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Structure of HDAC3 bound to corepressor and inositol tetraphosphate

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Summary

Histone deacetylase enzymes (HDACs) are emerging cancer drug targets. They regulate gene expression by removing acetyl groups from lysine residues in histone tails resulting in chromatin condensation. The enzymatic activity of most class I HDACs requires recruitment to corepressor complexes. We report the first structure of an HDAC:corepressor complex - HDAC3 with the deacetylase-activation-domain (DAD) from the SMRT corepressor. The structure reveals two remarkable features. First the SMRT-DAD undergoes a large structural rearrangement on forming the complex. Second there is an essential inositol tetraphosphate molecule, Ins(1,4,5,6)P_4, acting as an 'intermolecular glue' between the two proteins. Assembly of the complex is clearly dependent on the Ins(1,4,5,6)P_4, which may act as a regulator – potentially explaining why inositol phosphates and their kinases have been found to act as transcriptional regulators. This mechanism for the activation of HDAC3 appears to be conserved in class I HDACs from yeast to man and opens novel therapeutic opportunities.

The acetylation of lysine residues in the tails of histone proteins plays an important role in the regulation of gene expression in eukaryotic cells[1,2]. The level of lysine acetylation is controlled through the opposing actions of histone acetyl transferases (HATs) and histone deacetylases (HDACs). Although chromatin is the best understood substrate for these enzymes, lysine acetylation is emerging as a general regulatory mechanism in a diverse array of cellular processes[3].

There are four classes of HDACs in mammalian cells (reviewed in[4]). Class I HDACs are Zn-dependent enzymes and include HDACs 1, 2, 3 and 8. Of these, only HDAC8 is a fully functional enzyme in isolation[5,6]. HDACs 1, 2 and 3 require association with large multisubunit corepressor complexes for maximal activity[7-12]. These corepressor complexes bring about the repression of gene expression when recruited to repressive transcription factors, but also contribute to the 'resetting' of chromatin after rounds of transcriptional activation[13-16].

In recent years HDACs have become important targets for the treatment of a number of cancers[17]. Cancer cell lines treated with HDAC inhibitors undergo terminal differentiation, growth arrest and/or apoptosis. Several HDAC inhibitors are at various stages in clinical trials and two drugs, vorinostat and romidepsin, have been approved for the treatment of cutaneous T-cell lymphomas[18].

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Author Contribution PJW expressed, purified and crystallised the protein and performed the biochemical studies. PJW, LF & JWRS performed the structural determination and wrote the paper. GMS & LF performed early expression/purification trials in insect cells. JWRS conceived the study.

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HDACs 1 and 2 are found in three repression complexes: NuRD[7,19], CoREST[20,21] and Sin3A[22,23]. In contrast, HDAC3 appears to be uniquely recruited to the SMRT/NCoR complex where it interacts with a conserved deacetylase-activation-domain (DAD) within SMRT or NCoR[8,10,12,24,25,26]. The DAD both recruits and activates HDAC3[10-12]. Recruitment of HDAC3 to the DAD is essential for repression by certain nuclear receptors and for the maintenance of normal circadian physiology[27-29]. It has been proposed that the assembly of the HDAC3 and SMRT-DAD requires a chaperone complex, since when these proteins are expressed in bacteria they do not form a complex[30].

The DAD contains an extended SANT-like domain with an amino-terminal DAD-specific motif. Deletion of this motif results in both loss of binding and failure to activate HDAC3[11].We have previously reported the structure of the isolated DAD from SMRT[31]. This revealed that part of the DAD-specific motif forms an extra helix that is folded against the three helices of the SANT domain to form a four-helix bundle. The amino-terminal portion of the DAD-specific motif is unstructured in solution[31].

Here we report the structure of HDAC3 in complex with the SMRT-DAD. This structure not only reveals the specificity and mechanism through which SMRT-DAD recruits and activates HDAC3, but also identifies inositol-(1,4,5,6)-tetraphosphate as a key component of the complex that has the potential to regulate assembly of HDACs with their corepressors.

Overall architecture of the complex

Since HDAC3 and SMRT-DAD do not form a complex when expressed in bacterial cells, full-length HDAC3 and FLAG-tagged SMRT-DAD (aa: 389-480) were expressed in suspension grown mammalian HEK293 cells. The complex remained tightly associated during a three-step purification including size exclusion chromatography (Supplementary figure 1). Interestingly at salt concentrations higher than 50mM the complex dissociated and the HDAC3 predominately took the form of aggregate or oligomers (Supplementary figure 2). Such oligomerisation of HDAC3 has also been reported by others[32]. During crystallisation, the HDAC3 tail is proteolysed but HDAC3 remains bound to SMRT-DAD and retains deacetylase activity (Supplementary figure 3). Small crystals (15μm - Supplementary figure 1) diffracted to 2Å and the structure was solved by molecular replacement with an HDAC8 structure[33]. Model building and refinement yielded an excellent map to 2.1Å resolution with clear density for both the HDAC3 and the SMRT-DAD (Supplementary figure 1).

Overall the HDAC3 structure is similar to the previously determined class I HDAC structures of HDAC8[34] and HDAC2[35] and consists of an eight-stranded parallel beta-sheet surrounded by a number of alpha-helices. The active site lies at the base of a tunnel leading from the surface of the protein. A solvent exposed tyrosine residue is located on the surface of the enzyme immediately adjacent to the active site tunnel. This tyrosine is unique to HDAC3 and it seems that this residue might interact with substrate and possibly contribute to substrate specificity (Supplementary figure 4).

Structural rearrangement of the SMRT-DAD

On forming a complex with HDAC3, the amino terminal helix of the DAD undergoes a major structural rearrangement, from that seen in the NMR structure, such that it no longer forms part of the core structure, but lies along the surface of HDAC3 making extensive intermolecular interactions (figure 1A & B). Along with a further extended region, this DAD-specific motif buries a surface of 1,178Å^2. The remaining 3-helix bundle resembles a canonical SANT domain and buries a further 1,160Å^2 at the interface with HDAC3.

The SMRT-DAD interacts with the amino terminal region of HDAC3 (residues 9-49) that forms helix H1, loop L2, helix H2 and strand S2. This region differs between HDAC8 and HDAC3. Indeed, in HDAC3, helix H1 is distorted and can only be considered to be a pseudo-helix. These differences may explain why HDAC8 is active in the absence of an interacting corepressor.

An essential role for Ins(1,4,5,6)P_4

At the earliest stages of refinement, the electron density difference map revealed a well-ordered small molecule bound at the interface between HDAC3 and the DAD (figure 2A). The electron density was sufficiently well defined that the small molecule could be readily identified as inositol tetraphosphate. During further refinement it could be unambiguously assigned as D-myo-inositol-1,4,5,6-tetrakisphosphate (based on the axial orientation of the hydroxyl group on carbon 2) and is hereafter termed Ins(1,4,5,6)P_4 (figure 2B).

The Ins(1,4,5,6)P_4 molecule is sandwiched between HDAC3 and the DAD, in a highly basic pocket, making extensive contacts to both proteins, burying a surface of 407Å^2 (figure 2C and D). HDAC3 and the SMRT-DAD each contribute five hydrogen bonds and salt bridges to the Ins(1,4,5,6)P_4 (His17, Gly21, Lys25, Arg265 & Arg301 and Lys449, Tyr470, Tyr471, Lys474 & Lys 475 respectively) (figure 2E).

It is notable that the Ins(1,4,5,6)P_4 is sufficiently tightly bound to the complex that it is retained through the entire purification process. It is also striking that the binding is highly specific for Ins(1,4,5,6)P_4 since the electron density shows that the ligand is uniquely Ins(1,4,5,6)P_4 rather than a mixture of inositol phosphates.

A careful examination of the structure suggests that Ins(1,4,5,6)P_4 binding is an essential requirement for the interaction between SMRT and HDAC3 acting as a ‘intermolecular glue’ that cements the complex together. If the Ins(1,4,5,6)P_4 ligand were not present, then the many basic residues on either side of the binding cleft would likely prevent interaction through charge repulsion. Indeed, at the base of the cleft, the ζ–nitrogen atoms of SMRT Lys449 SMRT, and HDAC3 Lys25 are just 4.4Å from each other. Consequently the assembly of the 3-way SMRT:HDAC3:Ins(1,4,5,6)P_4 complex appears to be mutually interdependent, such that both the SMRT-DAD and the Ins(1,4,5,6)P_4 are required for activation of the HDAC3 enzymatic functionality.

These conclusions are supported by our previous mutagenesis study of the SMRT-DAD in which we looked at the effect of mutations on both the interaction with, and the deacetylase activity of, HDAC3. All mutations that failed to activate HDAC3 also abolished, or significantly impaired, interaction. These include mutations of Lys449, Tyr470 and Tyr471 which play a key role in binding the Ins(1,4,5,6)P_4[31].

The requirement for Ins(1,4,5,6)P_4 to enable complex formation may contribute to the explanation as to why recombinant proteins expressed in bacteria fail to interact, since bacteria probably do not contain sufficient Ins(1,4,5,6)P_4 to support complex formation.

Having discovered that Ins(1,4,5,6)P_4 plays a key role in HDAC3 activation we asked whether inositol phosphates might contribute to the assembly and activation of other class I HDAC complexes. Significantly, the residues that mediate interaction with the Ins(1,4,5,6)P_4 and the SANT domain from SMRT are conserved in both HDAC1 and 2, but not in HDAC8 (figure 3A), fitting with the observation that only HDAC8 is fully active in isolation.

Similarly, the corepressor partners for HDAC1 and 2, MTA(1-3) and CoREST(1-3), contain SANT domains that are analogous to the SMRT-DAD. The key Ins(1,4,5,6)P_4 binding residues are almost entirely conserved (figure 3B). It is likely that the specificity for the particular HDAC is conferred by the region amino-terminal to the SANT domain i.e. the DAD specific motif in SMRT/NCoR and the ELM2 domains in the MTA and CoREST proteins.

Taken together this suggests that inositol phosphate activation of HDACs is a general mechanism and, given that the key residues are also found in the yeast HDAC, Rpd3, and the Snt1 corepressor, it would appear that this mechanism is also evolutionarily conserved.

Mechanism of activation of HDAC3

The HDAC3:SMRT-DAD structure provides insight into the mechanism through which the DAD and Ins(1,4,5,6)P_4 contribute to the activation of HDAC3. In the crystal, the active site of the HDAC3 resembles a product complex (figure 4A and B). An acetate molecule (present during purification) is bound at the active site, making hydrogen bonds to the catalytic zinc and side chains of Ty298 and His134. Furthermore, a methionine sidechain (from an adjacent SMRT-DAD in the crystal lattice) is bound in the active site tunnel mimicking a lysine residue.

The binding surfaces for the DAD and the Ins(1,4,5,6)P_4 are located to one side of the HDAC3 active site involving pseudo helix H1 and the loops L1 and L6 (figure 4A,B,C). We propose that changes in both conformation and dynamics occur when the DAD and Ins(1,4,5,6)P_4 bind to HDAC3 and that these facilitate substrate access to the active site resulting in enhanced enzyme activity.

There is a key interaction between the Ins(1,4,5,6)P_4 and Arg265 in loop L6 (coloured orange in figure 4B). This loop seems to be very important for access to the active site since Leu266 forms one wall of the active site tunnel and in the absence of the Ins(1,4,5,6)P_4 this loop is likely to be relatively mobile.

Comparison of the HDAC3 structure with that of HDAC8, which does not require activation by complex formation, reveals that HDAC8 differs significantly in the region where HDAC3 interacts with the SMRT-DAD and Ins(1,4,5,6)P_4 (figure 4D). In HDAC8 the pseudo helix H1 has a regular stable helical structure, loop L1 is two amino acids shorter and loop L6 contains a proline residue that partly orientates the loop away from the active site (figure 4D). We suggest that together these differences give substrate better access to that active site of HDAC8 than would be possible in the uncomplexed HDAC3. The pattern of crystallographic temperature factors for the various structures supports this interpretation (Supplementary figure 5).

To test the importance of Arg265 as well as loops L1 and L6, we co-expressed mutant HDAC3 constructs with SMRT-DAD in mammalian cells. Strikingly, all the designed mutations resulted not only in total loss of deacetylase activity (figure 5A) but also abolished interaction with the SMRT-DAD (figure 5B). These findings support our interpretation of the importance of these residues in the activation of HDAC3.

To support the conclusions that Ins(1,4,5,6)P_4 is essential for HDAC3 activity and to test the specificity for Ins(1,4,5,6)P_4, we sought to establish an *in vitro* reconstitution assay. We expressed and purified C-terminally FLAG-tagged HDAC3 from mammalian cells and incubated the anti-FLAG resin with bacterially expressed SMRT-DAD and various concentrations of Ins(1,4,5,6)P_4, Ins(1,4,5)P_3 and Ins(1,2,3,4,5,6)P_6 (figure 5C). HDAC activity was very sensitive to inositol phosphate concentration and higher concentrations do

not support reconstitution. This can be explained through high levels of free inositol phosphate competing with complex formation. This is analogous to moderate monovalent salt concentrations causing complex dissociation during purification (Supplementary figure 2).

A similar pattern of activation was seen using both Ins(1,4,5)P_3 and Ins(1,2,3,4,5,6)P_6, but in both cases a 10-fold higher concentration was required to achieve a similar level of HDAC activity to that seen with Ins(1,4,5,6)P_4. This supports the interpretation that Ins(1,4,5,6)P_4 is the physiologically relevant assembly partner.

Does Ins(1,4,5,6)P_4 regulate HDACs?

The finding that Ins(1,4,5,6)P_4 is essential for the assembly of class 1 HDAC repression complexes raises the question as to whether it is a signalling molecule with a role in regulating complex assembly, or if it is simply an essential structural cofactor.

The fact that Ins(1,4,5,6)P_4 remained bound during purification would seem to suggest that it is a structural cofactor. However, to retain an intact complex it was necessary to use low ionic strength buffers. At physiological ionic strengths the Ins(1,4,5,6)P_4 is likely to dissociate readily and thus could regulate complex assembly.

Importantly, several studies have previously implicated Ins(1,4,5,6)P_4 as a regulator of gene expression and chromatin remodelling - although no molecular mechanism was identified. Yeast Arg82 is a transcriptional regulator of genes involved in arginine metabolism. Odom et al showed that Arg82 is an inositol phosphate kinase that converts Ins(1,4,5)P_3 to Ins(1,4,5,6)P_4 and that kinase activity is required for its role in transcriptional regulation (figure 5d)[36]. Several further studies have supported the importance of Arg82 kinase activity in transcriptional regulation and chromatin remodelling [37-39], together suggesting that Ins(1,4,5,6)P_4 has an important regulatory role in yeast.

IPMK, the mammalian homologue of Arg82, has been reported to phosphorylate Ins(1,4,5)P_3 to form Ins(1,3,4,5,6)P_4 and then Ins(1,3,4,5,6)P_5[40,41]. Consequently, in mammalian cells, a phosphatase is required to generate Ins(1,4,5,6)P_4. The most likely enzyme is the well-known tumour suppressor gene PTEN[42,43], which is known to be active in the nucleus and to play a role in chromosome stability (reviewed in[44]). It is tempting to speculate that loss of HDAC complex function might be one of the routes through which PTEN mutations contribute to oncogenesis.

A final important question is whether the levels of Ins(1,4,5,6)P_4 are regulated in the cell. This is not easy to establish since regulation could be temporally and/or spatially compartmentalised. However there is some evidence that both PTEN and InsP_4 levels change with progression through the cell cycle[45]. It is also possible that changing levels of Ins(1,4,5,6)P_4 may contribute to the circadian regulation of HDAC3 activity [46]. Further research will be needed to clarify these important questions.

Conclusions

We present the first structure of a histone deacetylase in complex with its activating corepressor. The structure reveals a striking structural rearrangement of the corepressor and an unexpected inositol tetraphosphate molecule (Ins(1,4,5,6)P_4) acting as an ‘intermolecular glue’ contributing to the stabilisation and activation of HDAC3.

Sequence conservation suggests that Ins(1,4,5,6)P_4 plays a key role in corepressor assembly and activation of class I HDACs from yeast to man and is likely to act as a regulator of

HDAC complex assembly. Whilst it remains to be established how the availability of Ins(1,4,5,6)P_4 might be controlled, the requirement of Ins(1,4,5,6)P_4 for corepressor HDAC assembly presents novel opportunities for therapeutic intervention that may complement existing HDAC inhibitors. It may be possible to develop molecules that target the Ins(1,4,5,6)P_4 binding site itself, but it may also be possible to target the enzymes responsible for Ins(1,4,5,6)P_4 synthesis.

Methods Summary

HDAC3 and FLAG-tagged SMRT-DAD (residues 389–480) were expressed using transient transfection in suspension grown HEK293F cells. The HDAC3:SMRT-DAD complex was purified by FLAG affinity chromatography, followed by TEV protease cleavage and size exclusion chromatography. Crystals of the HDAC3:DAD complex were grown by sitting drop vapour diffusion with a 10% propan-2-ol precipitant. The structure was determined by molecular replacement based on HDAC8 (PDBcode 3EW8). Ligands, including Ins(1,4,5,6)P_4, zinc, potassium, acetate and glycerol were added during the refinement process.

Methods

Protein expression, purification and crystallisation

The DAD domain (SMRT 389 – 480) and full length HDAC3 were cloned into pcDNA3 vector. The DAD domain construct contained a N-terminal 10xHis-3xFLAG tag and a TEV protease cleavage site. HEK293F cells (Invitrogen) were co-transfected with both constructs using Polyethylenimine (PEI) (Sigma). To transfect 250 ml of cells at 1×10^6 cell/ml final density, 0.25 mg DNA total was diluted in 25 ml of PBS (Sigma), vortexed briefly, 1 ml of 0.5 mg/ml PEI added, vortexed briefly, incubated for 20 min at room temperature then added to the cells. For larger volumes multiple flasks were used. Cells were harvested 48 h post transfection and lysed by sonication in buffer containing 50 mM Tris pH 7.5, 100 mM potassium acetate, 5 % v/v glycerol, 0.3 % v/v Triton X-100, Roche complete protease inhibitor (buffer A), the insoluble material was removed by centrifugation. The lysate was pre-cleared using Sepharose 4B (Sigma) and the complex was then bound to FLAG resin (Sigma), washed three times with buffer A, three times with buffer B (50 mM Tris pH 7.5, 300 mM potassium acetate, 5 % v/v glycerol) and three times with buffer C (50 mM Tris pH 7.5, 50 mM potassium acetate, 5 % v/v glycerol, 0.5 mM TCEP). The complex was eluted from the resin by overnight cleavage at 4°C with TEV protease in buffer C. The eluted protein was further purified by gel filtration on a Superdex S200 column (GE healthcare) in buffer containing 25 mM Tris/HCl pH 7.5, 50 mM potassium acetate, 0.5 mM TCEP. The purified complex was concentrated to 7.5 mg/ml for crystallisation trials.

Crystals were grown by sitting drop vapour diffusion at 4°C using 0.1 M HEPES pH 7.5, 0.2 M NaCl, 10 % v/v propan-2-ol. Crystals were cubic in nature, grew to a final dimension of 15 X 15 X 15 μm and belong to the space group C 2 2 2_1.

Comparison of a fresh protein sample with protein from within the crystallisation drops (after 3 months) by SDS-PAGE showed the presence of a truncated form of HDAC3 (Supplemental figure S2). Analysis by LC-MS/MS showed that this HDAC3 was truncated at the C-terminus to residue Q376.

Structure determination

Crystals were flash frozen in mother liquor containing 40% glycerol as a cryoprotectant. Diffraction data were collected on a single crystal in two 45° wedges at the Diamond synchrotron microfocus beamline I24 and processed using XDS[47]. The structure was solved

by molecular replacement using HDAC8 (PDB code 3EW8)[33] as a search model in Phaser[48]. Initial model building was performed with ARP/wARP (http://www.embl-hamburg.de/ARP/), which was able to automatically build 95% of the HDAC3 protein chain, and two helices from the DAD. The additional HDAC3 and DAD sequences were fitted following multiple rounds of refinement and building using REFMAC and Coot[49,50]. Fo-Fc density consistent with the ligand, zinc/potassium ions and acetate/glycerol molecules observed during the refinement/ model building process were fitted and refined as they became apparent. The final model contains amino acids 2-370 chain A and 2-370 chain B of HDAC3, amino acids 408-476 chain C and 408-475 chain D of the DAD. The model also contains two inositol-1,4,5,6 tetraphosphate molecules, two zinc ions, four potassium ions, 2 acetate molecules, and 4 glycerol molecules. The final model has 97.8% residues in the favoured region, 2.0% in the allowed region and 0.2% in the outlier region of the Ramachandran plot.

HDAC Activity assays

C-terminally FLAG-tagged HDAC3 and Myc-tagged DAD were co-expressed in HEK 293 cells as described above. Cells were lysed in 50 mM Tris pH 7.5, 50 mM potassium acetate, 5 % v/v glycerol, 0.3 % v/v Triton X-100, Roche complete protease inhibitor. In order to standardise the assay 800 μg total protein was bound to 40 μl FLAG resin (Sigma) for 2 hrs at 4°C, then washed 4 times with lysis buffer. HDAC activity was measured using the HDAC Assay Kit (Active Motif) and read using a Victor X5 plate reader (Perkin Elmer).

Reconstitution assays

C-terminally FLAG-tagged HDAC3 was expressed in HEK 293 cells and purified as described above. His6-tagged SMRT-DAD was expressed in *E. coli* strain Rosetta (Novagen) and initial purification was carried out using Nickel NTA agarose (Qiagen) followed by gel filtration chromatography using a Superdex-S200 26/60 column (GE Healthcare) in buffer containing 50 mM Tris/HCl pH 7.5, 50 mM potassium acetate. 40 μl of HDAC3-FLAG resin was mixed with His-DAD, with or without phosphoinositide as required, in a final volume of 1ml of buffer D (50 mM Tris/HCl pH 7.5, 50 mM potassium acetate, 5% glycerol, 0.3 % v/v Triton X-100), and incubated for 2hrs at 30 °C. The resin was then washed extensively with buffer D and HDAC activity was measured using the HDAC Assay Kit (Active Motif) and read on a Victor X5 plate reader (Perkin Elmer).

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Supplementary Material

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Acknowledgments

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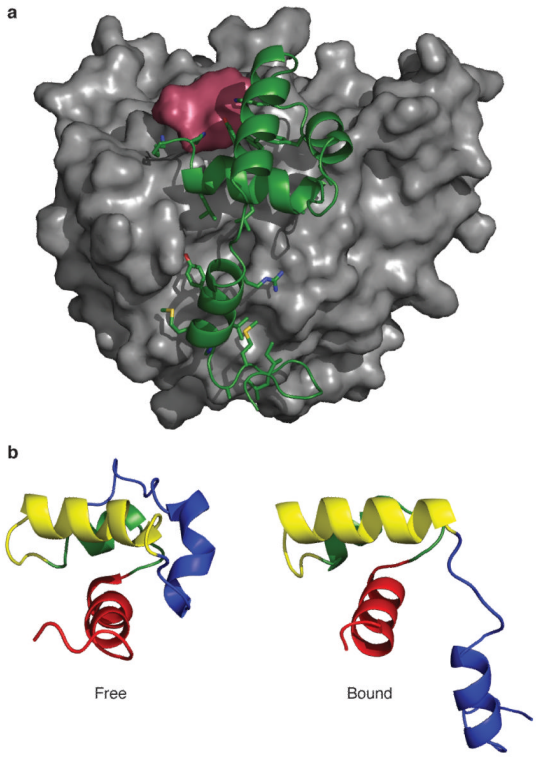

Figure 1. Structure of the HDAC3:SMRT-DAD complex

a, Interaction of the SMRT-DAD (green ribbon) with the HDAC3 (grey surface). The Ins(1,4,5,6)P_4 at the interface is shown as a raspberry coloured surface. Side chains in the DAD that mediate interaction with HDAC3 and Ins(1,4,5,6)P_4 are shown as sticks. **b,** Structure of the DAD domain in solution (PDBcode 1XC5) compared with that bound to HDAC3 (helices are individually coloured to facilitate comparison).

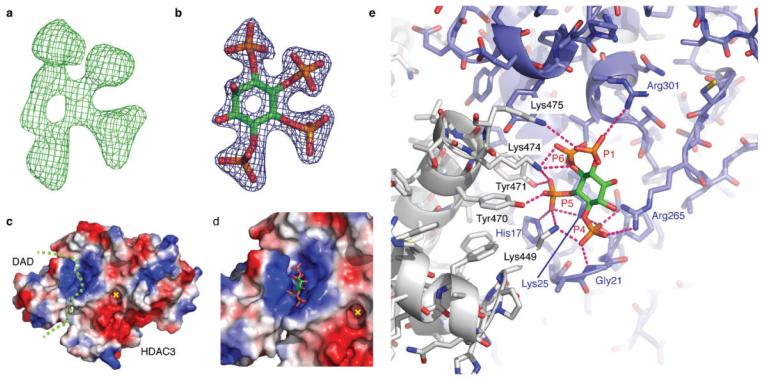

Figure 2. Ins(1,4,5,6)P_4 binding to the HDAC3:SMRT-DAD complex

a, A striking feature in the difference electron density map (Fo-Fc at 3σ,) observed following molecular replacement **b,** Electron density corresponding to the Ins(1,4,5,6)P_4 ligand following refinement (2Fo-Fc at 2.25σ). **c,** Electrostatic surface representation of the HDAC3/SMRT-DAD complex. A strikingly basic pocket is located at the HDAC3:SMRT-DAD interface (indicated by a dashed green line). The active site pocket of HDAC3 is indicated by a yellow cross. The Ins(1,4,5,6)P_4 is not shown for clarity. **d,** Ins(1,4,5,6)P_4 binding in the basic pocket at the HDAC3:SMRT-DAD interface. **e,** Detailed interactions of Ins(1,4,5,6)P_4 with HDAC3 (blue) and SMRT-DAD (grey).

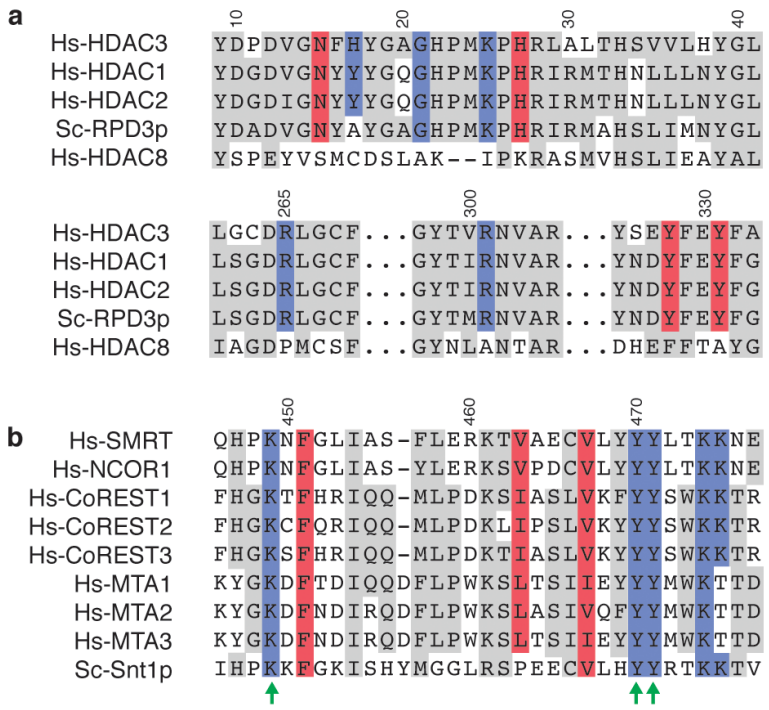

Figure 3. Sequence conservation suggests that class I HDACs, from yeast to man, require inositol phosphates for assembly and activation

a, Alignments of key regions of class I HDACs from H.sapiens and S.cerevisiae. Residues that mediate interaction with the Ins(1,4,5,6)P_4 and SMRT-DAD are highlighted in blue and red respectively. Other conserved residues are highlighted grey. **b,** Alignment of SANT domains from known interaction partners for class I HDACs. Residues that mediate interaction with the Ins(1,4,5,6)P_4 and HDAC3 are highlighted in blue and red respectively. Green arrows indicate residues that impair HDAC3 recruitment and activation when mutated to alanine[31].

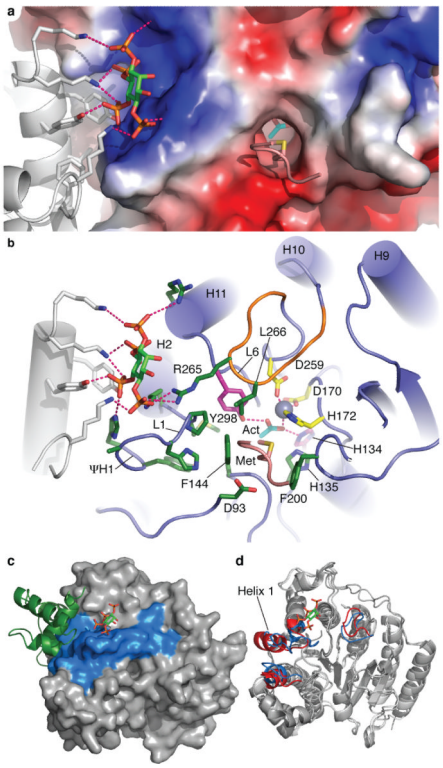

Figure 4. Mechanism of activation of HDAC3 by binding SMRT-DAD and Ins(1,4,5,6)P_4

a, The SMRT-DAD (grey cartoon) and the Ins(1,4,5,6)P_4 bind adjacent to the HDAC3 (charged surface representation) active site. Acetate and a methionine (lysine mimic) are located in the active site. **b,** Details of the HDAC3 active site. Key residues and loops are labelled – see text for details. **c,** Pseudo helix H1 and loops L1 and L6 are shown in blue on the surface of HDAC3. These regions are influenced / stabilised by SMRT-DAD and Ins(1,4,5,6)P_4 binding. **d,** Comparison of the structures of HDAC3 and HDAC8. Regions of significant difference are coloured blue (HDAC3) and red (HDAC8).

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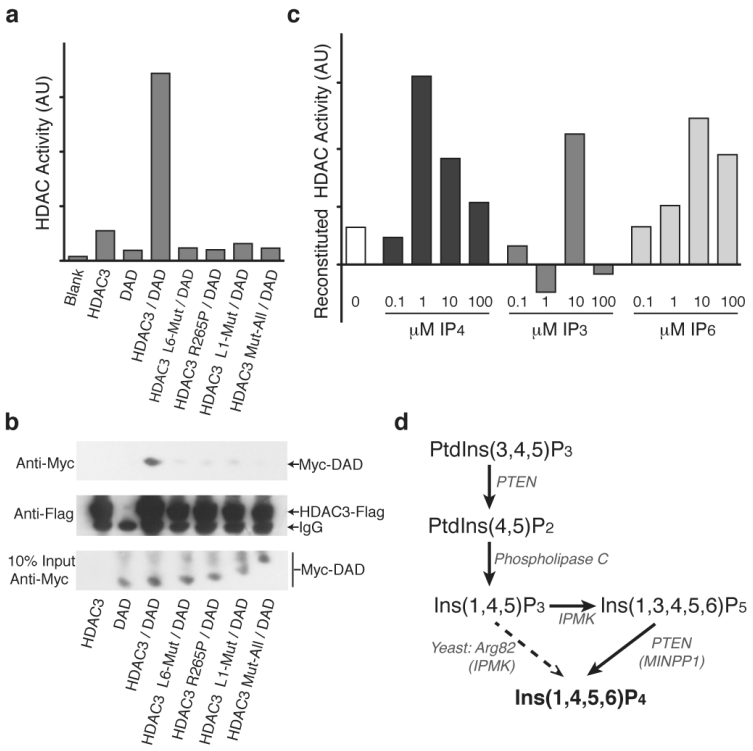

Figure 5. Exploring the role of Ins(1,4,5,6)P_4 in complex assembly and HDAC3 activation

a, Effect of HDAC3 mutations on deacetylase activity of complexes with SMRT-DAD. Residues are mutated to their equivalents in HDAC8. Loop 1 (L1) mutation is H17C/G21A/K25I. Loop 6 (L6) mutation is R264P/L265M. Mut-All mutant is H17C/G21A/K25I/R264P/L265M/R301A. **b,** Immunoblots showing that the HDAC3 mutations perturb interaction with the SMRT-DAD. **c,** Deacetylase assays of HDAC3-FLAG reconstituted with bacterially-expressed SMRT-DAD in the presence of various inositol phosphates. IP4, IP3 and IP6 are Ins(1,4,5,6)P_4, Ins(1,4,5)P_3 and Ins(1,2,3,4,5,6)P_6 respectively. **d**, Synthesis pathway for Ins(1,4,5,6)P_4 from PtdIns(3,4,5)P_3. Yeast Arg82 converts Ins(1,4,5)P_3 to Ins(1,4,5,6)P_4. In mammals, both IPMK and PTEN are required to make Ins(1,4,5,6)P_4.