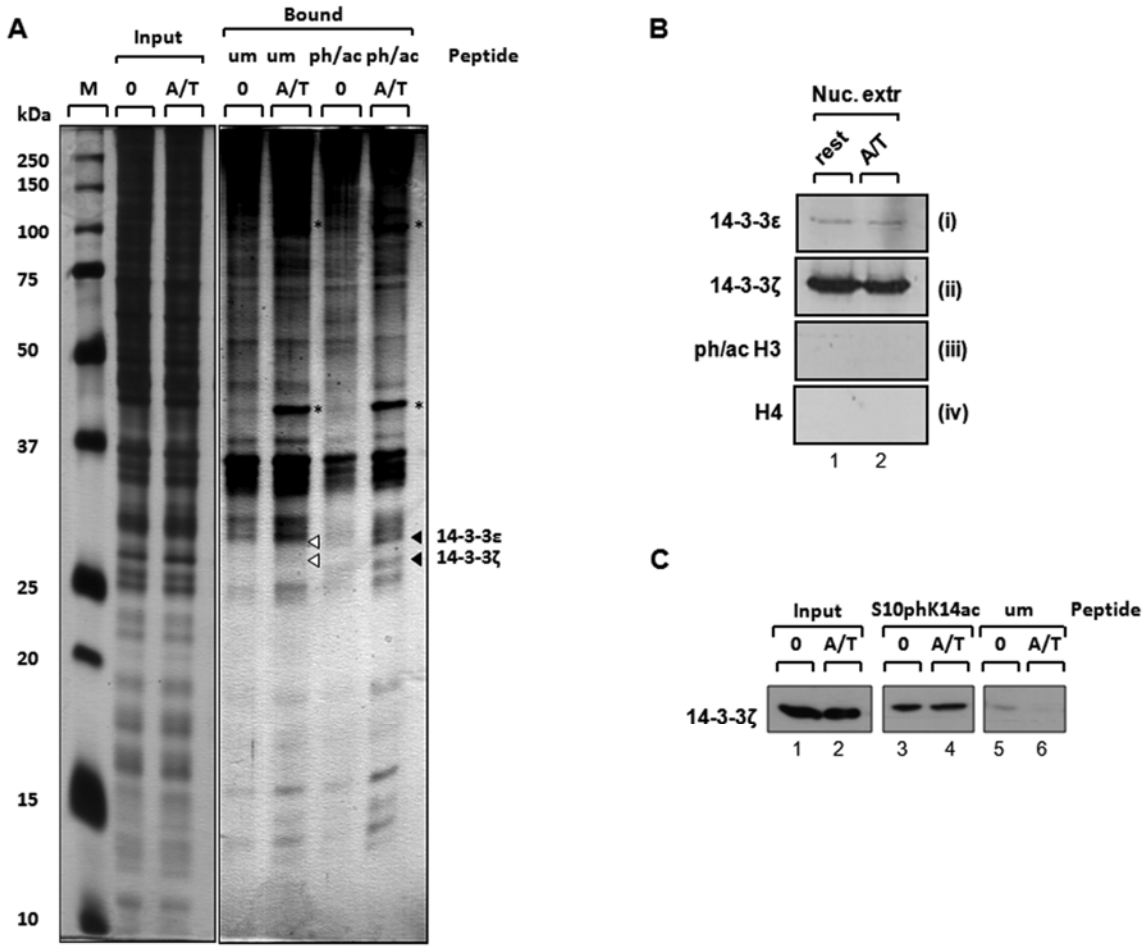


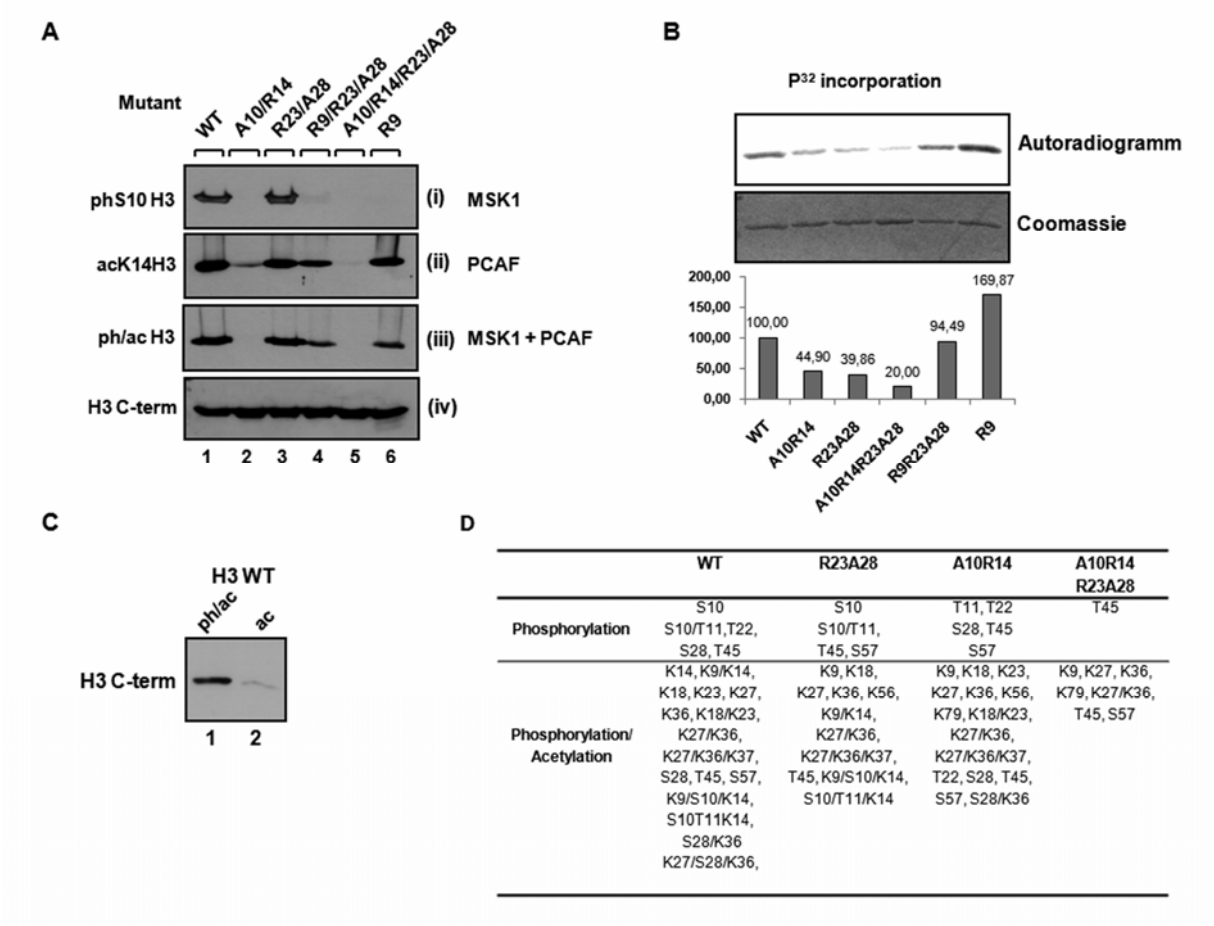
Figure S1. Affinity purification of 14-3-3 proteins as phosphorylation dependent histone H3 interacting factors.

(A) Nuclear extracts from proliferating HeLa cells which were either left untreated (lanes 2, 4 and 6) or treated with anisomycin and TSA (50 ng/ml) (lanes 3, 5 and 7) for one hour were incubated with unmodified (um) or H3S10phK14ac (ph/ac) histone H3 peptides. Bound proteins were separated by SDS-PAGE and visualized by silver staining. Factors specifically interacting with the S10phK14ac peptide (black triangles) were excised from the gel and identified by mass spectrometry. The corresponding regions of the unmodified peptide incubations were used as control (white triangles). The asterisks denote factors that showed modification independent peptide interactions only after anisomycin and TSA treatment.

(B) Analysis of 14-3-3 protein localization. 3T3 mouse fibroblasts were arrested by serum deprivation for 72h (lane 1) and were stimulated with anisomycin and TSA (lane 2). Nuclear extracts were prepared and analyzed by SDS-page and immunoblotting with antibodies specific for 14-3-3ε (panel i), 14-3-3ζ (panel ii), ph/ac histone H3 (panel iii) and histone H4 (panel iv). Both 14-3-3 proteins are clearly detected in nuclear extracts (lanes 4 and 5).

(C) Anisomycin and TSA treatment does not significantly alter the binding properties of 14-3-3 proteins to the unmodified or the H3S10phK14ac histone H3 peptide. Whole cell lysates

were prepared from HeLa cells treated as described for panel A and incubated with the unmodified or the S10phK14ac peptide. Binding of 14-3-3ζ from either untreated (lanes 3 and 5) or stimulated (lanes 4 and 6) cells was analyzed by immunoblotting. An input aliquot of either non-treated (0) (lane 1) or stimulated (AT) (lane 2) lysate was used to control equal loading. One representative experiment is shown indicating that the binding of 14-3-3ζ is not considerably changed upon drug treatment.



	WT	R23A28	A10R14	A10R14 R23A28
Phosphorylation	S10 S10/T11,T22, S28,T45	S10 S10/T11, T45,S57	T11,T22 S28,T45 S57	T45
Phosphorylation/ Acetylation	K14,K9/K14, K18,K23,K27, K36,K18/K23, K27/K36, K27/K36/K37, S28,T45,S57, K9/S10/K14, S10T11K14, S28/K36 K27/S28/K36,	K9,K18, K27,K36,K56, K9/K14, K27/K36, K27/K36/K37, T45,K9/S10/K14, S10/T11/K14	K9,K18,K23, K27,K36,K56, K79,K18/K23, K27/K36, K27/K36/K37, T22,S28,T45, S57,S28/K36	K9,K27,K36, K79,K27/K36, T45,S57

Figure S2. Analysis of *in vitro* modification of Histone H3 mutants.

(A) Wildtype histone H3 (lane 1) or the indicated histone H3 mutants (lanes 2-6) were in vitro modified as described for figure 1B. *In vitro* modification was monitored by immunoblotting with the indicated antibodies. Mutation of lysine 9 to arginine (lanes 4 and 6) resulted in impaired recognition by the H3S10phspecific antibody and reduced recognition of the H3K14ac and ph/ac specific antibodies.

(B) Incorporation of ^{32}P via MSK1 mediated phosphorylation of WT and mutant histone H3. The indicated H3 mutants were phosphorylated using P^{32}-ATP (1µCi/µl final concentration) and P^{32} incorporation was normalized to total histone H3 loading. The histogram depicts normalized incorporation efficiencies relative to wild type histone H3. Mutation of lysine 9 to arginine resulted in strongly increased ^{32}P incorporation despite the reduced affinity of modification specific antibodies as described for panel A, suggesting that this mutation increases MSK1 mediated phosphorylation *in vitro*.

(C) Histone H3 acetylation via PCAF does not mediate binding to 14-3-3 in the absence of additional phosphorylation. WT recombinant histone H3 was *in vitro* phosphoacetylated (lane 1) or acetylated only (lane 2) as described in material and methods section and incubated with GST-14-3-3ζ. Bound histones were analyzed by immunoblotting with C-terminal H3 antibodies.

(D) Mass spectrometry analysis of amino acid positions phosphorylated by MSK1 or acetylated by PCAF in histone H3 mutants *in vitro* (S: serine, T: Threonine, K: Lysine)

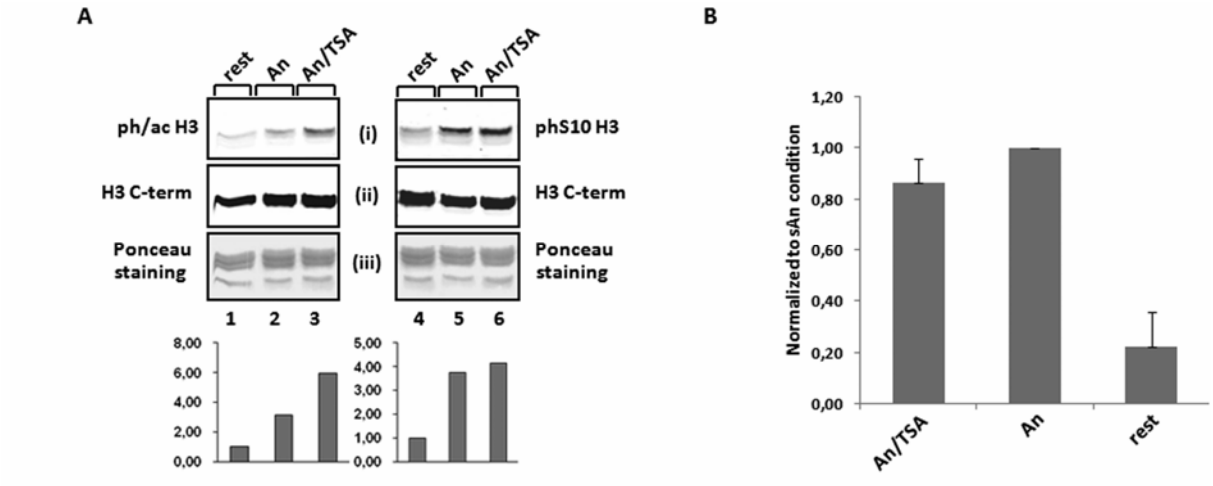


Figure S3. Anisomycin mediated phosphorylation of H3S10 is not increased by TSA.

(**A**) Quantitative analysis of histone H3 phosphorylation and phosphoacetylation upon differential stimulation. Histones were extracted from resting 3T3 fibroblasts that were left untreated (rest) or treated with anisomycin (An) or anisomycin plus TSA (An/TSA). Levels of histone H3 phosphorylation and phosphoacetylation were analyzed by quantitative immunoblotting with the indicated antibodies (panel i). Signals for histone H3 phosphorylation and phosphoacetylation were normalized to histone H3 C-terminal antibodies signals (panel ii). As additional control Ponceau staining of the blot is shown (panel iii). The panel depicts one representative experiment and relative levels of histone modifications are depicted as histogram under the panel.

(B) Additional TSA treatment does not increase the anisomycin generated phospho-histone H3 pool. Histones were isolated as described for panel A and histone H3 was used for mass spectrometry analysis. Intensities for all identified S10 phosphorylated histone H3 peptides (neglecting additional modifications) were summarized and normalized to an unmodified H3 control peptide. Values are depicted relative to the anisomycin condition as average of four independent measurements (mean±SD).

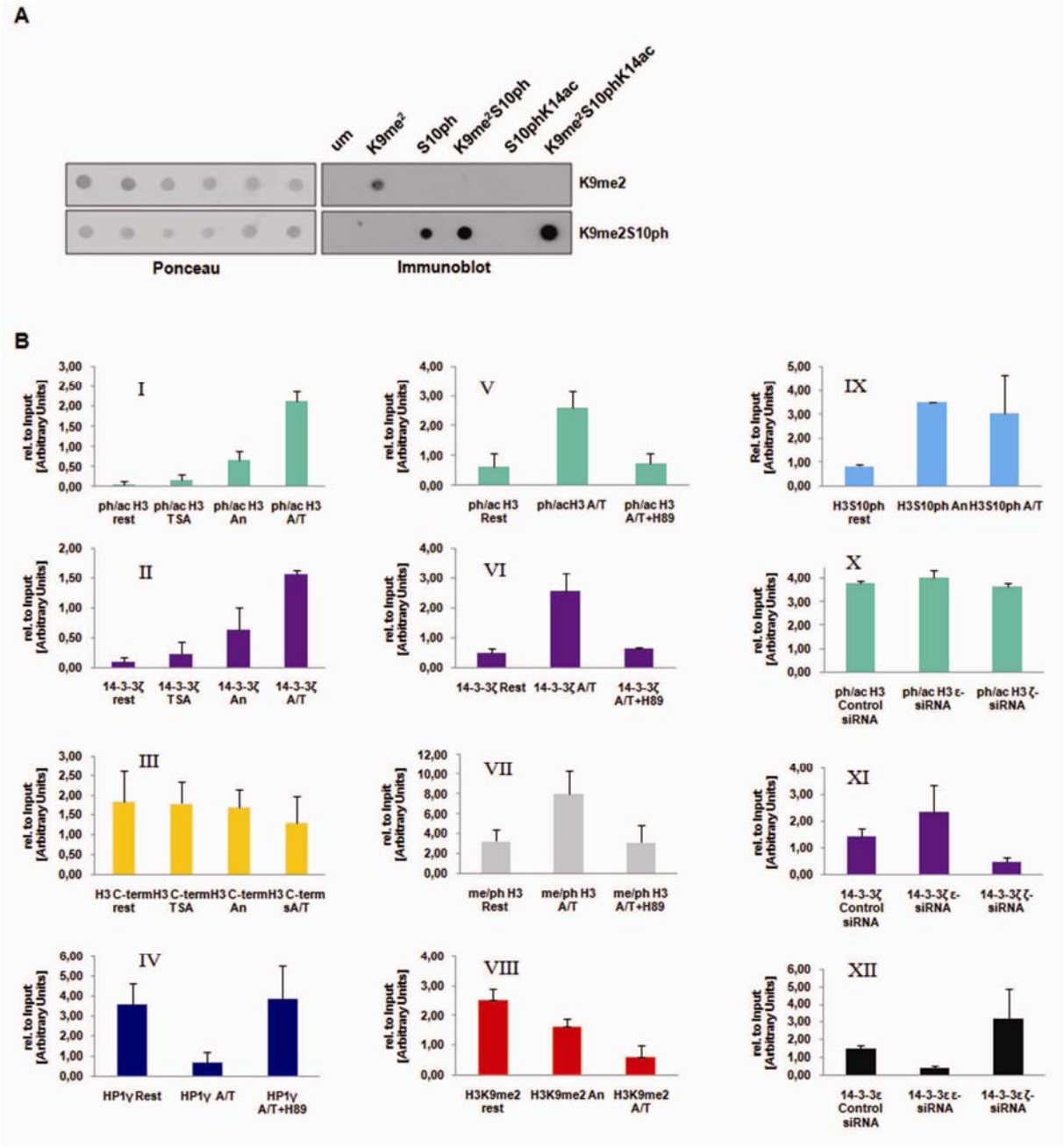


Figure S4: (A) Anti-me^2 antibody is occluded by additional S10 phosphorylation. The reactivity of the indicated antibodies towards differentially modified H3 peptiodes was investigated by dot blotting.

(B) Summary of ChIP-assays performed in the study. The panels indicate the relative amount of precipitated DNA relative to the Input. (I) ph/ac H3 n=3 (II) 14-3-3ζ n=3 (III) H3 C-term. N=2 (IV) HP1γ n=3 (V) ph/ac H3 n=5 (VI) 14-3-3ζ n=2 (VII) me/ph H3 n=4 (VIII) H3K9me2 n=2 (IX) H3S10ph n=2 (X) ph/ac H3 n=2 (XI) 14-3-3ζ n=2 (XIII) 14-3-3ε n=2.

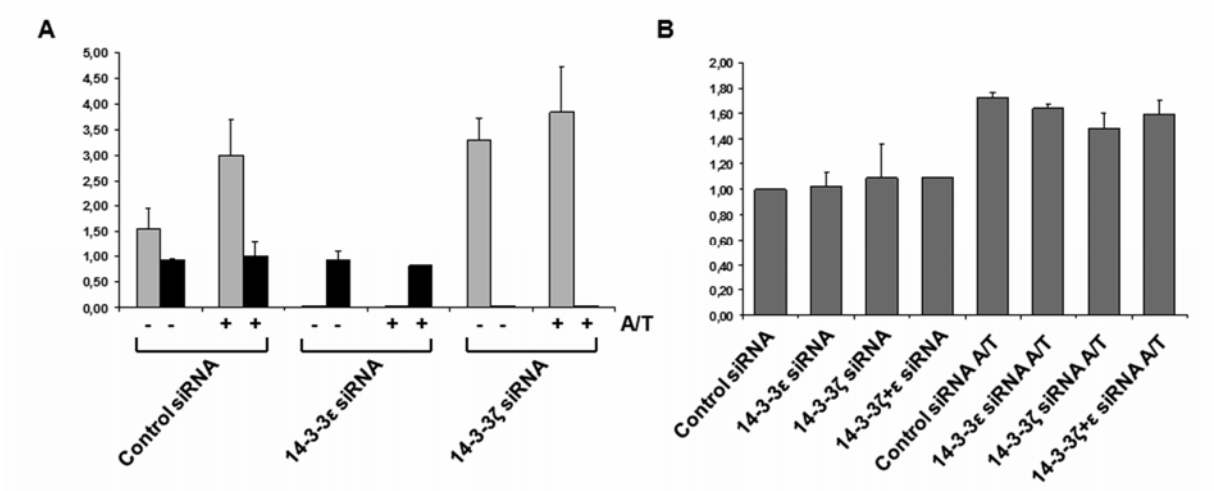


Figure S5. 14-3-3 knock down specificity and histone H3 phosphoacetylation in 14-3-3 knock down cells.

(**A**) HeLa cells were transfected with siRNAs as described for Fig. 5A and RNA was prepared as described for Fig. 5C. 14-3-3 expression levels were analyzed by quantitative Real-time PCR and normalized to GAPDH expression levels. Expression of 14-3-3ε (grey bar) and ζ (black bar) are depicted as diagram. Transfection with 14-3-3ε siRNA did not interfere with 14-3-3ζ expression and *vice versa* indicating strong isoform specificity of the knock down (compare lanes 1, 3 and 5). Treatment with anisomycin and TSA did not affect efficiency or specificity of the knock down (lanes 2, 4 and 6).

(**B**) 14-3-3 knock down does not influence histone H3 phosphoacetylation. Histones prepared from 14-3-3 knock down cells were analyzed for presence of phosphoacetylated histone H3 by quantitative immunoblotting. Results are depicted as diagram, summarizing three independent experiments (mean±SD).

SUPPLEMENTARY METHODS

Affinity purification of phosphoacetyl histone binding proteins

For affinity purification 200-500µg of purified nuclear extracts were diluted 1/8 with ΔX buffer (50 mM Tris-HCl pH 7.4, 150mM NaCl, 10mM EDTA, 1 mM PMSF, Complete-Protease inhibitor cocktail (Roche), PPI, 10 mM sodium butyrate) and incubated with 40µl of pre-equilibrated peptide-slurry over night (14h) at 4°C on a roller. Beads were collected by centrifugation and washed sequentially with ΔX buffer, RIPA buffer 300 (300 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40), RIPA buffer 500 (500 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) and ΔX buffer. Bound proteins were resolved by SDS-PAGE and visualized by silver staining. Proteins specifically interacting with the phosphoacetylated histone H3 peptide were identified by MALDI-TOF peptide mass fingerprinting.

Mass Spectrometry

Histone H3 containing gel bands were excised and cut into small pieces. The gel pieces were washed with ultrapure water and destained with 44% acetonitrile in 50mM $(NH_4)HCO_3$. Unmodified and mono-methylated lysine residues were propionylated as described (Peters et al., 2003). Briefly, samples were incubated for 90 minutes at room temperature with 50µl of a mixture of 70% propionic anhydride in methanol and 20µl 50mM $(NH_4)HCO_3$, followed by two washing steps with 100mM $(NH_4)HCO_3$. Disulfide bridges were reduced with DTT and alkylation of cystein residues was performed using iodoacetamide. Proteins were digested with trypsin overnight at 37°C. Extracted peptides were separated on a reversed phase nano-HPLC (Ultimate 3000, Dionex, Sunnyvale CA, USA; trapping column: PepMap C18, 300µm×5mm, 3µm, 100Å, flow rate 20µl/min; separation column: PepMap C18, 75µm×150mm, 3µm, 100Å, flow rate 300nl/min, gradient: 0-25% B in 120 min, 25%-50%B in 60 min, 90% B for 18 min, solvent A: 5% acetonitrile, 0.1% formic acid in water, solvent B: 80% acetonitrile, 0.08% formic acid in water).

The HPLC was coupled online via a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark) to an LTQ or an LTQ-FT (Thermo Electron, Bremen, Germany) linear ion trap Fourier Transform hybrid mass spectrometer. The mass spectrometer was operated in data dependent mode, each high-resolution full scan (m/z 300 to 1800, resolution at m/z 400 set to 100.000) in the ICR cell was followed by 5 product ion scans in the linear trap, preferentially for precursor masses from a parent mass list containing the calculated masses of different modifications of the H3 K9-R17 peptide. For each MS2 scan the neutral loss algorithm in the Xcalibur 2.0 software was enabled. In this mode an MS3 scan is triggered, if a neutral loss of phosphoric acid is detected among the 5 most intense fragment ions in the preceding MS2 scan. Dynamic exclusion (exclusion duration 30 s, exclusion window ±5 ppm) was enabled to allow detection of less abundant ions. Data analysis was performed using the SEQUEST search algorithm (BioWorks 3.2, Thermo Electron). A custom-built database, containing the histone variants of *Mus musculus* and common contaminants, was searched with a precursor mass accuracy of 20 ppm. Carbamidomethylation of cysteine and propionylation of lysine were set as static modifications, monomethylation of lysine and arginine, acetylation, di- and trimethylation of lysine, phosphorylation of serine, threonine and tyrosine as well as the loss of water from serine and threonine were set as variable modifications. All search results were subjected to stringent manual validation.

GST Pull-down assay

Beads coated with GST fusion proteins (5µg) were incubated with 20µg acid extracted histone proteins in 200µl ΔX buffer at 4°C for 2h on a roller. Reactions were washed sequentially with ΔX buffer, RIPA buffer 300mM NaCl, RIPA buffer 500mM NaCl and finally twice with ΔX buffer. Bound proteins were separated by 16% SDS-PAGE and visualized by Western blotting. Recombinant histone H3 was expressed in BL21 (DE3) RIPL cells and purified as described (Sunke *et al.* 1999), MSK1 and PCAF were purchased from Upstate Biotechnology (Lake Placid, N.Y.). *In vitro* histone modifications were performed according to the provider instructions. *In vitro* phosphoacetylation of histone H3 was carried out by initial phosphorylation and subsequent acetylation for 45 min at 30°C each after adjustment of

buffer conditions to PCAF assay requirements. For monitoring modification status of histones, modification reactions were precipitated as described previously (Wessel and Flugge, 1984), and analyzed by immunoblotting or subjected to mass spectrometry.

In vitro peptide pull-down assay

Peptides were covalently coupled to agarose beads via free sulfhydryl-groups (SulfoLink Kit, Pierce Biotechnology) at a concentration of 2.5 µg peptide per µl solid gel volume and stored as 50% slurry at 4°C. Equilibration and coupling efficiency was monitored by peptide dot-blotting and Ponceau staining. 10µl of peptide-slurry were equilibrated with HERR buffer (20mM HEPES pH 7.9, 50mM KCl, 2mM EDTA, 0.1% NP-40, 10% glycerol (v/v), Complete-Protease inhibitor cocktail (Roche), PPI, 10 mM sodium butyrate) prior to incubation with 2.5µl *in vitro* translated, ^{35}S-methionine labeled 14-3-3 proteins (TNT rabbit reticulocyte lysate system, Promega) in 50µl total reaction volume. Binding reactions were incubated for 14h at 4°C on a roller. Reactions were washed twice with HERR buffer, RIPA buffer 300mM NaCl (see above) and finally twice with HERR buffer. Bound proteins were resolved by 16% SDS-PAGE. Gels were incubated with ENHANCE^3 solution and dried under vacuum. Proteins were visualized by fluorography on storage phosphor plates. Signals were scanned with a Typhoon 8600 Imager (Amersham Biosciences) and analyzed with Image Quant software.

Fluorescence polarization binding measurements

Histone peptides (residues 1-20 or 25-38) were labeled using fluorescein-5-EX succinimidyl ester (Molecular Probes). Fluorescinated peptides were purified by gel filtration and reversed phase chromatography. The identity and purity of all peptides was verified by mass spectrometry. After GSH Agarose affinity chromatography and cleavage of the GST tag with factor Xa protease, 14-3-3 zeta was further purified by anion exchange (Mono Q, Amersham), and gel filtration (Superdex 75, Amersham) chromatography. Fluorescence polarization binding measurements were performed under conditions of 50 mM HEPES pH 7.5, 50 mM KCl, 2 mM DTT, and 100 nM fluorescein-labeled peptide (Fischle et al., Nature

2005). Data were recorded on a HIDEX Chameleon II plate reader at room temperature. Anisotropy values were normalized to the bound and unbound state (fraction bound). Data sets obtained from at least three independent measurements were averaged.