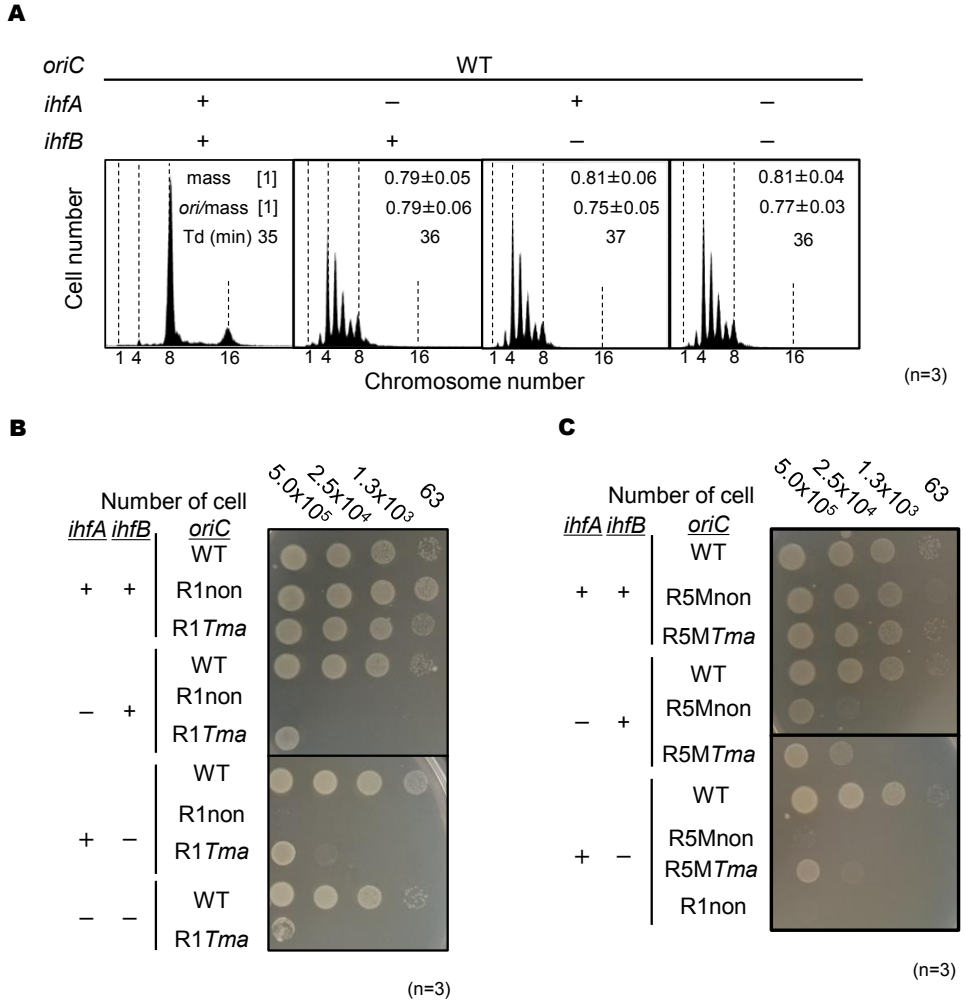

**Supplementary Figure S1.** Construction of a phylogenetic tree using the webtool iTOL (Letunic, I., & Bork, P., 2021) Presence or absence of genes homologous to E. coli IHF or HU in the representative bacterial species are based on a previous report (Kamashev, D., *et al.,* 2017). + IHF/HU conserved; - IHF/HU not conserved.

**Supplementary Figure S2**



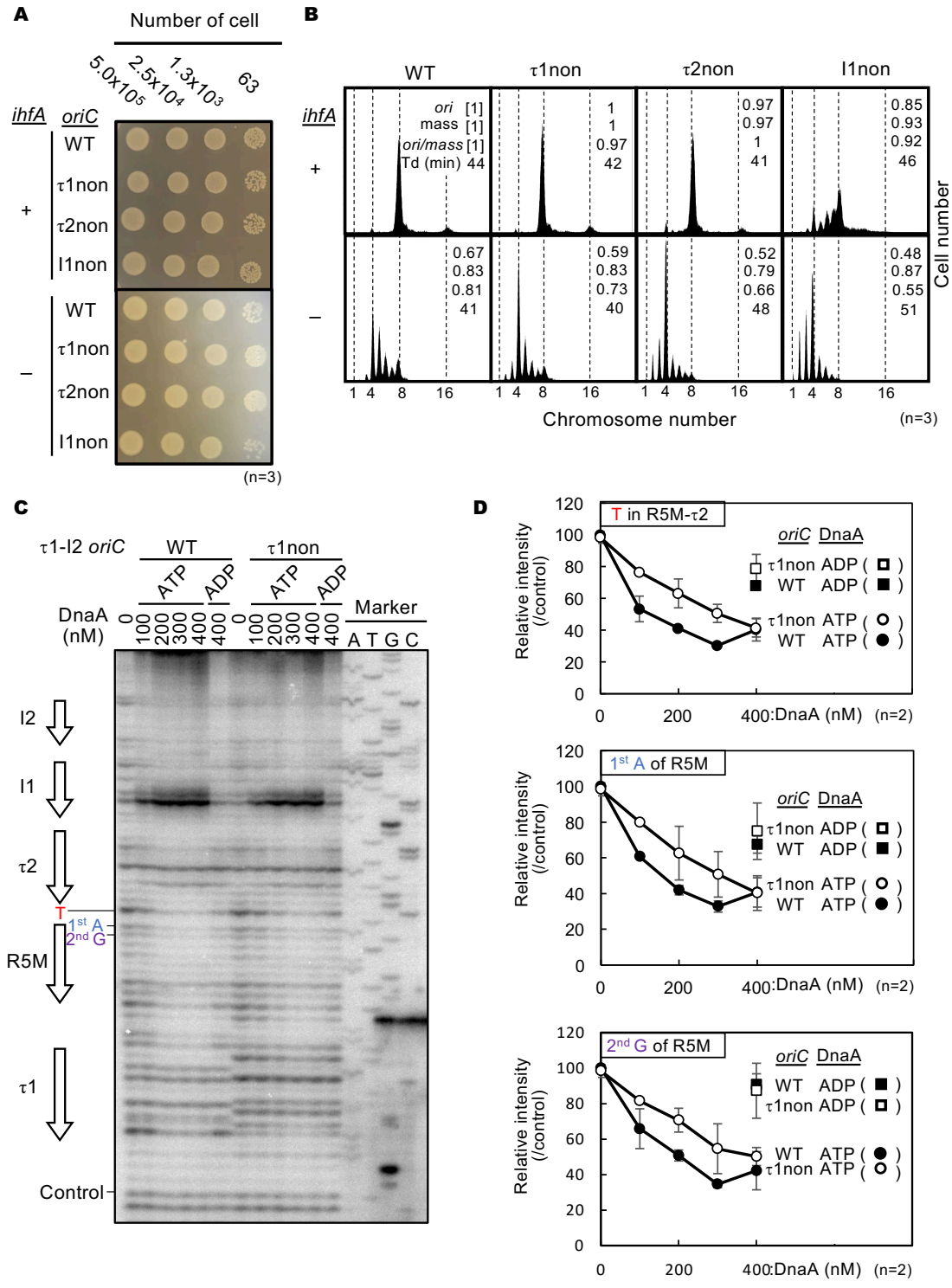

**Supplementary Figure S2.** DUE unwinding assay using *oriC* plasmids bearing nonsense boxes (A-F) The *oriC* plasmid (M13*oriC*MS9 WT) and its derivatives (M13*oriC*MS9 R1non, M13*oriC*MS9 τ1non, M13*oriC*MS9 R5Mnon, M13*oriC*MS9 τ2non, or M13*oriC*MS9 I1non) (1.3 nM) were incubated with ATP-DnaA (0-50 nM) in the presence (+) or absence (–) of HU (42 nM) or IHF (36 nM), followed by P1 nuclease assays. DNA products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. Gel images are representative of two independent experiments.

**Supplementary Figure S3**




**Supplementary Figure S3. Loss of IHF function by a single deletion of the *ihfA* or *ihfB* gene.**
(A) Flow cytometry analysis using cells with deletion mutations in *ihfA* and/or *ihfB* (NY20-frt, NY20-dihfA, NY20-dihfB, and NY20-dihfAB) grown at 30°C in LB medium, followed by further incubation with 0.3 mg/mL rifampicin and 0.01 mg/mL cephalexin for run-out replication. Cell sizes (mass) at the time of drug addition were measured using a Coulter counter. Mean mass, *ori*/mass ratio, and the doubling time (Td) of each strain are indicated at the top right of each panel. (B) Spot test using R1-box mutant cells with deletion mutations of *ihf* genes. Cells with wild-type *oriC* (WT) or with R1non or R1*Tma* substitution mutation carrying wild-type (+) (NY20-frt, SYM25-frt, and NY24-frt) or deletion (–) mutations of *ihfA* (NY20-dihfA, SYM25-dihfA, and NY24-dihfA), *ihfB* (NY20-dihfB, SYM25-dihfB, and NY24-ihfB) or both (NY20-dihfAB, NY24-dihfAB) were cultured to ~$10^9$ cells/mL, and 20-fold serial dilutions of each were spotted onto LB agar medium and the plates incubated for 14 h at 30°C. (C) Spot test using R5M-box mutant cells with deletion mutations in *ihf* genes. Cells with wild-type *oriC* (WT) or with R5Mnon or R5M*Tma* substitution mutations carrying wild-type (+) (NY20-frt, SYM6-frt, SYM24-frt) or deletion (–) mutations of *ihfA* (NY20-dihfA, SYM6-dihfA, SYM24-dihfA), or *ihfB*(NY20-ihfB, SYM6-ihfB, SYM24-ihfB) were incubated as described above.

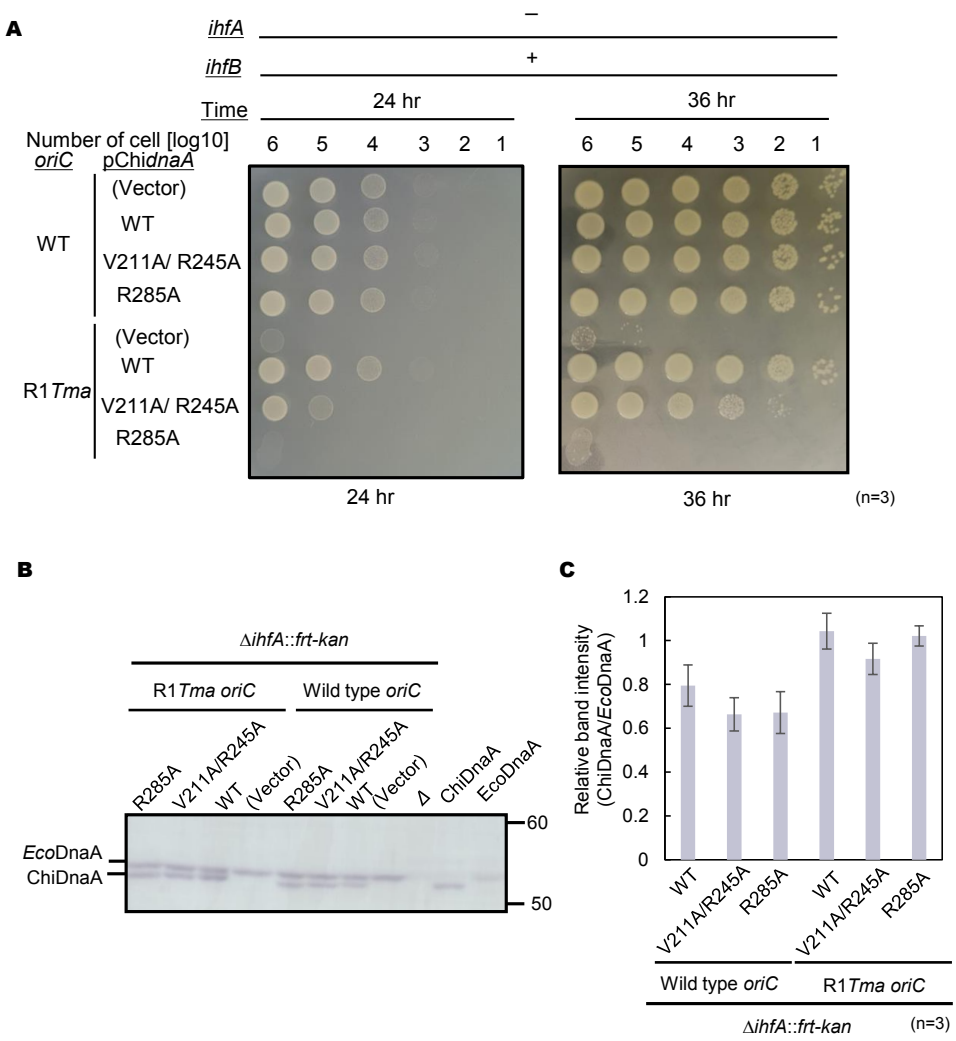
**Supplementary Figure S4**



**Supplementary Figure S4. Cell growth and replication initiation of *oriC* mutant strains bearing τ1non, τ2non, or I1non.**

(A) Spot test using cells with wild-type *oriC* (WT) or mutant *oriC* bearing τ1non, τ2non, or I1non in the presence (NY20-frt, SYM5-frt, SYM7-frt, SYM9-frt) or absence of *ihfA* deletion (NY20-ihfA, SYM5-dihfA, SYM7-dihfA, SYM9-dihfA). 20-fold serial dilutions of the full-growth cultures (~$10^9$ cells/mL) were incubated for 14 h at 30℃ on LB agar medium. Triplicate experiments were performed. + wild-type *ihfA*; – Δ*ihfA*::*frt-kan.* (B) Flow cytometry analysis of these cells. The cells were grown at 30℃ in LB medium, followed by further incubation with 0.3 mg/mL rifampicin and 0.01 mg/mL cephalexin for run-out replication. DNA contents and cell mass were analyzed as described in the legend to *Supplementary Figure S3A.* Triplicate experiments were performed. (C-D) DNaseI footprint experiments using the τ1-I2 *oriC* fragment. $^{32}$P-labeled τ1-I2 *oriC* fragment or its τ1non derivative (2.4 nM) was incubated at 30℃ for 10 min with ATP- or ADP-DnaA (0-400 nM) and competitors poly (dI-dC) and poly (dA-dT), followed by further incubation at 30℃ for 4 min with DNase I. Resultant DNA fragments were analyzed by 6% sequencing gels. A representative gel image is shown in *panel C*. Relative band intensities of the indicated T residue in R5M-τ2 region, 1st A and 2nd G residues of R5M box were calculated referring to a control band and normalized by the intensity of WT fragment without DnaA defined as 100 (D). Results are shown as the mean and standard deviations (SDs) (n=2).

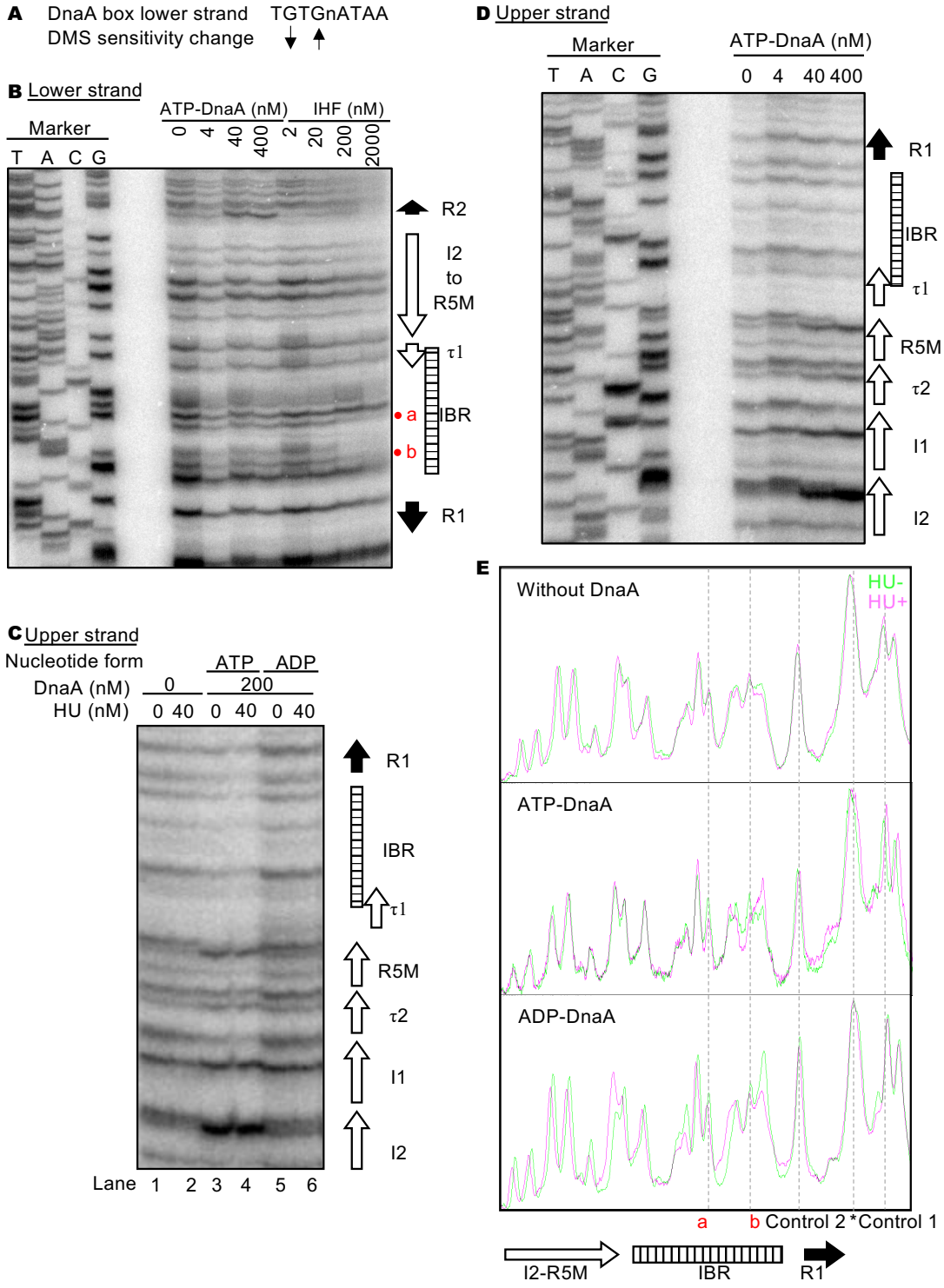
**Supplementary Figure S5**

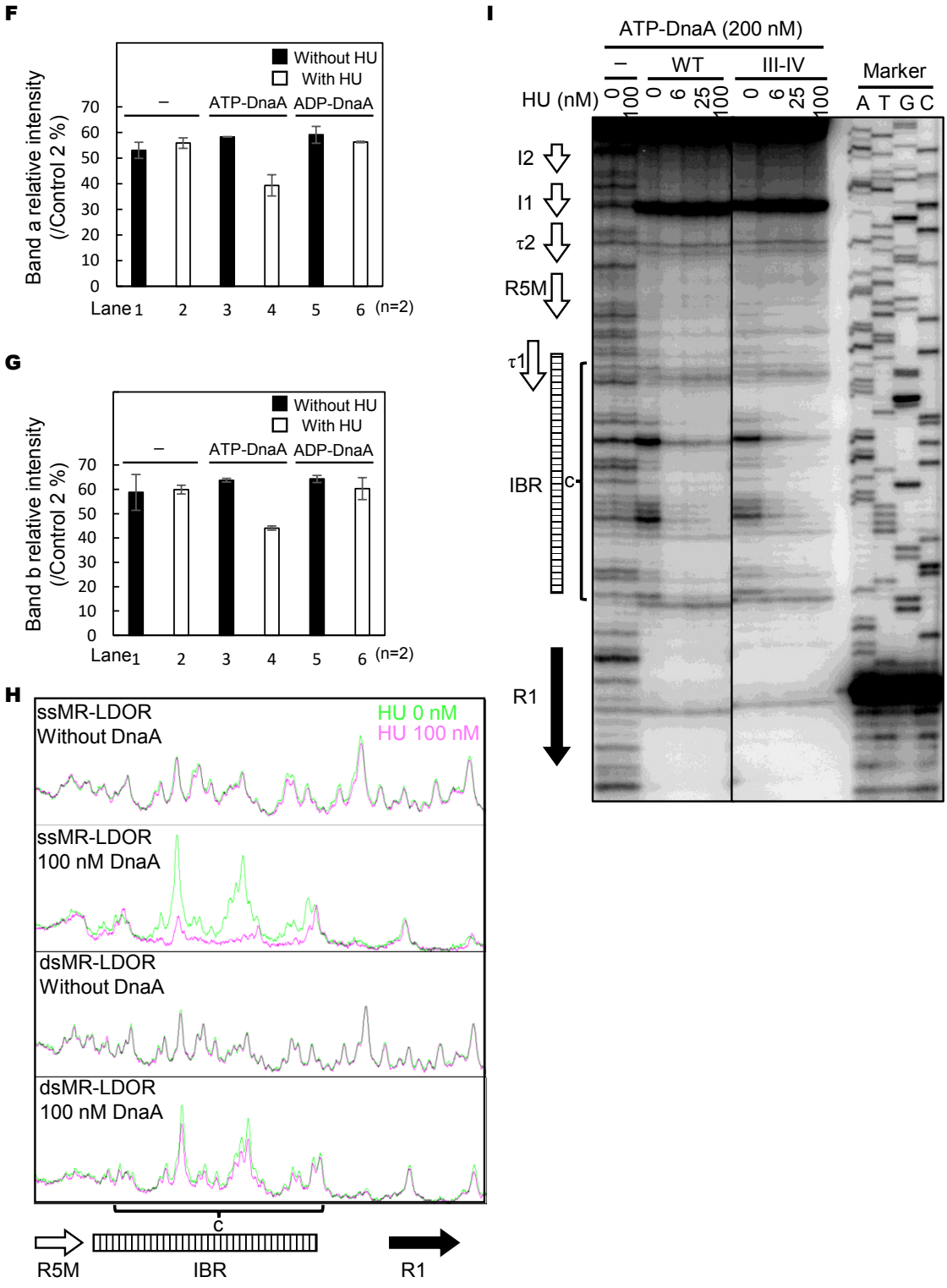



**Supplementary Figure S5.** Cell growth and DnaA contents of Δ*ihfA*::frt-*kan* cells with wild-type *oriC* or R1Tma *oriC* bearing plasmids

(A) Colony forming abilities. Δ*ihfA*::frt-*kan* cells with wild-type *oriC* or R1Tma *oriC* were transformed with pING1 plasmid (Vector) or its derivatives expressing ChidnaA (WT), ChidnaA V211A/R245A (V211A/R245A), or ChidnaA R285A (R285A). The transformants were grown overnight at 30˚C and 20-fold serial dilutions of the cultures (~$10^9$ cells/mL) were incubated on LB agar plates at 25°C for 24 h or 36 hr. +, wild-type; –, deletion. (B and C) Δ*ihfA*::frt-*kan* cells with wild-type *oriC* (NY20-dihfA) or R1Tma *oriC* (NY24-dihfA) bearing pING1 (Vector), pChidnaA WT, pChidnaA V211A/R245A, or pChidnaA R285A were grown at 30°C in LB liquid medium including ampicillin and were harvested for immunoblot analysis with polyclonal anti-DnaA antiserum. A representative gel image is shown in *panel B*. Band intensities of each lane in the gel image were analyzed using scanning densitometry. The relative band intensities of ChiDnaA to *Eco*DnaA are shown as "Relative intensity (ChiDnaA/EcoDnaA)" (C). Means and standard deviations (SDs) are also shown (n=3).

**Supplementary Figure S6**

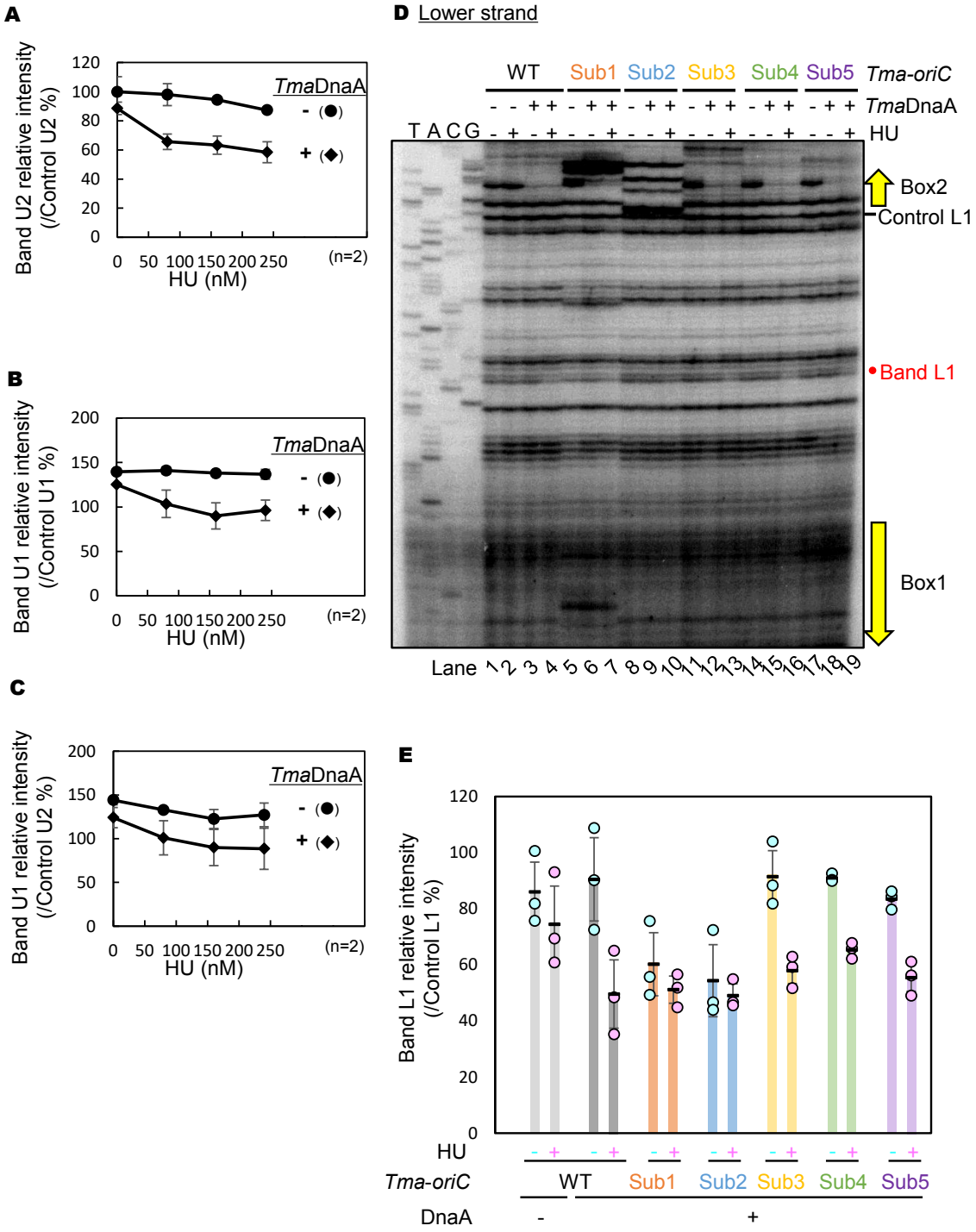


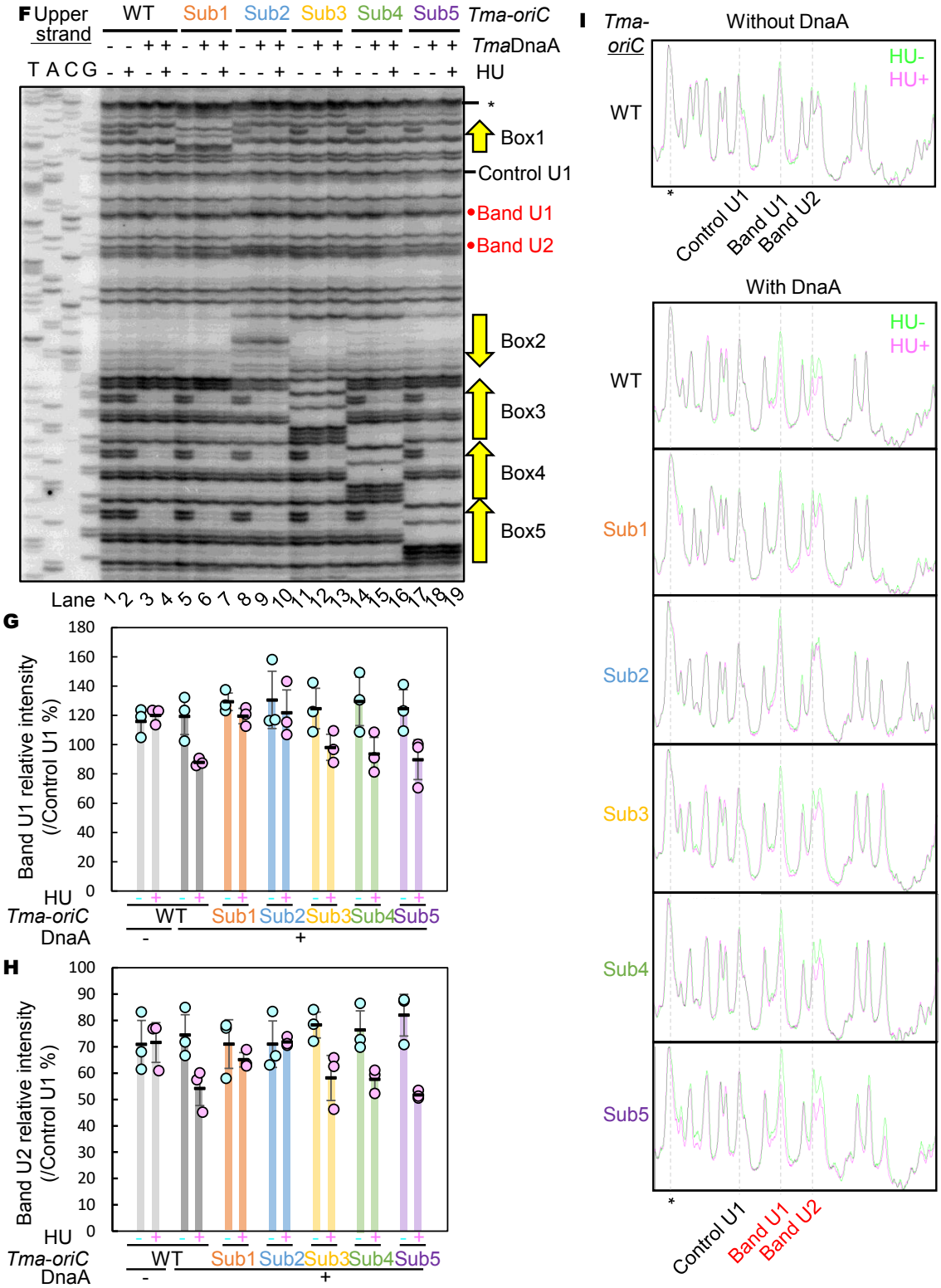

F
Band a relative intensity (/Control 2 %)
Without HU
With HU
–
ATP-DnaA
ADP-DnaA
Lane 1 2 3 4 5 6 (n=2)
G
Band b relative intensity (/Control 2 %)
Lane 1 2 3 4 5 6 (n=2)
H
ssMR-LDOR
Without DnaA
HU 0 nM
HU 100 nM
ssMR-LDOR
100 nM DnaA
dsMR-LDOR
Without DnaA
dsMR-LDOR
100 nM DnaA
c
R5M
IBR
R1
I
ATP-DnaA (200 nM)
–
WT
III-IV
Marker
HU (nM)
A T G C
I2
I1
τ2
R5M
τ1
IBR
c
R1

**Supplementary Figure S6. DnaA binding to supercoiled *oriC*.**

(A) DMS modification pattern by ATP-DnaA. ATP-DnaA decreased the DMS sensitivity of guanines in the second position, while increasing the DMS sensitivity of guanines in the fourth position. (B) The supercoiled form of *oriC* plasmid (M13*oriC*MS9) was incubated at 38ºC for 5 min with the indicated amount of ATP-DnaA (200 nM) or IHF, followed by further incubation for 5 min with 0.25% DMS and cleavage by 1 M piperidine. The resultant DNA fragments were analyzed by primer extension assays using the $^{32}$P-labeled primer KWsmaIoriCFwd for the upper strand modification. Bands were annotated by Sanger sequence marker and the positions of each DnaA box are shown on the right side of the gel image. Primer sequences are shown in Supplementary Table S1. Bands a and b are indicated by red dots. (C-D) The supercoiled form of *oriC* plasmid (M13*oriC*MS9) was incubated at 38ºC for 5 min with the indicated amount of HU in the presence or absence of ATP-DnaA or ADP-DnaA (200 nM), followed by further incubation for 5 min with 0.25% DMS and cleavage by 1 M piperidine. The resultant DNA fragments were analyzed by primer extension assays using $^{32}$P-labeled primer ori2 for the upper strand (*panel C*). Bands were annotated by Sanger sequence marker (*panel* D). (E) Histogram of Figure 7A. Each lane was scanned by image J and calibrated using the band indicated by asterisk (*). Histograms of with and without HU lanes are overlayed in each DnaA condition. (F and G) Intensities of bands a and b relative to the intensity of control band 2 (shown in Figure 7) were quantified and shown as “Band s relative intensity” (F) and “Band b relative intensity” (G). Results are the means and standard deviations (SD) of two independent experiments. Analysis of DNaseI-treated ssMR-Left DOR and dsMR-Left-DOR using the M28-R1 f primer on 6% sequencing gel with the Sanger sequence marker. (H) Histogram of IBR in Figure 7E. Each lane is scanned by image J and the resultant histograms are overlayed depending on experimental conditions as indicated. The position of R1/R5M boxes and IBR are shown in the bottom of the histograms. (I) DNase I footprint analysis using ssMR-left DOR with HU and DnaA WT or DnaA III-IV. $^{32}$P-labeled ssMR-left DOR was incubated at 30ºC for 10 min with the indicated amount of HU in the presence (200 nM) or absence (-) of ATP-DnaA WT or ATP-DnaA II-IV, followed by further incubation for 4 min with DNase I (10 mU). DNase I-digested products were analyzed on 6% sequencing gels. The positions of DnaA boxes and IBR are shown on the left side of the gel image.

**Supplementary Figure S7**




F
Upper strand
WT
Sub1
Sub2
Sub3
Sub4
Sub5
Tma-oriC
TmaDnaA
HU
T A C G
*
Box1
Control U1
Band U1
Band U2
Box2
Box3
Box4
Box5
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
G
Band U1 relative intensity (/Control U1 %)
HU
Tma-oriC
DnaA
H
Band U2 relative intensity (/Control U1 %)
I
Tma-oriC
Without DnaA
HU-
HU+
WT
Control U1
Band U1
Band U2
With DnaA
Sub1
Sub2
Sub3
Sub4
Sub5

**Supplementary Figure S7. Annotation of the bands to specific positions within *Tma-oriC*.**
(A-C) The relative intensities of band U2 to control U2 and of band U1 to control U1 or U2 were quantified as "Band U1 relative intensity (/Control U2%)" in *panel* A, and "Band U2 relative intensity (/Control U1%) in *panel B*, and "Band U2 relative intensity (/Control U2%)" in *panel C*. Results are the means and standard deviations (SD) of two independent experiments. (D-I) DMS footprint using *Tma-oriC* plasmid. The supercoiled form of *Tma-oriC* plasmid pOZ14 (WT) and its derivatives with substitution of one of *Tma*DnaA boxes 1-5 (Sub1-5) were incubated at 48ºC for 5 min with 400 nM HU in the presence or absence of ATP-*Tma*DnaA (400 nM), followed by further incubation with 0.25% DMS for 5 min and cleavage by 1 M piperidine. The resultant DNA fragments were analyzed by primer extension assay using $^{32}$P-labeled primer TMA28 for lower strand modification (*D-E*) and $^{32}$P-labeled primer 306 for upper strand modification (*F-H*). The primer extension products were resolved on 6% sequencing gels with the Sanger sequence marker. Positions of the *Tma*DnaA boxes are shown on the right side of the gel images (*D* and *F*). The relative intensities of band L1 to control L1 and of bands U1 and U2 to control U1 were quantified as "Band L1 relative intensity (/Control L1%)" in *panel* E, and "Band U1 relative intensity (/Control U1%) in *panel G*, and "Band U2 relative intensity (/Control U1%)" in *panel H*. Results are the means and standard deviations (SD) of three independent experiments. Intensities of the bands shown in *panel F* were quantified and the resultant histograms are shown in *panel I.* Each histogram obtained in the presence (+) or absence (-) of HU were calibrated by the band indicated by an asterisk (*) and overlayed for each plasmid template as indicated in the left side of the histograms.

**Supplementary references**

Kasho,K., Fujimitsu,K., Matoba,T., Oshima,T. and Katayama,T. (2014) Timely binding of IHF and Fis to DARS2 regulates ATP-DnaA production and replication initiation. *Nucleic Acids Res.*, **42**, 13134–13149.

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Ozaki,S., Fujimitsu,K., Kurumizaka,H. and Katayama,T. (2006) The DnaA homolog of the hyperthermophilic eubacterium *Thermotoga maritima* an open complex with a minimal 149-bp origin region in an ATP-dependent manner. *Genes to Cells*, **11**, 425–438.

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