Structure *16*

Supplemental Data

Structural Basis for the Recognition of Methylated Histone H3K36 by the Eaf3 Subunit of Histone Deacetylase Complex Rpd3S

Chao Xu, Gaofeng Cui, Maria Victoria Botuyan, and Georges Mer

Supplemental Experimental Procedures

Protein and Peptide Preparation

The cDNA corresponding to the chromo barrel domain (1 – 113 aa) of budding yeast Eaf3 was amplified from a DNA library by polymerase chain reaction (PCR). The cDNA was next cloned into the NdeI/XhoI sites of pET-22b(+) vector (Novagen) in-frame with a DNA sequence encoding a C-terminal hexahistidine tag (LEHHHHHH), and used to transform *E. coli* BL21(DE3) cells. To produce nonlabeled Eaf3 protein, the cells were initially grown in LB media at 37ºC. Once the cells reached an A_{600} of ~0.8, they were transferred to a shaker incubator set at 15ºC. Forty five min later 1 mM IPTG (final concentration) was added to the culture to commence protein expression. Incubation was continued for 16 to 20 hr after which the cells were harvested.

The cells were resuspended in 50 mM sodium phosphate (NaPi), pH 7.5, 300 mM NaCl (resuspension buffer) and 1-2 mM PMSF, and lyzed with a high pressure microfluidizer Emulsiflex C5 (Avestin). Cell debris were spun down by centrifugation and the clarified supernatant loaded onto a column with Ni-NTA resin (Qiagen) pre-equilibrated with the resuspension buffer. The column was then washed with the resuspension buffer containing 20 mM imidazole, and the protein eluted with similar buffer but having 500 mM of imidazole. Further purification was performed using a preparative Superdex 75 (GE Healthcare) size exclusion chromatography column.

The preparation of isotopically labeled Eaf3 followed similar steps as above except that instead of LB media, M9 media containing 1 g/L ^{15}N NH_4Cl, 4 g/L [$^{12}C_6$]-D-glucose and 1 g/L ^{15}N Isogro (Isotec) (for preparing ^{15}N-labeled samples); and 1 g/L ^{15}N NH_4Cl, 2 g/L [$^{13}C_6$]-D-glucose and 1 g/L $^{15}N/^{13}C$ Isogro (Isotec) (for producing $^{15}N/^{13}C$-labeled samples) were used.

To make the fused Eaf3–H3K_C36me2 complex, the cysteines 14 and 76 of Eaf3 (1 – 115 aa) were first mutated to a valine and a serine, respectively, by Quickchange protocol (Stratagene), producing the plasmid, Eaf3-C14V/C76S. The plasmid was next recloned into a modified pET15b vector (designated as pTEV) through NdeI/BamHI sites, adding to the expressed protein an N-terminal hexahistidine tag that can be cleaved by tobacco etch virus (TEV) protease. Preparation of nonlabeled and isotopically labeled proteins cloned into pTEV followed a procedure similar to that used for pET-22b(+) cloned proteins but with an additional step of overnight cleavage at room temperature by TEV protease after Ni-NTA elution. Removal of the tag left a GH sequence preceding the actual protein sequence.

Next, the double mutant Eaf3-C14V/C76S plasmid was used as a template in a PCR, employing appropriate overlapping primers to extend the C-terminal end of Eaf3-C14V/C76S by a 4 amino acid linker (GSTG) followed by the histone H3K36 sequence

encompassing residues 28 – 42. This new construct is designated as Eaf3–H3K36 where the additional amino acids from the linker and H3K36 are renumbered from 116 to 134.

Next, Lys128 of Eaf3–H3K36 (or H3K36) was mutated into a cysteine by Quickchange methodology (Stratagene) producing the Eaf3–H3C36 plasmid. After purification of the Eaf3–H3C36 protein and cleavage of the hexahistidine tag, the cysteine was converted chemically to an N-dimethylated aminoethylcysteine, a methyllysine analog, by an alkylation reaction (Simon et al., 2007) (Figure 4A). Depending on the alkylating reagent used, one can control the degree or state of methylation of the product (Simon et al., 2007). All alkylation steps were done in the dark. For dimethylation, 50 μL of 1M (2-chloroethyl)-dimethylammonium chloride or me2 was added to a 1 mL solution of 10 mg Eaf3–H3C36, 1M HEPES, pH 7.8 and 20 mM DTT. The reaction was allowed to proceed for 3 hr at room temperature, after which were added 10 μL of DTT (1M), and then, 30 min later, 50 μL of me2 (1 M). The reaction was continued for another 3 hr at room temperature and terminated by addition of 50 μL of β-mercaptoethanol (14.2 M). The dimethylated product, referred to here as Eaf3–$H3K_C36me2$, was purified by size exclusion chromatography using a preparative Superdex 75 column (GE Healthcare).

Four mutants of Eaf3–H3C36 harboring one of the following changes: Y23A, Y81A, W84A, and W88A were produced. The mutant proteins W84A and W88A were expressed, dimethylated at Cys128 and purified as described above. All the purified proteins (> 95% pure by SDS-PAGE) were concentrated and buffer exchanged according to the next experiments in line. Final protein concentrations were determined by UV spectrophotometry using extinction coefficients calculated based on the protein sequences.

All methylated histone peptides were chemically synthesized and HPLC-purified at the Mayo Clinic Protein Core Facility.

Supplemental Reference

Simon, M.D., Chu, F., Racki, L.R., de la Cruz, C.C., Burlingame, A.L., Panning, B., Narlikar, G.J., and Shokat, K.M. (2007). The site-specific installation of methyl-lysine analogs into recombinant histones. Cell *128*, 1003-1012.

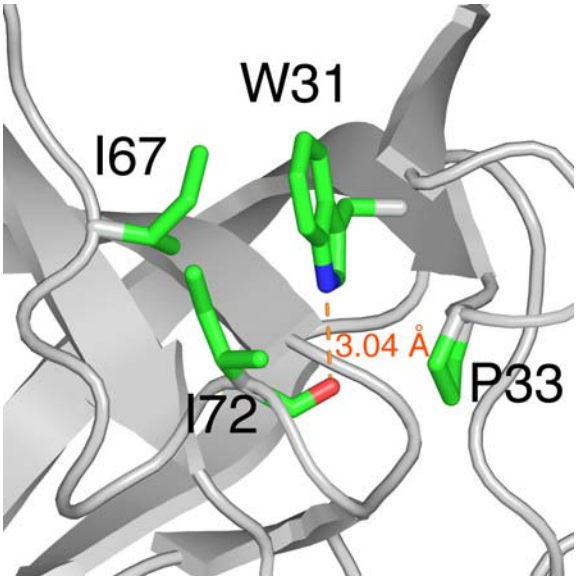

Figure S1. Close-Up View of a Hydrophobic Interaction Core Within Eaf3–H3K$_C$36me2
The hydrogen bond between Trp31 HE1 and the carbonyl group of Ile72 is shown.

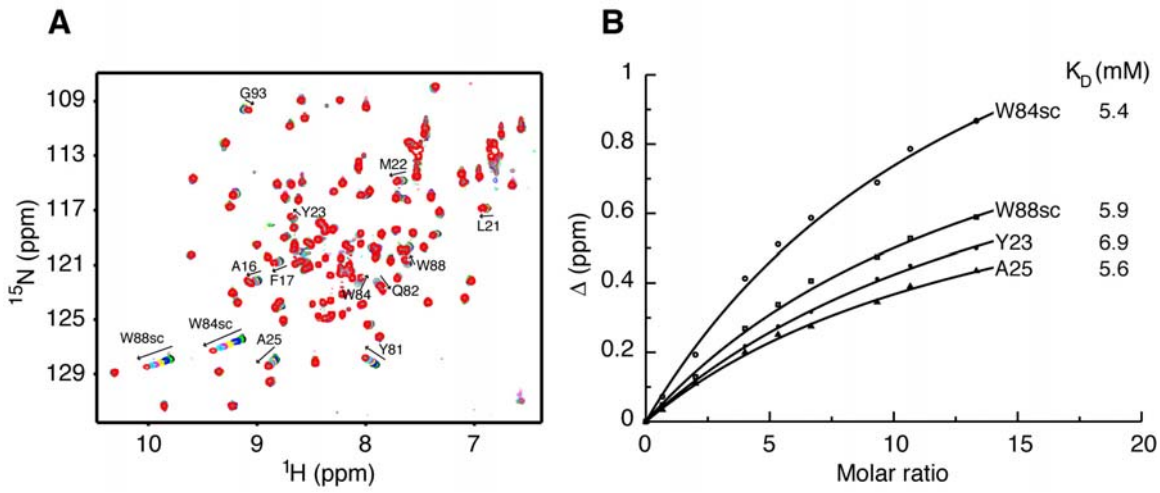

Figure S2. Interaction of Eaf3 Chromo Barrel Domain with an H3K$_C$36me3 Peptide
(A) ^{15}N-^{1}H HSQC titration spectra of ^{15}N-labeled Eaf3 (1 – 113 aa), free (black) and upon addition of increasing amounts of H3K$_C$36me3 peptide (31 – 42 aa). The side chain amide atom signals of Trp84 and Trp88 are labeled W84sc and W88sc, respectively.
(B) Estimates of K_D of Eaf3 for H3K$_C$36me3 peptide from the change in chemical shifts of selected ^{1}H-^{15}N Eaf3 resonances upon addition of nonlabeled H3K$_C$36me3 (31 – 42 aa).

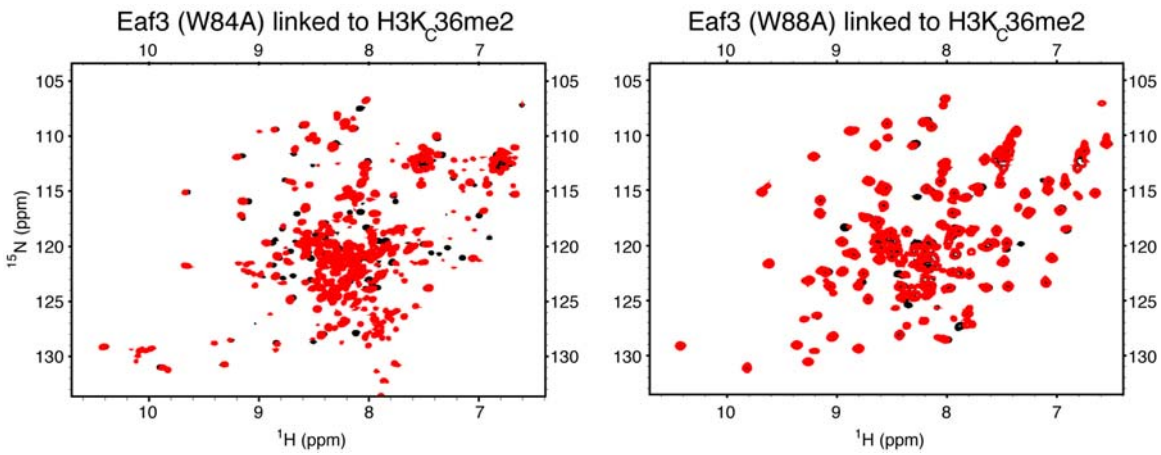

Figure S3. Mutation of Trp84 and Trp88 in the Chromo Barrel Domain of Eaf3
Shown are the overlaid ^{15}N-^{1}H HSQC spectra of the W84A and W88A mutants of Eaf3–H3C36, before (black) and after (red) chemical conversion of Cys128 (or H3C36) into a dimethyllysine analog.