Supplementary information of: Characterization of the effects of phosphorylation by CK2 on the structure and binding properties of human HP1β

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Supplementary material and methods

Samples preparation

Human Heterochromatin Protein 1 beta HP1β (2-185) and its fragments CSD (107-185), spanning the chromoshadow domain and the full C-terminal tail, and CD (19-79) that contains an isolated chromo domain, were cloned into pET expression vector with an N-terminal His-tag and TEV cleavage site. For NMR kinetics and relaxation experiments, HP1β (2-185) was prepared ^{15}N-perdeuterated by protein expression in D_20-based minimal medium, using $^{15}NH_4Cl$ and ^{2}H,^{12}C-glycerol as unique source of nitrogen and carbon, respectively. For backbone assignment experiments, we prepared a ^{15}N-^{13}C labeled HP1β (2-185) sample using $^{15}NH_4Cl$ and ^{1}H,^{13}C-glucose in H_20-based minimal medium. CSD (107-185) and CD (19-79) were prepared with a single ^{15}N-labeling scheme. HP1β (2-185) protein expression was carried out overnight at 24°C, in BL21 (DER) cells induced with 0.4 mM IPTG. CSD (107-185) expression was done at 37°C within 4 hours and CD (19-79) at 24°C overnight. For

SAXS and chromatin association studies, unlabeled HP1β (2-185) samples were prepared in standard LB medium. Protein purification was performed following standard procedures using nickel-affinity (Ni-NTA Qiagen or HisTrap GE Healthcare) ion-exchange (HiTrap GE Healthcare) and size exclusion (Superdex, GE Healthcare) chromatography. The His-tag was removed by TEV cleavage and a second step of nickel-affinity chromatography. Due to the cloning design, TEV left a GHM tripeptide and a single Gly at the N-terminus of CSD and CD, respectively. Protein samples were dialyzed and concentrated in different buffers depending on the experimental requirement.

The hinge region fragment was produced as a synthetic peptide spanning residues 80-109 of human HP1β. For CSD-shadock peptide binding studies, we used a chemically synthetized H3 (35-67) Xenopus core peptide containing the PXXVXL variant (shadock) that previous studies [1, 2] have shown to specifically bind to CSD dimer.

For HP1β-chromatin co-precipitation studies, *in vitro* reconstituted 12-mer oligonucleosomal arrays were prepared as described previously [3, 4]. In brief, 12x 601-200 DNA was prepared as described by digestion of 12x 601-200 containing pUC18 plasmid (a kind gift of Daniela Rhodes, University of Cambridge, UK) with restriction enzymes *Dde*I, *Hae*II, *BfuC*I and *EcoR*I to liberate the 12x 601-200 DNA piece and to fragment the vector backbone [5]. The intact 12x 601-200 DNA was purified from fragmented plasmid vector backbone by precipitation with PEG-6000. Purity of the purified 12-mer DNA was checked by agarose gel electrophoresis. Unmodified and modified core histones were prepared as follows. *Xenopus* core histones were expressed in *E.coli* and purified from inclusion bodies by tandem gel filtration and ion exchange chromatography [6]. To obtain the trimethyl-K9 analog (H3Kc9me3), histone H3K9C, C110A was expressed and purified as above. Alkylation of C9 to a trimethyl-K9 analog and purification of alkylated histone H3Kc9me3

was performed as described [7-9]. Alkylation status of histone H3K9C, C110A was confirmed by mass spectrometry (>95% alkylated to H3Kc9me3, [9]). Unmodified or modified core histones were aliquoted, lyophilized and kept in large stocks at -80°C. For histone octamer assembly, purified and lyophilized core histones were dissolved in unfolding buffer (7 M Guanidine-HCl, 20 mM Tris-HCl (pH 7.5), 1 mM DTT), mixed at a equimolar concentration and extensively dialyzed against high salt refolding buffer (10 mM Tris-HCl (pH 7.5), 2 M NaCl, 1 mM EDTA, 1 mM DTT) to assemble histone octamer [6]. Assembled octamers were purified by gel filtration chromatography using a Sephadex 200 column (GE Healthcare). To reconstitute 12-mer oligonucleosomal arrays, 50 μg 12x 601-200 DNA and purified unmodified or H3Kc9me3-containing histone octamers were mixed in a final volume of 100 μl high salt refolding buffer at 1.0:1.0 to 1.0:1.2 ratios. Samples were dialyzed against 400 mL high salt refolding buffer, which was continuously replaced by 2 L low salt refolding buffer containing 10 mM NaCl over 36 hrs [6]. The resulting oligonucleosomal arrays were dialyzed against TEAE buffer (10 mM triethanolamine-HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT). Efficiency of assembly and the comparability between unmodified and alkylated oligonucleosomal arrays were tested by digestion with *Ava*I restriction enzyme [10]. 250 ng 12x 601-200 DNA and 500 ng 12-mer oligonucleosomal arrays were digested with 20 U *Ava*I in 25 μl NEB buffer 4 (New England Biolab) for 3 hrs at 30°C. Digested samples were analyzed by 2% agarose/0.25x TBE gel electrophoresis and subsequent ethidium bromide staining (Figure S2). Oligonucleosomal arrays assembled at DNA:octamer ratio of 1.0:1.1 were used for chromatin co-precipitation assays.

Chromatin co-precipitation assay

Unmodified and H3Kc9me3 12-mer oligonuleosomal arrays from the same batch of preparation were used to assess the chromatin binding activity of non-phosphorylated and

CK2-phosphorylated HP1β. 1 μg (A_{260}) of reconstituted oligonucleosomal arrays were incubated with 1 μM HP1β in 100 μl binding buffer (10 mM triethanolamine-HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton-X, 0.1 mM EDTA) for 1 hr on ice. Samples were then centrifuged at 16100 x g for 30 min at 4°C to precipitate chromatin and bound HP1β. The pellet was washed once with 500 μl binding buffer, resuspended in 10 μl 1x SDS gel loading buffer and analyzed by SDS-PAGE followed by Coomassie blue staining.

NMR Spectroscopy

For all NMR experiments (with the exception of kinetic measurements done in CK2 buffer), protein samples (unmodified and phosphorylated) were buffer-exchanged and measured in 20 mM sodium phosphate (pH 6.5), 50 mM NaCl, 2 mM DTT.

Sequence-specific backbone resonance assignment of full-length HP1β was obtained as described previously [9]. Due to the high correspondence of chemical shifts, the assignment of CSD (107-185) and CD (19-79) could be transferred from the full-length protein and shorter constructs previously assigned and used in [9]. 6D APSY-seq-HNCOCANH [11], HNCA and TROSY-HNCO were measured at 303 K, 700 and 600 MHz, on ^{15}N-^{13}C-labeled HP1β (2-185) modified by CK2, to assign the peaks shifted upon phosphorylation.

^{13}C-resonances were referenced to internal DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid).

Steady-state ^{1}H-^{15}N heteronuclear nuclear Overhauser enhancement (^{1}H-^{15}N hetNOE) experiments were measured at 600 MHz, 298 K, using ^{15}N-perdeuterated labeled samples. Experiments were performed twice with recycle delays of 5 s (Figure S1C) and 10 s (Figure 3C). NOE values were obtained from the ratio of the peak intensities between saturated and reference spectra.

Processing and analysis of NMR data were carried out using NMRPipe [12] and Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco).

The PDB structure shown in Figure 4 was visualized with PyMOL (The PyMOL Molecular Graphics System, Version 1.1r1, LLC).

Mass Spectrometry

Proteins and peptide samples were purified by reversed-phase HPLC prior to MS measurements to efficiently remove small molecules and buffer salts. A 250x4.6 mm C18 column (Eurospher) and an acetonitrile gradient in 0.1% TFA were used. The HPLC system was connected to an Electrospray ionization (ESI) Micromass ZQ 4000 MS instrument (Waters) equipped with a single quadrupole detector. MS measurements were carried out in positive mode. MS data were analyzed with MassLynx MS Software (Waters).

Small angle X-ray scattering

SAXS data were gathered at X33 beamline of European Molecular Biology Laboratory at Deutsche Elektronen Synchrotron in Hamburg, Germany [13, 14]. Samples of 50 μl were exposed to 8 frames of 15 seconds each at 1.5Å wavelength. Automatic data processing pipeline AutoPilatus [15] was used to evaluate radiation damage and for signal averaging and processing. Data analysis was performed with PRIMUS software [16]. Data exhibited no concentration effect between concentrations of 0.64, 1.5, and 3 mg/ml, and no radiation damage. SAXS experiments were carried out at 288 K in 40 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 3 mM DTT, 0.02% NaN_3 and 0.4 mM ATP.

Supplementary figure

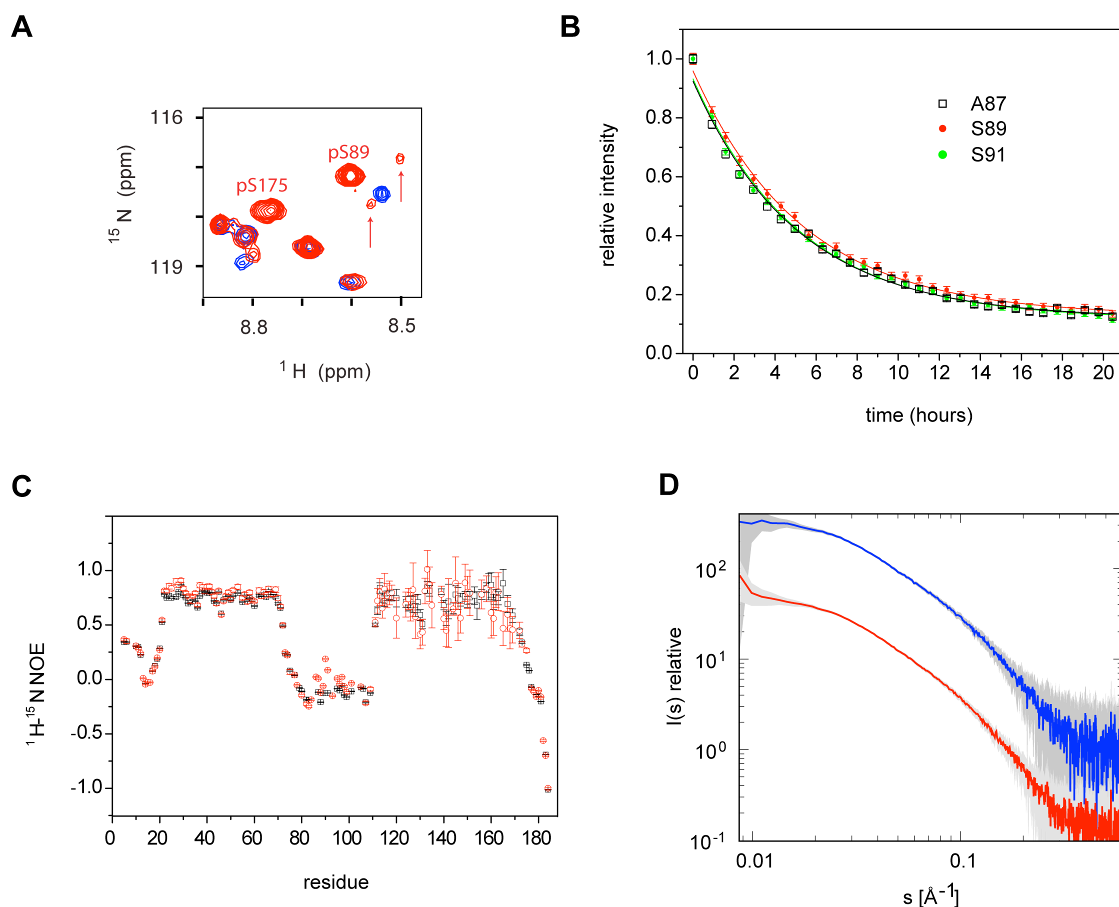

Figure S1. Analysis of phosphorylated HP1β (pHP1β). A) Details of the ^{1}H-^{15}N TROSY HSQC spectra of HP1β (blue) and HP1β after phosphorylation by CK2 (red). A lower contour level has been used with respect to Figure 2A to highlight (see the red arrows) two new small peaks most probably corresponding to a scarce phosphorylated HP1 isoform where the hinge region has more than one phosphorylated serine. **B)** Comparison of time course of intensity decrease of S89 (red) and S91 (green) signals during HP1β phosphorylation by CK2. The signal decay time of S91 was 5.2±0.2 hours. The correspondence with the S89 value indicates that the signal change observed for S91 is likely due to the conformational change upon phosphorylation of the neighbor S89. The value of A87 (5.1±0.2 hours) serves as additional reference. **(C)** Steady-state ^{1}H-^{15}N hetNOE measured on full-length HP1β (black) and pHP1β (red) using a recycle delay of 5 s. **D)** SAXS profiles of HP1β before

(blue) and after (red) phosphorylation by CK2. I(s) is the small-angle X-ray scattering intensity, s=4πsin(θ)/λ, where 2θ is scattering angle, and λ is the X-ray wavelength. Gray shadows show error bars for each curve. The derived radii of gyration (Rg) for HP1β and pHP1β at 3 mg/ml protein concentration are 4.7±0.3 nm and 4.5±0.2 nm respectively.

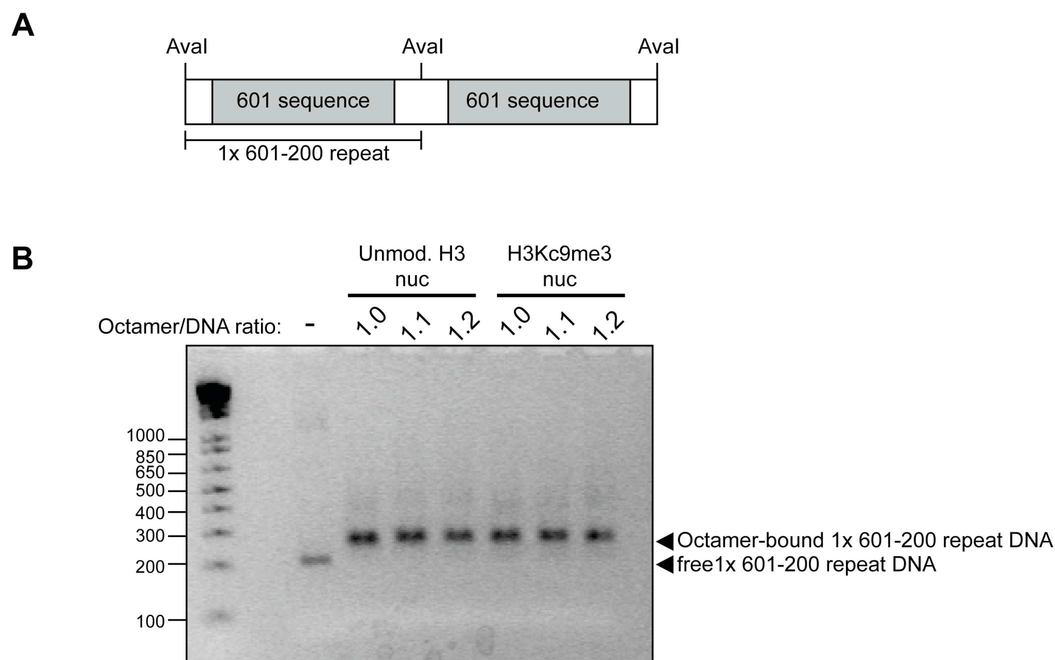

Figure S2. Analysis of 12-mer oligonucleosomal arrays. A. Scheme of two units of the 601-200 repetitive sequence. *Ava*I restriction sites are present in the middle of the linker region. **B.** Digestion of input DNA and *in vitro* reconstituted unmodified and H3Kc9me3 12-mer oligonucleosomal arrays by *Ava*I. Agarose gel stained with ethidium bromide is shown. Molecular size markers are indicated on the left. Running positions of free DNA and single 601-200 units complexed with histone octamers are indicated by arrow heads.

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