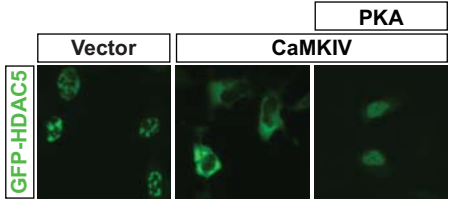

Figure S1. PKA reverses CaMKIV-mediated nuclear export of HDAC5

HEK293 cells were transiently transfected with an GFP-HDAC5 expression plasmid together with an empty vector, or with expression plasmids for CaMKIV and/or PKA as indicated.

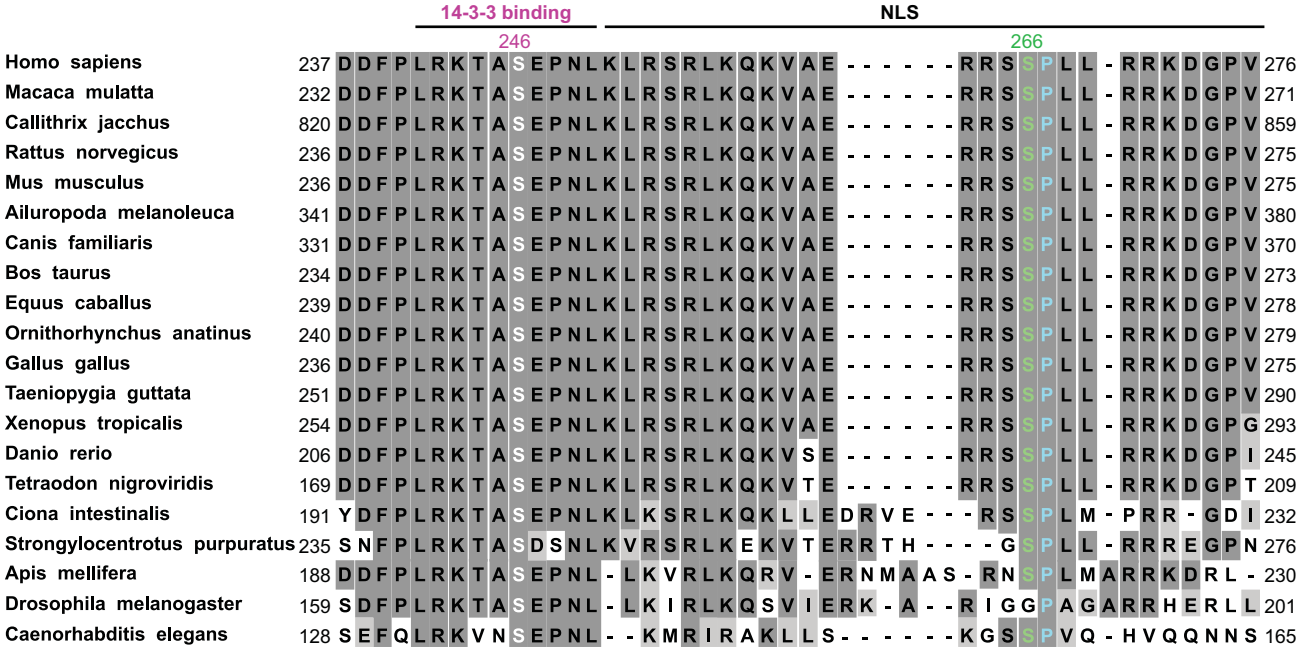

Figure S2. Comparison of the NLS region of HDAC4 from different organisms

After an initial BLAST search for HDAC4 homologues from different species, amino acid sequences encompassing the first 14-3-3 binding site and the adjacent NLS region were aligned using ClustalW. The aligned file was imported to Adobe Illustrator CS4 for further editing. Identical residues are highlighted by dark grey boxes while similar residues are shaded by light grey boxes. The S246 14-3-3 binding site is shown as white “S” while the cAMP-responsive residue S266 is depicted by green “S”. Genbank accession numbers used for the alignment are: *H. sapiens* (NP_006028); *M. mulatta* (XP_001099472); *C. jacchus* (XP_002806986); *R. norvegicus* (XP_001067733); *M. musculus* (NP_997108); *A. melanoleuca* (XP_002922520); *C. familiaris* (XP_849514); *B. taurus* (XP_002686643); *E. caballus* (XP_001497201); *O. anatinus* (XP_001510939); *G. gallus* (NP_989644); *T. guttata* (XP_002190608); *X. tropicalis* (XP_002932071); *D. rerio* (NP_001034447); *T. nigroviridis* (CAF98995); *C. intestinalis* (XP_002128142); *S. purpuratus* (XP_797761); *A. mellifera* (XP_391882); *D. melanogaster* (NP_727682); *C. elegans* (AAZ82858).

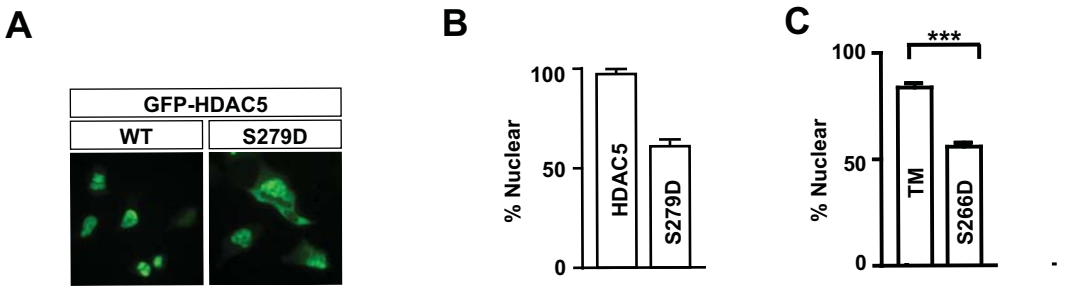

Figure S3. Subcellular localization of the S279D and S266D mutants

A) Representative fluorescence microscopy images of GFP-HDAC5 WT and the mutant S279D after transient transfection in HEK293 cells.

B) Quantification of GFP-HDAC5 and GFP-S279D subcellular localization post-transient transfection in C2C12 cells. At least 100 cells were counted for each condition over 2 independent experiments. Data are presented as mean +/- standard deviation (error bars).

C) Quantification of GFP-HDAC4 TM and GFP-TM/S266D subcellular localization post-transient transfection in HEK293 cells. Data are presented as mean +/- standard deviation (error bars). ***, p=0.0006.

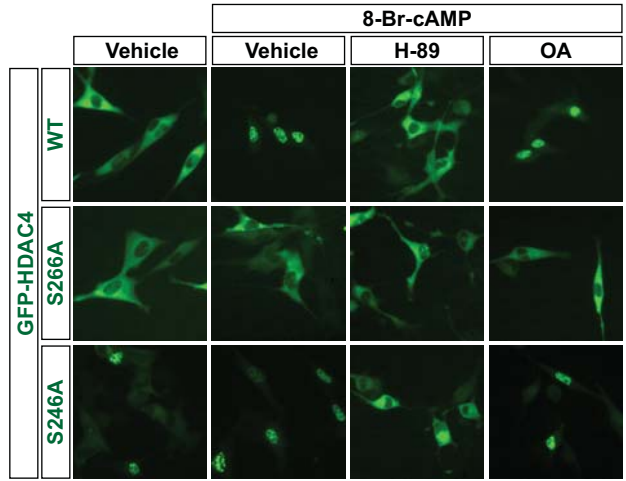

Figure S4. H-89 treatment inhibits cAMP-mediated nuclear localization of HDAC4

C2C12 cells were transiently transfected with expression plasmids for GFP-HDAC4, -S266A, or -S246A. Cells were treated with pre-treated with vehicle (H2O), the PKA inhibitor H-89 (10 μM), or the PP2A inhibitor okadaic acid (OA, 100 nM) for 1 h, followed by treatment with vehicle (H2O) or 1 mM 8-Br-cAMP for 30 min. Subcellular localization of the GFP-HDAC4 constructs was monitored by green fluorescence microscopy.

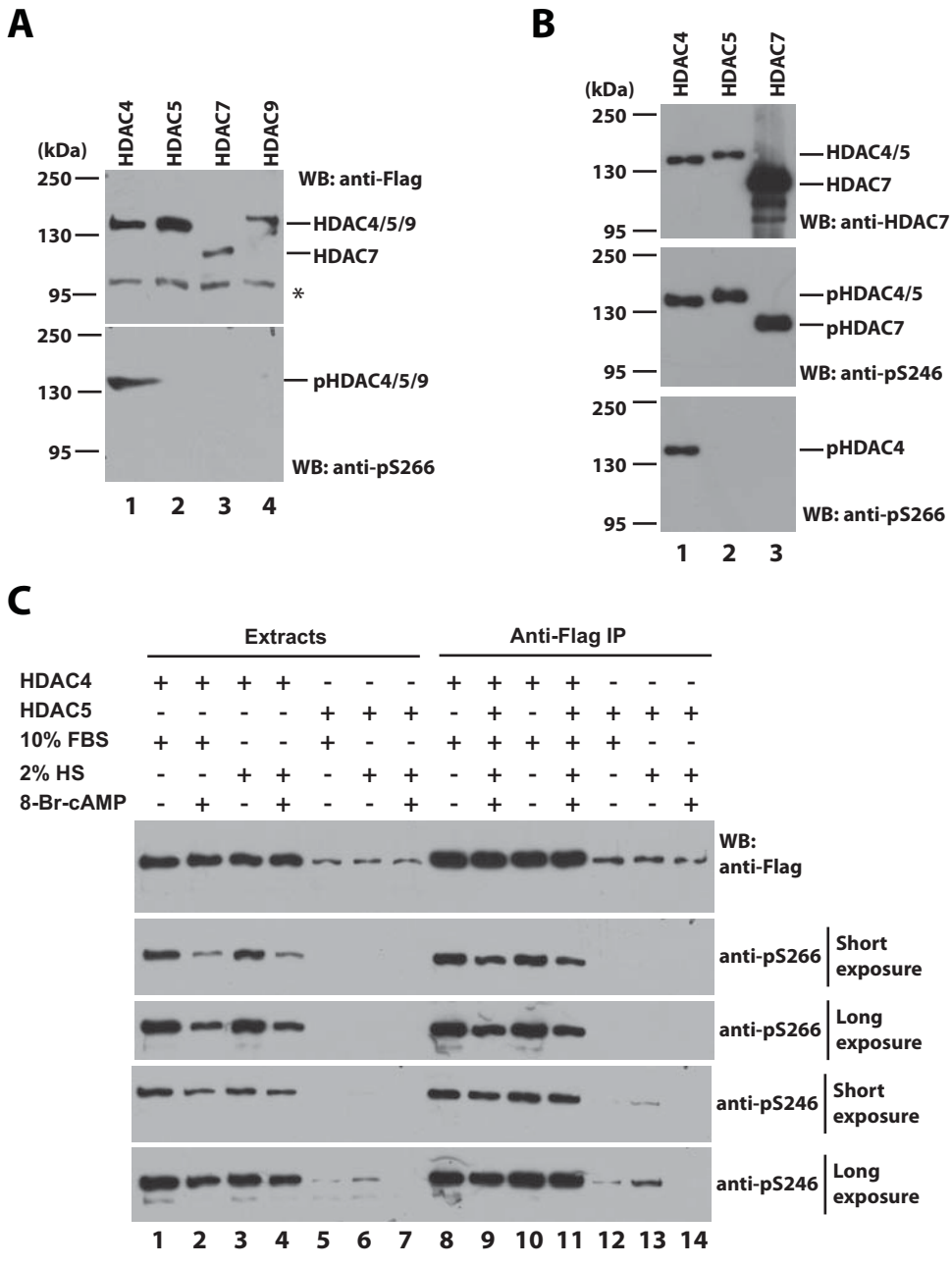

Figure S5. Differential phosphorylation of HDAC4, 5 and 9 at the conserved motif

A) HEK293 cells were transiently transfected with Flag-HDAC4, 5, 7 and 9 expression plasmids as indicated. Soluble extracts were analyzed by Western blotting with anti-Flag or phospho-S266 specific antibody. *, non-specific bands.

B) Same as A) except that only HDAC4, 5 and 7 were analyzed. For immunobloting, anti-HDAC7, anti-phospho S246 and anti-phosphoS266 antibodies were used. The anti-HDAC7 antibody crossreacts with HDAC4 and HDAC5.

C) C2C12 cells were transiently transfected with Flag-HDAC4 and -HDAC5 expression plasmids for analysis as in Fig. 5C. The blots in Fig. 6C are part of the blots presented in this supplemental figure (lanes 1-4, different exposure). Note that due to the predominantly nuclear localization of HDAC5, the phosphorylation levels remain low or undetectable. FBS, fetal bovine serum; HS, horse serum.

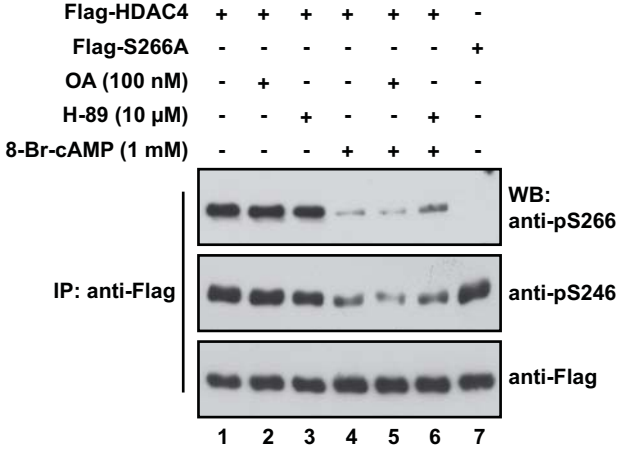

Figure S6. Effect of PP2A or PKA inhibition on S266 phosphorylation

C2C12 cells were transiently transfected with an expression plasmid for Flag-tagged wild-type HDAC4 or mutant S266A. The following day, cells were pre-treated with 100 nM okadaic acid (OA) or 10 μM H-89 for 1 h, followed by treatment with vehicle (H2O) or 1 mM 8-Br-cAMP for 30 min as indicated. Cells were harvested and Flag-tagged proteins were immunoprecipitated on M2 agarose beads. Immunoblotting was performed by use of antibodies as indicated.

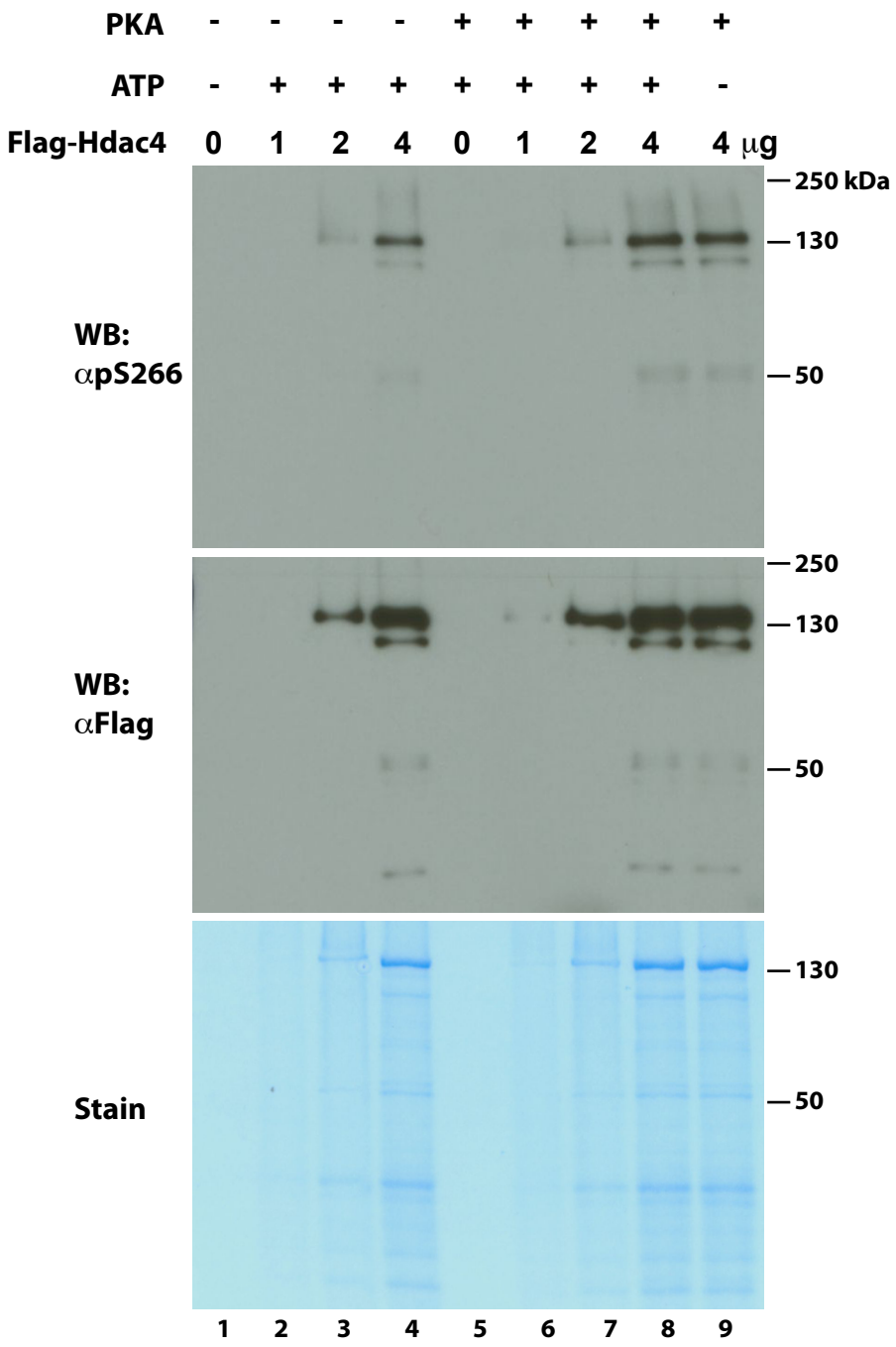

Figure S7. Phosphorylation of HDAC4 by PKA *in vitro*.

Different amounts of Flag-HDAC4, expressed in Sf9 cells and affinity-purified from the soluable extracts, were subject to phosphorylation assays in the presence or absence the PKA catalytic subunit *in vitro*, with or without ATP, as specified. A third of each reaction mixture was stained with Colloidal Coomassie blue (*bottom*) and the remaining mixture was used for immuno-blotting with anti-phospho-S266 (*top*) or anti-Flag antibody (*middle*). The residual signals detected by the anti-phospho-S266 antibody (lanes 2-4) could be due to phosphorylation already occured in Sf9 cells. The PKA catalytic subunit used for the particular experimnet shown here was from Calbiochem and has a specific activity of 20 units/ng, the amount of 50 ng is equivalent to 1,000 units per reaction. Even in the presence of this exessive amount, PKA had minimal effects on ATP-dependent phosphorylation, so PKA did not appear to phosphorylate HDAC4. We also tested a smaller amount of a PKA catalytic subunit from Sigma and did not find any phoshorylation either (data not shown).

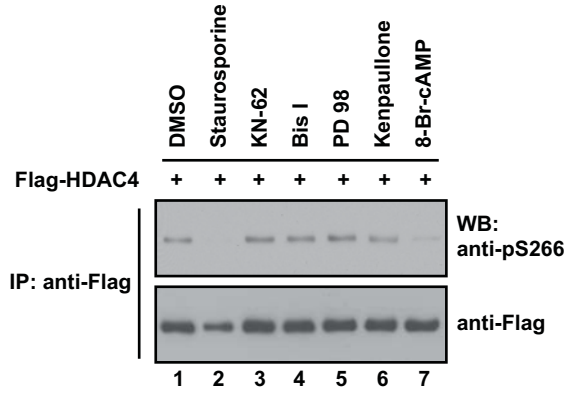

Figure S8. Effect of various kinase inhibitors on S266 phosphorylation

C2C12 cells were transiently transfected with an expression plasmid for Flag-HDAC4. The following day, cells were treated with either DMSO, 8-Br-cAMP, or inhibitors of the following kinases: general S/T kinases (Staurosporine, 1 μM), CaMKs (KN-62, 10 μM), PKC (Bis I, 10 μM), MEK/ERK (PD 98059, 1 μM) and GSK-3 (Kenpaullone, 5 μM). Cells were harvested for extraction and Flag-HDAC4 was immunoprecipitated on M2 agarose beads. Immunoblotting was performed with the anti-Flag antibody or the phospho-S266 specific antibody.