Supporting Information:

Analysis of phosphorylation-dependent protein-protein interactions of histone H3

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Experimental Section

General: Amino acid (AA) derivatives were purchased from GLS (Shanghai, China). Coupling reagents were bought from Merck Novabiochem (Darmstadt, Germany), Tentagel RAM resin from Rapp Polymere (Tuebingen, Germany). Other chemical were purchased from Sigma Aldrich (Germany) unless stated otherwise. Recombinant 14-3-3ζ was also purchased from Sigma Aldrich (Germany). Analytical and preparative HPLC were performed on a Varian ProStar 210 HPLC system with a Nucleosil C18 column (5 µm, 4.6 x 250 mm, Machery-Nagel) or Dynamax C18 column (10 µm, 21.4 x 250 mm, Varian), with 0.1% TFA in water (A) and 80% ACN, 0.1% TFA in water (B), as eluents. All peptides were analyzed by MALDI-MS on a MALDI-TOF-TOF, 4700 Proteomics Analyzer (Applied Biosystems) and by analytical RP-HPLC.

Synthesis of Fmoc-L-Pma: L-phosphonomethylenalanine (220 µmol, 40 mg, Biotrend, Germany) and Na_2CO_3 (220 µmol, 23 mg) were dissolved in 8 mL $AcOH/H_2O$ (1:1). N-(9-Fluorenylmethoxycarbonyloxy)-succinimide (210 µmol, 70mg) was added stepwise under stirring over 1 hour. After addition of 1 mM Na_2CO_3 the pH was adjusted to 10 and the mixture was stirred over night at RT. The clear solution was washed two times with 10 mL diethyl ether. The pH was adjusted to 1 by slow addition of concentrated HCL and the product was extracted with 20 mL of ethyl acetate. The ethyl acetate phase was intensively washed with water, dried over $MgSO_4$ and followed by removal of solvent under vacuum to yield Fmoc-L-Pma (101 µmol, 41 mg, 48%) as a white solid.

ESI-MS: m/z (Fmoc-L-Pma, $[M+H]^+$) = 406

^1H-NMR (DMF-d7): δ 7.9 ppm (d,^2H, CH CH, H-Ar); 7.8 (m, ^2H, CH=CH, H-Ar) 7.5-7.3 (m, 2H, CH=CH, H-Ar); 4.4 (m, 4H, 1x CH-CH_2/1xCH_2-O-C=O/1xNH-CH-CO_2H); 2.1 (q, CH_2, CH_2-CH_2-PO_3H_2); 1.9 (m, CH_2, CH_2-PO_3H_2),

^{13}C-NMR (DMF-d7): δ 174.4 (Cq, CO_2H); 157.6 (Cq, CO_2R); 145,1 (Ct, Ar-C); 142.1 (Ct, Ar-C); 128.7 (Ct, Ar-C); 128.1 (Ct, Ar-C); 126.5 (Ct, Ar-C); 121.0 (Ct, Ar-C); 67.2 (Cs, C-CO_2-); 55.5 (Cp, N-C-CO_2H); 48.1 (Cp, Ar-C-Ar); 26.6 (Cs, C-PO_3H_2); 24.5 (Cs, C-C-PO_3H_2).

Synthesis of H3 tail peptides: The H3 tail peptides were synthesized in 25 μmol scale using standard Fmoc-based solid-phase chemistry on a Intavis Respep XL synthesizer (Intavis, Cologne, Germany). TentaGel R RAM resin (cap. 0.19 mmol/g, Rapp Polymere GmbH, Germany) was used as solid support, and amino acid side chains were protected as follows: Arg(Pbf), Cys(Trt), Gln(Trt), Lys(Boc), Ser(tBu), and Thr(tBu). To enhance synthetic efficiency the following Dmb dipeptide and pseudo proline dipeptides were used: Fmoc-(Dmb)Gly-OH, Fmoc-Gly-(Dmb)Gly-OH, Fmoc-Ala-Thr(psiMe,Mepro)-OH, Fmoc-Gln(Trt)-Thr(psiMe,Mepro)-OH and Fmoc-Lys(Boc)-Ser(psiMe,Mepro)-OH (Novabiochem, Germany). Coupling reactions were achieved by using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU)/ N-Hydroxybenzotriazole (HOBt) as activation agent mixture and N-methylmorpholine (NMM) in DMF/NMP as base. Each amino acid was doubly coupled in 5-fold molar excess. Monobenyzl protected phosphoamino acids derivatives were used in 4-fold molar excess and were coupled twice manually using HBTU/HOBt and DIPEA in DMF as base for 2 h. The Fmoc-L-Pma double couplings were

performed in 2-fold molar excess for 2 h and over night. The H3Pma10 synthesis were continued manually and each double couplings step was followed by a capping step using 2,6-Lutidin/Ac_2O in DMF. Fmoc group removal was carried out with 20% piperidine in DMF. Cleavage and removal of the side chain protection groups were performed with TFA/phenol/triisopropylsilane/H_2O (85:5:5:5) for 4 h. Cleaved peptides were precipitated and washed in cold diethyl ether, dissolved in H_2O, and lyophilized. Crude peptides were HPLC-purified on a semipreparative polymer column to ≥ 95% purity.

Peptide immobilization: The C-terminal cysteine peptides were coupled covalently to SulfoLink agarose resin (Thermo Scientific GmbH) by incubation of 1 mM peptide solution in coupling buffer (50 mM TrisHCl, 5 mM EDTA·2Na) in 7-fold excess for 1 h at RT. Afterwards unreacted iodoacetyl groups were blocked by incubation with 50 mM β-Mercaptoethanol. Subsequently, the peptide-loaded beads were washed 6 times with 1 M NaCl, 2 times with H_2O and 4 times with storage buffer (50% ACN). The bait material was aliquoted in form of 20 µL 50% suspensions in storage buffer. Storage was performed at -20°C.

Loading of beads was determined by quantitative amino acid analyses (Genaxxon, Ulm, Germany).

Cloning of RBBP7 and HAT: The genes encoding HAT1 and RBBP7 were expressed as C-terminal His-6-tag and N-terminal GST fusion proteins, respectively. Briefly, the cDNA (ImaGenes, Berlin, Germany) encoding RBBP7 from mus musculus was cloned into pET42b (Novagen, Merck KGaA,

Darmstadt, Germany) and the gene encoding HAT1 was cloned into pET23b using standard PCR methods. Restriction sites for XmaJI and Cfr42I or NdeI and XhoI (RBBP7-forward primer: TTT CCG CGG AAA ACC TGT ATT TTC AGT CTG CGA GTA AAG AGA TGT TTG AAG, RBBP7-reverse primer: AAA CCT AGG TTA AGA TCC TTG CCC CTC CAG, HAT1-forward primer: TTT CAT ATG GCG GCC TTG GAG AAA TTT C, HAT1-reverse primer: AAA CTC GAG CTC TTG AGC AAG TCG CTC AAT G) were introduced in addition. E. coli BL21(DE3)pLysS or E. coli BL21(DE3) Rosetta competent cells (Stratagene, Agilent Technologies, Germany) were transformed with the resulting constructs.

Expression of WDR5, HAT1, RBBP7 and HSC70: Cells transformed with the His_6-WDR5, HAT1-His_6, GST-RBBP7 and His_6-SUMO-HSC70 expression plasmids were cultivated in Luria/Miller (LB) broth (Carl Roth GmbH, Germany) at 37°C. At an OD_{600} of 0.5-0.6 protein production was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Afterwards the cells were cultivated for 19-21 h at 20 °C (WDR5, HAT1 and RBBP7) for 4 h at 30°C (HSC70). In the following the cells were harvested by centrifugation, subsequently resuspended in lysis puffer (50 mM TrisHCl, 300 mM NaCl 12.5 mM Imidazol, 0.5 mM PMSF, pH: 8) and lysed in a EmulsiFlex-C3 high pressure homogenizer (Avestin, Germany). Cleared lysates of cells expressing WDR5, HAT1 and HSC70 were incubated with NiNTA agarose (Qiagen GmbH, Germany). After washing with lysis buffer bound proteins were eluted with 250 mM Imidazol in lysis buffer (pH 8.8). Fractions were pooled and dialyzed against 50 mM TrisHCl, 100 mM NaCl, pH 7.6 overnight at 8°C. His-SUMO tag of HSC70 was cleaved with His-

tagged ULP1 protease. The cleavage tags and ULP1 was afterwards removed by incubation with NiNTA agarose. GST-RBBP7 was purified on Glutathion Sepharose 4B (GE Healthcare, Germany). After incubating the cleared lysate and resin for 3h at 8°C, the beads were washed with lysis puffer and proteins were eluted five times with 10 mM Glutathion in 50 mM TrisHCl, 1 mM PMSF, pH 8. Proteins were supplied with additional 20 % glycerol and stored at -80°C until usage.

Cell culture: Swiss 3T3 cells (DSMZ no.: ACC 173) were cultured in DMEM (GlutaMAX™) medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% FBS (Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2. Cells were passaged every 2-3 days using 0.05% Trypsin with 0.2 g/L of EDTA·4Na (Invitrogen).

Preparation of nuclear extracts: 1-2 x10^8 cells were harvested and suspended 1-2 ml of PBS. Nuclear extracts (NE) were prepared according to the method described by Dignam et al.(*1*) 4-(2-aminoethyl)-benzensulfonylfluorid (AEBSF) was added to all buffers instead of phenylmethylsulfonylfluorid (PMSF).

Pull-Down with endogenous proteins: Each 20 µL peptide bead aliquot (50% suspension) were equilibrated with 20 mM HEPES, 100 mM KCl, 20% Glycerol, pH 7.9. The beads were incubated with 3T3 NE (SILAC1: 650 µL 6 mg/mL, SILAC2: 900 µL 2 mg/mL, unlabeled: 300 µL 0.1 or 1 mg/ml) for 1 h at RT in the presence of 0.2% Triton X-100. NE for pull downs with phosphorylated peptides were previously treated with (1x) phosphatase

inhibitor (Phosphatase Inhibitor Cocktail Set V (50x), Calbiochem, USA). Afterwards the beads were washed five times with 500 µL washing buffer (20 mM HEPES, 300 mM NaCl, 20% Glycerol, 0.2% Triton X-100, pH 7.9). Then the beads were combined and resuspended in 20 µL SDS sample buffer (Fermentas), and heated to 95 °C for 5 min. The chilled beads were filtered off and the filtrate loaded onto a SDS polyacrylamid gel. In case of the SILAC pull-down the filtrate was first incubated with 15 mM iodoacetamide for 30 min at RT and then loaded onto a Tris-glycine gradient gel (4–20%, BIO RAD Mini-Protean TGX, Munich, Germany). Gels were stained with Coomassie overnight and destained for several hours.

Pull-Down of recombinant proteins: After equilibration with pull down buffer (50 mM TrisHCl, 100 mM NaCl, pH 7.6) the 20 µL bead aliquots were pre-incubated with 500 mM bovine serum albumin (BSA) or myoglobin in pull down buffer for 30 min at RT. Then the beads were incubated with 20 µL of 5 µM target protein in 500 µM BSA or myoglobin in pull down buffer for 1h at RT. Washing steps and sample preparations were the same as previously described.

Fluorescence Polarization: Fluorescence polarization (FP) experiments were essentially performed and analyzed as described in FP buffer (25 mM Tris pH 7.5, 50 mM NaCl, 5% v/v glycerol) using a HIDEX Chameleon II plate reader at 4°C.(*2*) Briefly, dilution series of recombinant proteins dialyzed against FP buffer were prepared in 384 well plates (10 µL volume). H3 peptides containing D- or L-amino acids were fluorescinated using NHS-$(CH_2)_6$-fluorescein in 100 mM KPi buffer (Molecular Probes). Reaction

products were separated on C18 RP HPLC (Vydac). N-terminally labeled species were identified by MS analysis. Products were lyophilized and resuspended in water (1 mM). Final peptide concentration for FP measurements was 100 nM. Raw anisotropy data were analyzed implying a one site binding model using the equation $A = A_f + (A_b – A_f)([R]/(K_d – [R]))$ with A, Anisotropy; A_b, anisotropy of the bound state; A_f, anisotropy of the unbound state; K_d, dissociation constant; [R], protein concentration. Curves were fitted by least square fitting implemented in the KaleidaGraph Software (Synergy). Anisotropy readings were converted to fraction bound using the equation $F_b = (A - A_b)/(A_b - A_f)$; with F_b, fraction bound. Readings from multiple independent (minimum of 3) measurements were averaged after normalization and plotted.

Western blot analyses: Proteins were separated on 15%-SDS polyacrylamide gels. The proteins were transferred to nitrocellulose membranes (Roti®-NC, Carl Roth GmbH). The membranes were blocked with 2% (w/v) nonfat dry milk in buffered saline. Primary antibodies were used as follows: 14-3-3ζ (sc-1019, Santa Cruz biotechnology, Heidelberg, Germany), 14-3-3β (sc-628, Santa Cruz), 14-3-3γ (sc-731, Santa Cruz), WDR5 (ab22512, abcam), HAT1 (sc-8752, Santa Cruz), RBBP7 (ab3535, abcam), HSC70 (ab1427, abcam). Protein bands were detected via secondary horseradish peroxidase-conjugated secondary antibodies (santa cruz biotechnology) and visualized by Enhanced Chemiluminescence detection (ECL Western Blotting Substrate, Pierce) and a Luminescence Imager (Lumi-Imager F1 Workstation, Boehringer-Mannheim GmbH, Germany).

Supporting Figure S1

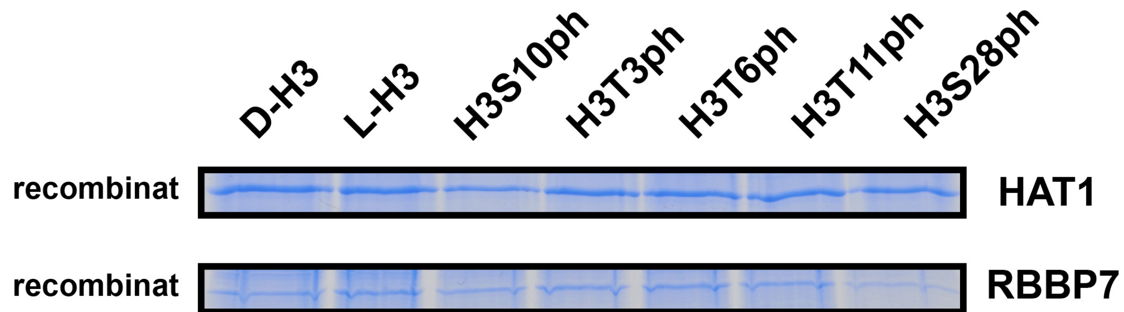

Figure S1: Recombinant HAT1 and RBBP7 were used as input at a concentration of 5 µM in presence of 500 µM BSA and in a volume of 20 µL. Experiments were analyzed by Coomassie Brilliant Blue stained SDS-PAGE.

Supporting References:

1. Dignam, J. D., Martin, P. L., Shastry, B. S., and Roeder, R. G. (1983) Eukaryotic Gene-Transcription with Purified Components, *Method Enzymol 101*, 582-598.
2. Jacobs, S. A., Fischle, W., and Khorasanizadeh, S. (2004) Assays for the determination of structure and dynamics of the interaction of the chromodomain with histone peptides, *Chromatin and Chromatin Remodeling Enzymes, Pt B 376*, 131-148.