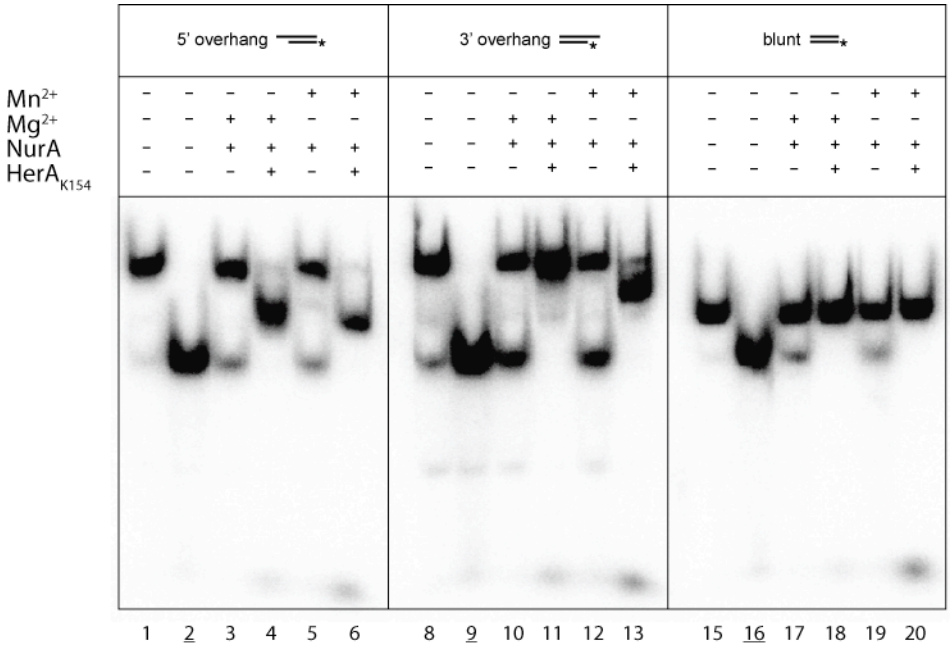
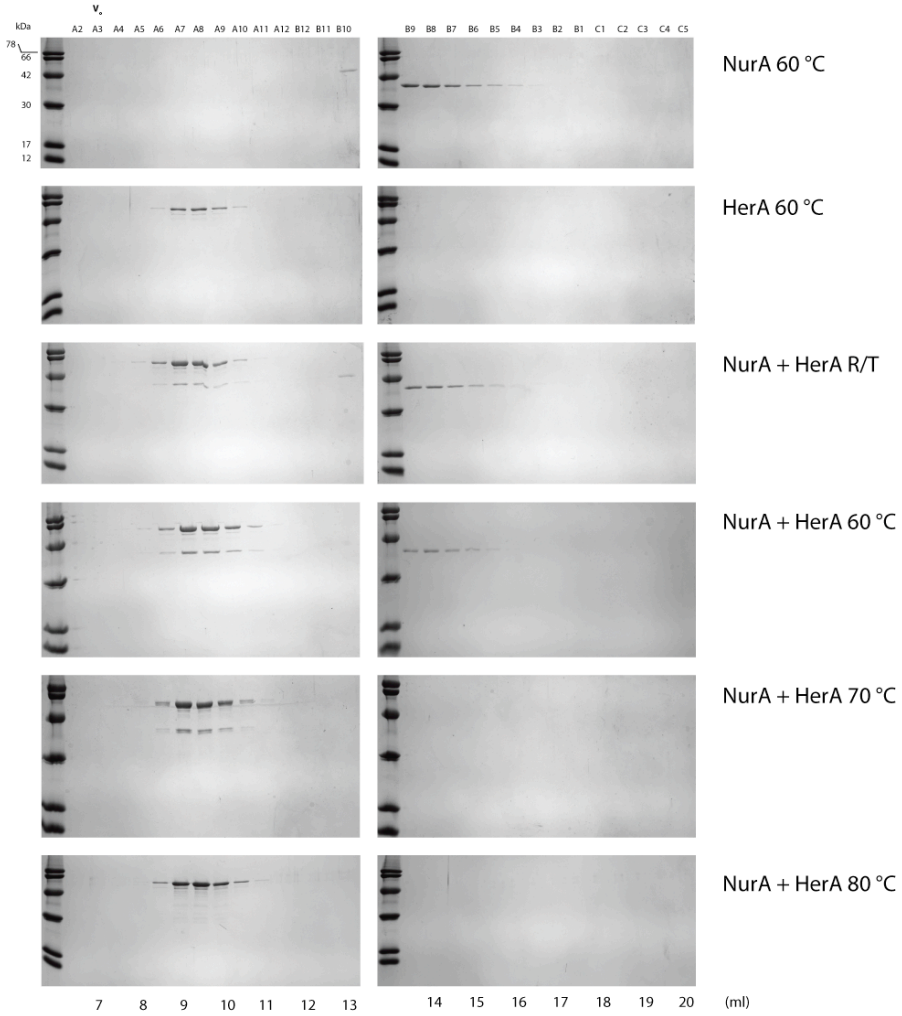
Supplementary Materials

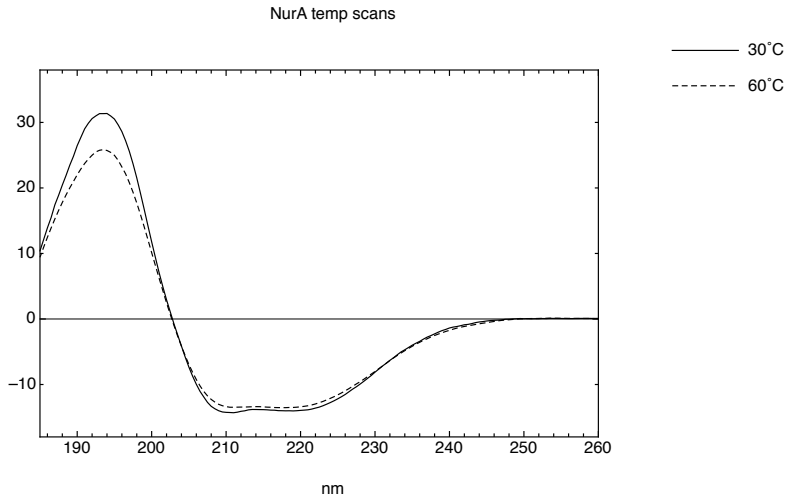
Supplementary Data



Supplementary Figure S1: Processing of 5' and 3' ssDNA overhangs by NurA alone or NurA-HerA K154A in the presence of Mg^{2+} or Mn^{2+}. Combined helicase-nuclease assays using ^{32}P-radiolabelled 54 bp dsDNA substrates, possessing either blunt ends, or a 25 nt single-stranded overhang on either the 5' or 3' end. Asterisks indicate the ^{32}P-radiolabel on one strand of each substrate. Substrates were incubated at 60 °C for 30 mins with wtNurA (2 μM dimer) in the presence of Mg^{2+} (lanes 3, 10 and 17), wtNurA-HerA K154A complex (2 μM hexamer and 2 μM dimer) in the presence of Mg^{2+} (lanes 4, 11 and 18), wtNurA (2 μM dimer) in the presence of Mn^{2+} (lanes 5, 12 and 13) and wtNurA-HerA K154A complex (2 μM hexamer and 2 μM dimer) in the presence of Mn^{2+} (lanes 6, 13 and 20). Lanes 1, 8 and 15 represent no protein controls. Lanes 2, 9 and 16 (underlined) represent the substrates after boiling at 99 °C for 5 mins. Products were visualised by phosphoimaging.

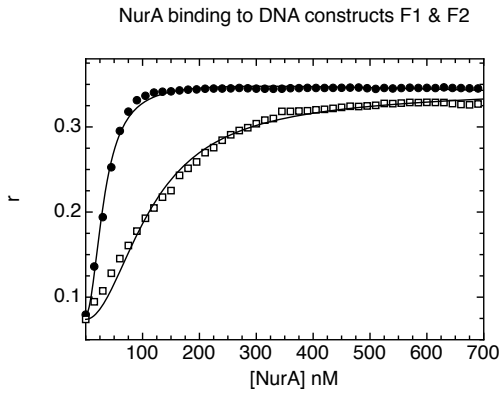


Supplementary Figure S2: Temperature dependence of optimal wtHerA-wtNurA complex formation. Analytical gel filtration analysis using an S200 Superdex HR 10/30 column. HerA (0.89 nmol, hexamer) and NurA C52A (1.8 nmol, dimer) were heated individually or together as a function of temperature (temperature range: room temperature (R/T – 25 °C) to 80 °C, 10 °C increments) for 20 mins, centrifuged 14 000 rpm for 5 minutes and resolved by size exclusion chromatography. Protein fractions were separated by SDS-PAGE and visualised with coomassie stain. Elution volumes are displayed below the final panels. V_o denotes column void volume.

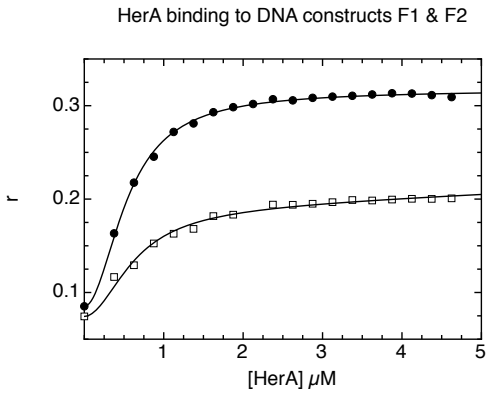


Supplementary Figure S3: CD spectra of NurAwt (0.14 mg.ml^{-1} monomer) as function of temperature (see methods for details). CD spectra at 30˚C and 60˚C and analysing the percentage contribution of secondary structure elements to the spectra. Between 30- and 60˚C a loss of α-helical content of 4% (from 40% to 36%) is detected and this is mirrored by an increase in disordered elements of 4% for the same temperature change. An increase in the content of β-sheet and turns of 1% and 2% respectively, was also observed. However, this data is less reliable because of the weak signal of these secondary structure elements.

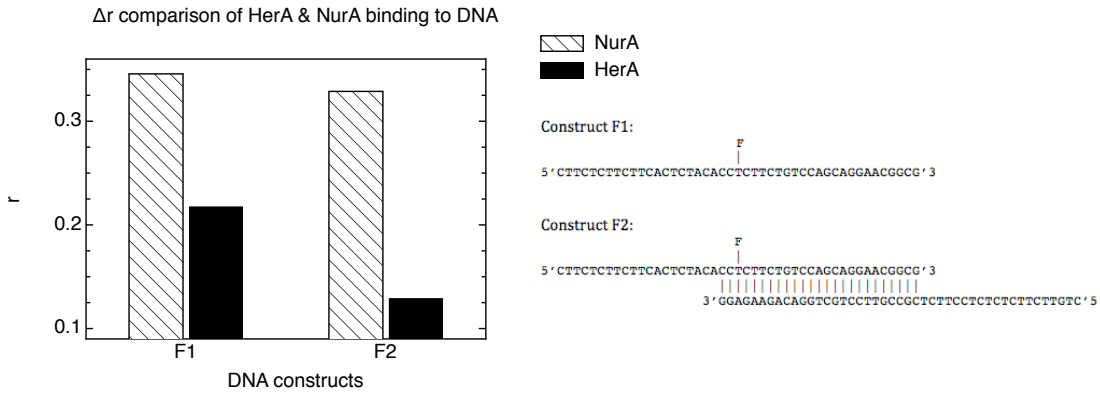
A.



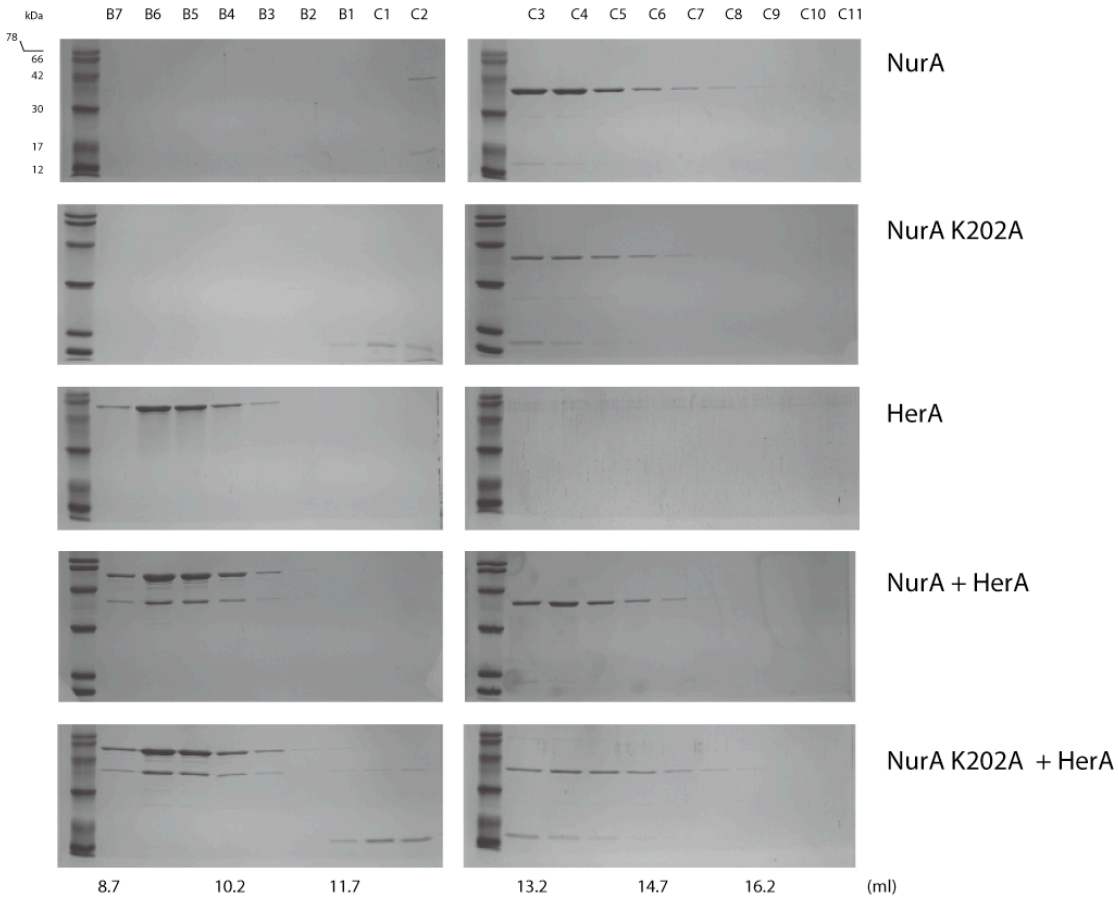
B.



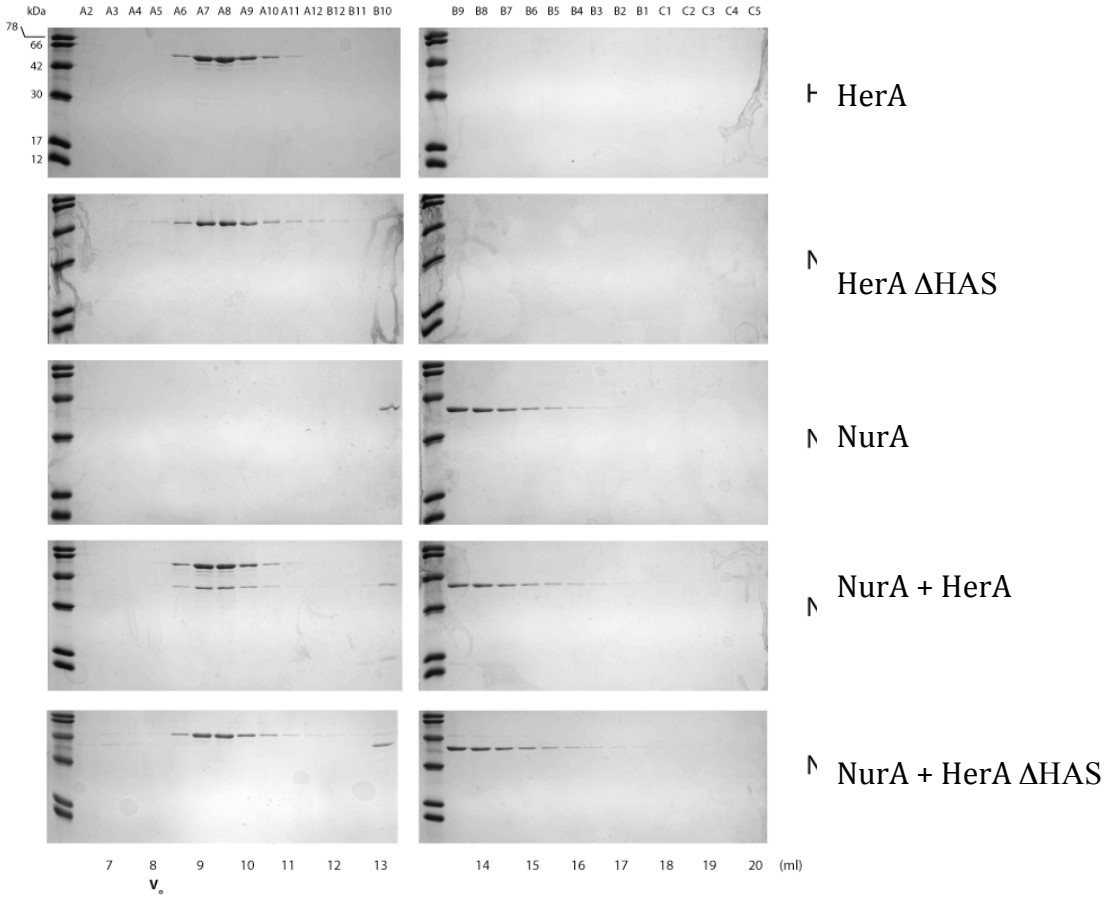
C.



Supplementary figure S4: **(A)** Fluorescence anisotropy measurements of binding of NurA to ssDNA oligo (F1) – filled circles, and to 5' overhangs dsDNA (F2) – open squares. The lines through the plots are fit to a binding model of two identical independent binding sites as described in the methods section. Apparent K_d for NurA binding to F1 and F2 are 33 ± 6 nM and 114 ± 29 nM respectively. **(B)** Binding of HerA to ssDNA oligo (F1) – filled circles, and to 5' overhangs dsDNA (F2) – open squares. Apparent K_d for HerA binding to F1 and F2 are 0.54 ± 0.01 μM and 0.62 ± 0.05 μM respectively. **(C)** Comparison of HerA and NurA binding to F1 and F2 constructs at protein concentrations of 0.62 μM.



Supplementary Figure S5: Stable formation of wtHerA-NurA K202A complex. Analytical gel filtration analysis using a S200 Superdex HR 10/30 column. HerA (2 nmol, hexamer), NurA K202A (7.2 nmol, dimer) and NurA (7.2 nmol, dimer) were heated individually or together at 60 °C for 20 mins, centrifuged 14, 000 rpm for 5 minutes and resolved using size exclusion chromatography. Protein fractions were separated by SDS-PAGE and visualised with coomassie stain. Protein elution volumes (ml) are displayed below the final panels.



Supplementary Figure S6: Formation of HerA-NurA complex is mediated by the HAS (HerA and ATP synthase) domain of HerA. Analytical gel filtration analysis using a S200 Superdex HR 10/30 column. HerA (0.89 nmol, hexamer), N-terminally truncated HerA (HerA ΔHAS) lacking HAS domain (0.89 nmol, hexamer) and NurA C52A (1.8 nmol, dimer) were heated individually or together at 60 °C for 20 mins, centrifuged 14 000 rpm for 5 minutes and resolved using size exclusion chromatography. Protein fractions were separated by SDS-PAGE and visualised with coomassie stain. Elution volumes are displayed below the final panels. V_o denotes column void volume.

Supplementary Table 1: Oligonucleotides used in the study

Primer	Sequence (5′ – 3′)
BLUNT_ REV	GTACAAATCATATAAAGTATTTATTTATTTTCAATTCAACATATGATTTAACTG
Blunt_ FOR	CAGTTAAATCATATGTTGAATTGAAAATAAATAAATACTTTATATGATTTGTAC
5PRI_ FOR	AATTGTGATCTCTTCTCCTTGTCAACAGTTAAATCATATG TTGAATTGAAAATAAATAAATACTTTATATGATTTGTAC
3PRI_ FOR	CAGTTAAATCATATGTTGAATTGAAAATAAATAAATACTT TATATGATTTGTACAATTGTGATCTCTTCTCCTTGTCAA
Match Bluntfor	GGGTCGGATGAGATATGCCCAGTTATTTTAACTAAGGTAGTAAAGGAACCGGGC
Match Bluntrev	GCCCGGTTCCTTTACTACCTTAGTTAAAATAACTGGGCATATCTCATCCGACCC
Mismatch Bluntrev	ATGGAATTCCTTTACTACCTTAGTTAAAATAACTGGGCATATCTCATCCGACCC
HH1a	GAGTTTTGAGTTTACCCCGCCCGCGCCGGCGCGCACGCTACATGCGTGAAGACC AGCCCCGGCCCGCGCGCGCGCGCGCTTTTGCGCGCGCGCGCG
HH1b	CCCGCCCGCGCCGGCGCGCACGCTACATGCGTGAAGACCAGCCCCGGCCCGCGC GCGCGCGCGCTTTTGCGCGCGCGCGCG
HH2	CGCGCGCGCGCGCTTTTGCGCGCGCGCGCGCCACCACCGCCACCACTCGTGGCA TGTAGCGTGCGCGCCGGCGCGGGCGGG
HH3 Holliday Junction	GGGCCGGGGCTGGTCTTCACGTTCAGGACTATCGCTATAGTTGCGCCAGGAGCT TTTGCTCCTGGCGCAACTATAGCGATAGTCCTGAACCACGAGTGGTGGCGGTGG TG
JB87_F	CGCCGAAACAAGCGCTCATGAGC
JB87_R	TGAGTCACTTTTGTGGGCTCT
JB53 TFO	TTCTTTTCTTTCTTCTTTCTTT
JB102	AAGAAAAGAAAGAAGAAAGAAA
JB103	TTTCTTTCTTCTTTCTTTTCTT
MutNurA D58A	GAAACTTGTAAATTTGTAGCTATTGCCGGTGGATCTTTTGGTAGACCTATGAG
MutNurA C52A	AATACGTTATAAACCATGAACAGAGCGAAACTGCTAAATTTGTAGCTATTG
MutHerA K154A	GCTATTTTAGCAGCTACTGGTTCTGGGGCGTCAAATACAGTAGCAGTTCTTTC
MutNurA F300E	GGAGTAAGTATTAAGGGCTATCCAGAGCCACTCTTAAAAGCTCACATGGAT
MutNurA I295E	ATGGATCGTCTGAGTGGAGTAAGTGAGAAGGGCTATCCATTTCCACTCTTA
MutNurA K202A	GATGGCAACGTTTTGTGGATATCTGCTGTTAGTAGAGGGAGAGATCTATTT
NurAfor	CCTCATATGATAAGAAAAATATATGATAAGTTAGTAG
NurArev	CCTCTCGAGATAAAACTGGCTTGGCCACCATTCGATATC
HerAfor	CCTCATATGATAATTGGTTATGTAATTGGTCAAGCTAC
HerArev	CCTCTCGAGATCACCAATTTCTGTTCCAAAGTCAGC
ATP5P Fwd	TTGTGGAAGTCGTTGACAGTCACCTCTTACATTCCTCAACTGGACTGACG
ATP5P Rev	TAACCTCACACGTCAGTCCAGTTGAGGAATGTAAGAGGTGACTGTCAACG
ATP3P Fwd	CGTTGACAGTCACCTCTTACATTCCTCAACTGGACTGACGTTGTGGAAGT
ATP3P Rev	CGTCAGTCCAGTTGAGGAATGTAAGAGGTGACTGTCAACGTAACCTCACA
ATP BluntFwd	CGTTGACAGTCACCTCTTACATTCCTCAACTGGACTGACG
ATPBlunt Rev	CGTCAGTCCAGTTGAGGAATGTAAGAGGTGACTGTCAACG
ATP3TFwd	TTTCGTTGACAGTCACCTCTTACATTCCTCAACTGGACTGACG
ATP3TRev	TTTCGTCAGTCCAGTTGAGGAATGTAAGAGGTGACTGTCAACG

Supplementary Table 2:
Data processing and crystallographic refinement

	Overall	InnerShell	OuterShell
Low resolution limit	35.94	35.94	2.64
High resolution limit	2.5	7.91	2.5
Rmerge	0.098	0.027	1.326
Rmerge in top intensity bin	0.036	-	-
Rmeas (within I+/I-)	0.113	0.031	1.541
Rmeas (all I+ & I-)	0.128	0.057	1.525
Rpim (within I+/I-)	0.057	0.016	0.782
Rpim (all I+ & I-)	0.046	0.021	0.551
Fractional partial bias	-0.032	-0.019	-0.101
Total number of observations	184283	5861	26519
Total number unique	24087	804	3465
Mean((I)/sd(I))	13.2	35.5	1.8
Completeness	100	99.2	100
Multiplicity	7.7	7.3	7.7
Anomalous completeness	100	99.4	100
Anomalous multiplicity	3.9	3.9	3.9
DelAnom correlation between half-set:	0.62	0.842	0.015
Mid-Slope of Anom Normal Probability	1.283	-	-

Average unit cell: 67.74 80.28 67.67 90.00 107.17 90.00

Space group: P21

Average mosaicity: 0.41

Selenomethionine crystals Wavelength: 0.97940 Å

Resolution range (Å)	35.94 - 2.50
Number of unique reflections	24056
Number of non-H atoms	5250
R_{factor}	23.84
R_{free}	27.07
Average B-factor	73.6
Waters	66
Manganese ions	2
Rmsd bonds	0.015
Rmsd angles	1.396
Ramachandran plot:	
Outliers	0
Allowed	18 (2.88%)
Favoured	606 (97.12%)
Rotamer outliers	9 (1.63%)

Supplementary Methods

Expression constructs

The *Sulfolobus solfataricus herA* and *nurA* genes were amplified from *Sulfolobus solfataricus* P2 genomic DNA (using primers HerAfor and HerArev or NurAfor and NurArev (Supplementary Table 1), respectively) and cloned individually into pET30a using *Nde*I and *Xho*I, positioning the ORFs in frame with a C-terminal hexa-histidine tag. Site-directed mutagenesis (QuikChange, Stragene) was used to introduce a stop codon in both constructs after the *hera* or *nura* ORFs, generating the untagged constructs. Point mutations in *herA* and *nurA* were introduced using site-directed mutagenesis (QuikChange, Stragene) and were confirmed by DNA sequencing of the complete open reading frame. Site directed mutagenesis oligonucleotides are listed in Supplementary Table 1.

Protein Purification

S. solfataricus HerA and NurA proteins (untagged or c-terminally his-tagged) were expressed in Rosetta (DE3) pLysS cells (Novagen) *E. coli* cells. Briefly, cells were grown at 37 °C to an OD_{600} 0.3, temperature lowered to 25 °C, induced with 0.3 mM IPTG and grown over-night with shaking. Cells were harvested by centrifugation, resuspended in 2x TBS (200 mM Tris pH 8.0, 300 mM NaCl, 10 % glycerol, 1 mM DTT) and lysed by sonication. Insoluble material was removed via centrifugation (17, 000 x g for 12 mins at 4 °C). The soluble fraction was subsequently heated to 70 °C for 20 minutes and centrifuged (17, 000 x g for 12 mins at 4 °C) again to remove insoluble material. For NurA, the soluble, heat-treated, fraction was loaded onto a HiTrap HP column (GE biosciences) pre-equilibrated with 50 mls Low Salt Buffer

(50 mM Nacl, 200 mM Tris pH 8.0, 5 % glycerol, 1 mM DTT). For HerA, the soluble, heat treated fraction was diluted with Buffer A (200 mM Tris pH 8.0, 5 % glycerol, 1 mM DTT), reducing the NaCl concentration to 50 mM before loading onto a HiTrap HP column. The proteins were eluted from the heparin column with a gradient of 50-1000 mM NaCl, in 20 mM Tris pH 8.0, 300 mM NaCl, 5 % glycerol, 1 mM DTT. HerA eluted at 300 mM NaCl and NurA at 360 mM NaCl. Fractions containing the purified proteins were pooled and concentrated before running a final size-exclusion purification step over a Superdex 200 16/60 column (GE biosciences), in 20 mM Tris pH 8.0, 300 mM NaCl, 5 % glycerol, 1 mM DTT. Fractions containing the purified proteins were pooled, concentrated, aliquoted and flash frozen in liquid N_2. Protein concentrations were quantified by UV spectrophotometry.

Supercoiled and linear plasmid nuclease assay

Reactions were performed in a 20 μl reaction volume containing 20 mM Tris (pH 8), 5 % glycerol, 5 mM $MnCl_2$, 100 mM NaCl, 3 mM ATP, 1 mM DTT and 1x BSA (NEB). Proteins (HerA, 2 μM hexamer; NurA 2 μM dimer) were preincubated at 60 °C for 20 mins before addition to 100 ng (at 3 nM) ϕX174 DNA (RFI – circular, double stranded DNA, covalently closed; RFII – nicked circular double stranded DNA, covalently closed; RFI linearised with either *Xho*I, *Sac*II or *Stu*I) and incubated at 60 °C for 1 hour. Reactions were terminated by the addition of 0.4 % SDS, 1.6 mg/ml proteinase K and incubated at 37 °C for 1 hr. Products were separated on 1 % agarose in 1x TBE, stained with SYBR Gold (Invitrogen), briefly rinsed with water and DNA visualised under UV light.

Generation of ^{32}P radiolabelled dsDNA substrates.

Substrates for the radiolabelled helicase-nuclease assays were constructed as follows: 100 ng oligonucleotide DNA substrate (4.1 pmol) was 5' end labeled with 50 pmol [γ-^{32}P] ATP (Perkin Elmer) using 20 units of T4 Polynucleotide Kinase (NEB). The helicase assay substrates consisted of a 5' [γ-^{32}P-ATP]-labeled 54 mer oligonucleotide annealed to a 2 fold molar excess of unlabeled complementary oligonucleotide creating blunt substrate, or a substrate with either a 5' or 3' 25 nucleotide single-stranded overhang. Unincorporated radionucleotide was removed using G25 micro-spin columns (GE Healthcare) and annealed substrates were further purified using non-denaturing PAGE.

The 5' overhang substrate shown in Figures 1B, 1D, 5C and 6E consisted of 5'[^{32}P]-labeled BLUNT_REV annealed to 5PRI_FOR. The 3' overhang substrate shown in Figures 1B, 1D, 5C and 6E consisted of 5'[^{32}P]-labeled BLUNT_REV annealed to 3PRI_FOR. The Blunt substrate used in Figures 1B, 1D, 5C and 6E consisted of 5'[^{32}P]-labeled BLUNT_REV annealed to BLUNT_FOR. The splayed-end constructs shown in Figure 1C were prepared by annealing ^{32}P-labelled MatchBluntrev or MismatchBluntrev to unlabelled MatchBluntfor. All oligonucleotides are listed in Supplementary Table 1.

Combined DNA unwinding and nuclease assays using ^{32}P-radiolabelled substrates.

DNA unwinding and nuclease reactions were performed in a 20 μl reaction volume containing 20 mM Tris acetate (pH 8), 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 5 % glycerol, 100 mM NaCl, 5 mM $MgCl_2$, 3 mM ATP, 1x BSA]. 0.46 μM of hexameric HerA protein (native protein or K154A mutant) and 0.46 μM dimeric NurA protein (native protein or D58A, K202A, F300E, I295E, F300E:I295E mutants) were preincubated at 60 °C for 20 mins before addition of 10 nM ^{32}P-labelled oligonucleotide and incubated at 60 °C for 30 mins. Reactions were terminated by the addition of 20 μl stop buffer (100 mM EDTA, 0.5 % SDS, 0.1 % bromophenol blue and 50 % glycerol) and separated on 12 % SDS-PAGE in 1x TBE, 0.2 % SDS at 7.5 V/cm. Gels were dried and results were visualised by phosphoimaging (GE Healthcare Typhoon system).

Holliday junction resolution assays

100 ng oligonucleotide DNA substrate HH1a or HH1b was 5' end labeled with 50 pmol [γ-^{32}P]ATP (Perkin Elmer) using 20 units of T4 Polynucleotide Kinase (NEB). After radiolabeling, the HH1a and HH1b oligonucleotides were annealed to the two complementary oligonucleotides HH2 and HH3 (100 ng of each added together) to generate the synthetic Holliday junction substrates, in which 3 of the available four termini were blocked by hairpin structures (see Figure 2A). The remaining free-end possessed either a 5' single-stranded overhang, if the oligonucleotide HH1a was used, or alternatively was blunt-ended when the substrate was constructed from

oligonucleotide HH1b. The annealed substrates were purified on a 12% polyacrylamide gel in 1 x TBE, prior to use in the assays.

Holliday junction resolution reactions were performed in a 20 μl reaction volume containing 20 mM Tris acetate (pH 8), 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 5 % glycerol, 100 mM NaCl, 5 mM $MgCl_2$, 3 mM ATP, 1x BSA]. 0.92 μM of hexameric HerA protein (native protein or K154A mutant) and 0.92 μM dimeric NurA D58A protein were mixed and preincubated at 60 °C for 20 mins, before addition of 20 nM ^{32}P-labelled Holliday junction substrate, and incubated at 65 °C for 3, 9 or 15 mins. Reactions were terminated on ice with the addition of 10 μl ice-cold 6 x glycerol loading buffer (20 mM Tris, pH8, 30 % glycerol, 0.25 % bromophenol blue) and immediately separated by 12 % SDS-PAGE in 1x TBE, 0.2 % SDS at 7.5 V/cm. Gels were dried and results were visualised by phosphoimaging (GE Healthcare Typhoon system).

Triplex displacement assays

Triplex displacement assays were based on Firman *et al*., 2000 (26), but used a linear 500 bp PCR product containing a 22 bp triplex forming oligo (TFO) binding site starting at nucleotide 212. A 500 bp PCR was amplified from plasmid pLKS5 (kindly provided by Mark Szczelkun, Bristol University, School of Biochemistry) using primers JB87_F [5' CGCCGAAACAAGCGCTCATGAGC] and JB87_R [5' TGAGTCACTTTTGTGGGCTCT] using PFU polymerase and purified using Qiagen PCR Purification kit. An alternative shorter oligonucleotide based substrate was also designed that represented the 22 bp TFO binding site only. Two individual oligos (JB102 and JB103) were annealed as described earlier. The triplex forming

oligonucleotide (JB53 TFO) was end labeled with ^{32}P using polynucleotide kinase as described above. 50 nM of the linear PCR product, or the short duplex TFO binding site (oligonucleotide approach), were mixed in buffer TAB (25 mM MOPS, pH 5.5, 10 mM $MgCl_2$) with 25 nM $\gamma^{32}P$-labelled TFO, in a total reaction volume of 100 µl, and annealed at room temperature overnight.

230 nM or 94 nM HerA (or Her K154A) hexamer and NurA D58A dimer were pre incubated in reaction buffer (25 mM MOPS, 50 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM Spermidine, pH 5.5) at 60 °C for 30 mins and then cooled to 50 °C. 5 nM triplex was added and incubated at 50 °C for 0.5, 1, 1.5 or 5 minutes (or 3 minutes for the oligonucleotide derived control triplex). Reactions were terminated on ice, followed by the addition of x6 glycerol loading buffer (30 % glycerol, 20 mM Tris pH8, 0.25 % bromophenol blue and 0.6 % SDS). Resulting samples were analysed using either 6 % non-denaturating PAGE, or 1 % agarose (40 mM tris-acetate, 5 mM sodium acetate, 1 mM $MgCl_2$ pH 5.5) at 120 V for 2.5 hrs at 4 °C. Acrylamide gels were vacuum dried (2 hrs, 60 °C); agarose gels were first semi-dried using a crush method and fully dried using vacuum drier (1 hr, 60 °C). Crush method involved constructing the following assembly: glass plate followed by layer of cling film, agarose gel, layer of DE81 paper, 3x sheets of whatman paper, appropriate layer of tissue paper, another glass plate. A 4 kg weight was left on top of this assembly for 1 hr at room temperature, after which the gel was transferred to the gel-drier. Gels were analysed by PhosphorImager and ImageQuant software.

ATPase assays

ATP turnover was determined through the monitoring of inorganic phosphate (P_i) release, utilizing a proprietary malachite dye kit (P_i ColorLock™ Gold – Innova Biosciences). Prior to use, ATP stocks were treated with P_iBind™ resin (Innova Biosciences) to remove background free P_i. Standard curves (against which experimental [P_i] values were determined), were prepared as per manufacturer's instructions. Reactions were performed in 200 μl aliquots in a buffer containing 20 mM Tris-Cl (pH8), 100 mM NaCl, 5% glycerol, 5 mM $MgCl_2$, 0.1 mM DTT, 400 μM ATP, 400 nM DNA (where relevant) and 40 nM hexamer or dimer of the respective proteins (later reduced to 20 nM for those reactions that initially exceeded the linear range of the detection method – see Results). Reactions were incubated for 20 minutes at 60 °C prior to treatment with the proprietary malachite reagents as per manufacturer's instructions. Absorbance at 635 nm was determined using a PHERAstar *FS* microplate reader (BMG Labtech), utilizing 96-well UV plates (Costar/Corning, #3635) and 150 μl of each final reaction mixture. DNA substrates are listed in Supplementary Table 1. The duplex DNA species were generated by annealing ATP5PFwd and ATP5PRev (5' overhang substrate), or ATP3PFwd and ATP3PRev (3' overhang substrate), or ATPBluntFor and ATPBluntRev (blunt substrate), or ATP3TFor and ATP3TRev (substrate with a poly-thymidine 5' overhang. The length of this oligo-T 5' overhang was varied from 3 to 21 nucleotides – the ATP3T oligos have a 3T overhang). The negative control was performed in the absence of HerA, NurA and DNA, and was normalised to zero as a background measure.

Size exclusion chromatography (analytical gel filtration)

Physical interactions between HerA and NurA (native protein and F300E, I295E, F300E/I295E and K202A mutants) were investigated using analytical gel filtration. HerA (0.89 nmol, hexamer) and NurA (1.8 nmol, dimer) were mixed and pre-incubated at 60 °C for 20 mins in a total 150 µl gel filtration buffer (20 mM Tris pH 8, 300 mM NaCl, 5 % glycerol, 1 mM DTT). Reactions were then spun full speed in a benchtop centrifuge for 5 mins to remove any precipitated protein and loaded onto a GE Superdex S200 HR 10/300 analytical gel filtration column. 0.5 ml fractions were collected and analysed via 15 % SDS-PAGE.

His-tagged pull-downs

Per reaction, 100 µl nickel–NTA agarose slurry (Qiagen) was washed twice in 500 µl 100 mM NaCl, 5% glycerol and 20 mM Tris pH 8.0. 30 µg his-tagged NurA (3.8 µM dimer) and 50 µg untagged HerA (1.5 µM hexamer), or alternatively 35 µg his-tagged HerA or his-tagged ΔHAS-HerA (1.0 µM or 1.3 µM hexamer, respectively) and 18 µg untagged NurA (2.3 µM dimer) were then mixed at 60°C for 20 mins in 100 µl 100 mM NaCl, 5% glycerol and 20 mM Tris pH 8.0. The reaction mixture was then cooled to room temperature and added to the equilibrated nickel-agarose and incubated at room temperature for 5 minutes with gentle agitation. The reactions were then washed 4 x in 500 µl 100 mM NaCl, 5% glycerol and 20 mM Tris pH 8.0 (plus 20 mM imidazole for the tagged-HerA pulldowns), and finally resuspended in 50 µl 2X laemmli protein loading dye. The complexes were eluted by boiling at 95°C for 5 minutes, separated by SDS-PAGE on a 15% polyacrylamide gel and visualized by coomassie staining.

Immunoprecipitations

Protein A-sepharose beads (Amersham Biosciences) (50 μl per reaction) were washed twice in an excess of 1x TBS (150 mM NaCl, 10 mM Tris, pH 8) then incubated with 1 x TBS, 4 % BSA for 1 hour at 4 °C to reduce any non-specific binding. Beads were then washed three-times and finally resuspended in Reaction Buffer (1x TBS, 0.1 % β-mercaptoethanol, 5 % glycerol). HerA (0.19 μM hexamer), NurA (0.4 μM dimer), NurA I295E (0.4 μM dimer), NurA F300E (0.4 μM dimer), NurA I295E/F300E (0.4 μM dimer) were mixed as required in a total of 100 μl Reaction Buffer, incubated at 60 °C for 20 mins and allowed to cool to room temperature before addition of polyclonal IgG HerA specific polyclonal rabbit antisera (CovalAb). Reactions were incubated at 37 °C for 90 minutes with agitation. Anti-HerA antibodies, bound to HerA and any interacting proteins, were immunoprecipitated following the addition of protein A-sepharose beads for 90 minutes at 37 °C. Beads were washed four times in an excess of reaction buffer to remove unbound protein. Proteins were eluted from the beads following addition of 100 mM glycine pH 3 and incubation at 37 °C for 30 minutes. Supernatant was recovered using centrifugation and the pH neutralized using 1M Tris, pH 8. Samples were mixed with 2X laemmli protein loading dye, boiled for 5 mins, resolved on 15 % SDS-PAGE and stained with coomassie blue.

Crystallisation and X-ray structure determination

NurA crystals were grown by the vapour diffusion method, mixing equal volumes of concentrated protein at 7.0 mg/ml and crystallisation buffer (0.1 M sodium citrate pH 4.5, 1 M lithium chloride, 5% w/v PEG 6k, 50 mM manganese chloride). Resultant 2 µl drops were sealed against a 1 ml well of the crystallisation buffer and allowed to equilibrate at 19 °C. Crystals were observed within 3-5 days. Suitable crystals were transferred to a cryoprotection buffer (0.1 M sodium citrate pH 4.5, 1 M lithium chloride, 5% w/v PEG 6k, 300 mM sodium chloride, 25% v/v glycerol) and snap-frozen in liquid nitrogen. X-ray diffraction data were collected at beamline X06DA of the Swiss Light Source (Paul Scherrer Institut, Switzerland). The X-ray structure was solved in PHENIX (44), using phase information obtained from anomalous differences measured at the Se K edge on selenomethionine-containing crystals. The X-ray data could be merged equally well in $P2_1$ or $C222_1$. Initial automatic model building and refinement in Phenix led to lower R/R_{free} values for data processed in $P2_1$ (28.5% / 31.8%) over $C222_1$ (30.0% / 34.1%), and crystallographic refinement was therefore continued in $P2_1$ (cell dimensions: a=67.7 Å, b=80.3Å, c=67.7Å; b= 107.2°). The initial atomic model built by Phenix was extended and completed manually in Coot (45). Crystallographic refinement was carried out using REFMAC (46) and Buster (47), together with appropriate manual model rebuilding in Coot. The structure was refined against the 2.5Å seleno-methionine data, to final R and R_{free} values of 23.2% and 27.1% respectively. The final crystallographic model contains two NurA chains in the asymmetric unit, 638 amino acids, 66 water molecules and two manganese ions. 97.1% of residues are in the favoured regions of the Ramachandran plot and there are no outliers. The MolProbity score is 1.89 (97^{th} percentile). Residues 11 to 14, 47 to 50 and 238 to 245 of both NurA chains could not

be positioned reliably in the electron density map and were therefore omitted from the final model. The conformation of amino acids 1 to 10 in both chains must be considered tentative as the electron density is poor in this region of the map.

Circular Dichroism

Circular Dichroism (CD) spectra between 250 and 185 nm were recorded on an AVIV 410 spectropolarimeter, at 25˚C and 0.5 nm steps. Protein concentrations were determined by UV spectroscopy using theoretical extinction coefficients determined from the amino acids sequence (http://www.expasy.ch/tools/protparam.html). Spectra were measured in 5 mM sodium phosphate pH 7.8, 50 mM NaF, in a 1 mm path-length quartz cuvette, 1 nm slit-width and 1 second averaging time. Protein samples were centrifuged at 13,000 rpm for 5 minutes at 25˚C prior to measurements, to reduce noise due to scattering. The spectra were not corrected for the refractive index of the solvent. For each spectrum the raw data from at least 3 scans was averaged, smoothed, and transformed into mean residue ellipticity ($[\theta]$). The transformed data was deconvoluted and analysed by the CDSSTR algorithm (48), using the DichroWeb server (http://dichroweb.cryst.bbk.ac.uk). CD thermal melts at 222 nm were measured at a ramp rate of 1˚C/min with 2 minutes equilibration time, 0.2˚C dead-band, 1 nm slit-width and 1 second averaging time. Apparent T_m and van't Hoff enthalpy for NurAwt was calculated as described (49), assuming a simple two states transition model. Thermal melts were normalised by converting the raw data (millidegrees) to mean residue ellipticity ($[\theta]$) using standard equations.

Fluorescence anisotropy

Fluorescence anisotropy measurements were recorded in a PHERAstar FS plate reader (BMG Labtech; Germany) equipped with a fluorescence polarisation optic module (excitation = 485 nm; emission = 520 nM). The anisotropy was measured at 25˚C in black 96-well half area, flat bottom, NBS plates (Corning; USA). The instrument was set to top optic measurement mode and 200 flashes per well. The gain and focal height were set using the instrument software against free fluorescein (Fluorescein Sodium; Fluka) in 50 mM Tris-HCl pH 8.0 (mP = 35).

Fluorescent anisotropy DNA constructs

NurA and HerA binding to DNA was performed using an internally-labelled 45 bp-long oligo, (F1 construct - 5'CTTCTCTTCTTCACTCTACACCT(F)CTTCTGTCCAGCAGGAACGGCG'3), and a complementary 45 bp-long oligo that generate, upon annealing to F1 construct, a dsDNA construct with 20 nucleotides 5' overhangs on both sides of the construct (F2 construct - 5'CTGTTCTTCTCTCTCCTTCTCGCCGTTCCTGCTGGACAGAAGAGG'3).

The fluorescein label was on nucleotide T23 of F1 in order to minimise quenching of the fluorescence by bound proteins. NurA and HerA protein stock solutions were thawed, incubated at 60˚C for 20 minutes and spun at 13,000 rpm for 5 minutes at room temperature to remove aggregates. The clarified proteins were buffer exchanged into buffer B (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 5% glycerol, 1 mM DTT). Protein concentrations were determined by UV spectroscopy using theoretical extinction coefficients determined from the amino acids sequence (http://www.expasy.ch/tools/protparam.html). NurA and HerA proteins were titrated onto the DNA using the built-in injectors in a way that maintained the DNA concentration constant (10 nM). All measurements were in buffer B and 100 μl/well final volume. No loss of total fluorescence intensity was detected throughout the titration. The data was analysed using a two equivalent independent binding site model as described by Fersht and colleagues (8), assuming that the proteins bind to both ends of the DNA constructs. Each titration was repeated at least three times.

Titration of 0.62 μM HerA (final) with increasing NurA concentrations was performed using the PHERAstar FS built-in injectors. The proteins were in buffer B and at 70 μl/well volume. The HerA-NurA mixtures were transferred to eppendorf tubes and incubated at 60˚C for 20 minutes. After cooling the mixtures to 25˚C, 50 μl from each sample were transferred to wells containing 50 μl of 10 nM F2 construct in buffer B (10 nM final) and the anisotropy was measured.

NurA and HerA proteins were titrated onto the DNA using the built-in injectors in a way that maintained the DNA concentration constant (10 nM). All measurements were in buffer B and 100 μl/well final volume and each titration was repeated at least three times. No loss of total fluorescence intensity was detected throughout the

titration. The data was analysed using a two equivalent independent binding site model as described by Fersht and colleagues (50). The raw anisotropy data was fitted to the following equation using the curve fitting software '*profit*' (http://www.quansoft.com):

$$r = r_0 + \frac{\Delta r \bullet C^n}{K_d + C^n}$$

where, r is the observed anisotropy, r_0 is the anisotropy for free DNA, Δr is the total change in anisotropy, C is the concentration of the free protein, n is the number of equivalent independent binding sites and K_d is the equilibrium dissociation constant. We assume that the proteins bind to both ends of the DNA constructs and no translocation occurred in the absence of ATP and at 25°C.

Supplementary References

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