# Histone Deacetylase 6 (HDAC6) Is an Independent Deacetylase for α-Tubulin

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**Abstract:** Histone deacetylase 6 (HDAC6) is a cytosolic enzyme that catalyzes deacetylation of several proteins. Acetylated tubulin has been recently identified as a physiological substrate of HDAC6. However in previous reports, all *in vitro* binding and enzymatic assays were accomplished with only partially purified protein samples. Therefore, it still remained unclear whether HDAC6 alone could interact with tubulin and catalyze deacetylation. In this study, both binding and enzymatic assays were conducted using recombinant-derived HDAC6 and purified α/β tubulin to eliminate possible contamination. The results clearly demonstrated that interaction between HDAC6 and tubulin is independent of other proteins. In addition, HDAC6 can independently catalyze deacetylation of both tubulin dimer and microtubule polymer.

**Keywords:** HDAC6, tubulin, deacetylase, co-immunoprecipitation, enzymatic activity.

## INTRODUCTION

Microtubule is one of the main cytoskeletal structures found in almost all eukaryotic cell types and is involved in a great variety of cellular processes. Reversible acetylation on the ε-amino group of Lys40 in α-tubulin is related to microtubule stability and possibly contributes to the regulation of microtubule dynamics [1, 2]. However, the enzymes responsible for the reversible acetylation were unknown until recently. The tubulin acetyltransferase from *CHLAMYDOMONAS* flagella has been partially purified [3] while histone deacetylase 6 (HDAC6) [1, 4, 5] and sirtuin 2 (SIRT2) [6] has been assigned to the deacetylation function.

Histone deacetylases are named after their function in the deacetylation of histone, which consequently represses active transcription. To date, 18 human proteins have been identified to be members of the HDAC family [7]. HDACs have attracted a lot of attention, not only for their role in controlling transcription, but also due to their diverse functions in many physiological processes, such as cell differentiation and growth [1]. HDAC6 was first discovered in mouse [8] and classified as a member of class II HDAC [7, 9]. Besides histone and α-tubulin, several other proteins have been identified as substrates of HDAC6, such as heat shock protein 90 (Hsp90) [10] and cortactin [11]. In addition to its enzymatic activity, HDAC6 also participates in ubiquitin degradation pathways, responsible for recruitment of polyubiquitinated proteins to dynein via its ubiquitin binding zinc finger domain. HDAC6 therefore facilitates formation of aggresome and consequently promotes clearance of misfolded protein [12] .

As HDAC6 is localized exclusively in cytoplasm [13, 14], histone is not the best physiological substrate. It was recently reported that HDAC6 could bind to and deacetylate α-tubulin both *in vivo* and *in vitro*, and knockdown of HDAC6 by siRNA did increase the acetylation level of tubulin [1, 4]. Such evidence clearly confirms that tubulin is one of the physiological substrates of HDAC6. However, due to the relatively poor purity of protein samples (HDAC6, tubulin, or both) used in reported assays, it was unknown whether other protein partners were required for HDAC6-tubulin interaction and the following catalysis. Interestingly, HDAC6 and SIRT2, the two tubulin deacetylases, can form a tubulin-binding complex [6, 15], indicating that the results based on only partially purified samples need careful inspection. In this study by using purified HDAC6 and tubulin samples, direct interaction between these two proteins was confirmed and it was concluded that HDAC6 alone deacetylated both α/β tubulin dimers and assembled microtubules.

## MATERIALS AND METHODS

### Proteins and Microtubule Assembly

Recombinant human HDAC6 (GenBank Accession No. BC069243) with N-terminal GST tag was purchased from Echelon Biosciences Inc. Rat α/β tubulin was purified from brain tissue as elsewhere described [16]. The α/β tubulin dimer in G-PEM buffer (1mM GTP, 80mM $K^+$-PIPES, PH6.9, 1mM EGTA and 1mM $MgCl_2$) was polymerized at 37°C for 1 hour, in the presence of 40μM taxol, to assemble microtubules.

### Co-Immunoprecipitation of HDAC6 and Tubulin

In order to evaluate interactions of HDAC6 and tubulin, two portions of ~500 μg of extracts from HEK-293T cells were respectively incubated overnight with two primary antibodies (rabbit anti-HDAC6 from Santa Cruz (Santa Cruz, USA), rabbit anti α-tubulin from abcam) at 4°C with gentle agitation. After this, 20μl of protein A/G Sepharose slurry (Santa cruz) was added and samples were incubated for 1 h

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at 4°C with gentle agitation. Beads were washed three times with IP buffer (20mM HEPES pH 7.4, 0.5mM EDTA, 150mM NaCl and 0.1% Triton X-100) and subsequently resuspended and boiled in loading buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For *in vitro* assays, 1μM purified α/β tubulin dimer and recombinant HDAC6 were used in stead of cell extract.

### Deacetylation Assays of Recombinant HDAC6

100μM rat brain α/β tubulin dimer and 0.1μM recombinant HDAC6 were incubated in 80mM $K^+$-PIPES (PH6.9) at 37°C overnight. 100μM purified α/β tubulin dimer in G-PEM buffer was polymerized as described above in the presence of 40μM taxol. 0.1μM HDAC6 was added to the taxol-stabilized microtubule and incubated overnight at 37°C.

10μM α/β tubulin dimer and 0.001μM HDAC6 in 80mM $K^+$-PIPES(PH6.9) was incubated at 37°C and sampled at 0, 15, 30, 60, 120, 240 minutes to perform a time course. The above reactions were stopped by boiling samples in loading buffer for SDS-PAGE.

### Western Blotting Analysis

Samples were resolved by electrophoresis on 8% SDS-polyacrylamide gels and subsequently blotted to nitrocellulose membranes (Bio-Rad). HDAC6, α-tubulin and acetylated α-tubulin were detected by rabbit anti-HDAC6 (Santa cruz), mouse anti-α-tubulin (Sigma) and anti-acetylated α-tubulin (abcam) antibodies respectively.

## RESULTS AND DISCUSSION

### HDAC6 Directly Interacts with Tubulin

Interaction between HDAC6 and tubulin has been confirmed by co-immunoprecipitation with either cell extract or *in vitro* translated protein samples [1]. Due to the impurity of such materials, it remained unknown whether the interaction required other proteins as cofactors. In order to eliminate contamination, purified rat α/β tubulin dimer and recombinant HDAC6 were tested in co-immunoprecipitation assay (Fig. **1C**). Meanwhile, lysate of HEK-293T cells was examined as a control (Fig. **1B**). In both cases, HDAC6 and tubulin were able to co-precipitate, demonstrating their interactions. This is the first time to successfully reconstitute the HDAC6-tubulin complex using pure components. Such results clearly excluded the requirement of adaptor proteins between HDAC6 and tubulin. In contrast, another tubulin deacetylase SIRT2 might require HDAC6 for effective interaction with tubulin [15].

### Recombinant Human HDAC6 Deacetylates Purified α/β Tubulin Dimers and Assembled Microtubule

Although acetylated α-tubulin was identified as a substrate of HDAC6 deacetylase both *in vivo* and *in vitro* [4, 5], it remained unclarified whether accessory proteins were needed for HDAC6 deacetylase activity. In addition, there was debate on whether α/β tubulin dimer is a suitable substrate of HDAC6, as contrary results had been reported [4, 6]. To answer the above questions, recombinant HDAC6 and purified tubulin were examined in the deacetylation assay. Obviously, HDAC6 alone was able to catalyze deacetylation of α-tubulin, in both polymerized and unpolymerized forms (Fig. **2A**). In order to confirm that the deacetylation of α/β tubulin dimer was not an experimental artifact, a time course assay was conducted (Fig. **2B**). After addition of HDAC6, there was a lag phase until 60 minutes, after which α tubulin began to be deacetylated. The acetylation level was moderately reduced at 120 minutes and approached a minimum at 240 minutes. Therefore, the deacetylation under unpolymerized condition was not just an incident. This certainly promoted a careful comparison of the previous [4, 6] and present studies. The following factors could attribute to the apparently different efficiency of deacetylation: 1. Recombinant and purified HDAC6, which was used in this study, might lack some cofactors in partially purified HDAC6 that modulate deacetylation. This was also supported by the re-

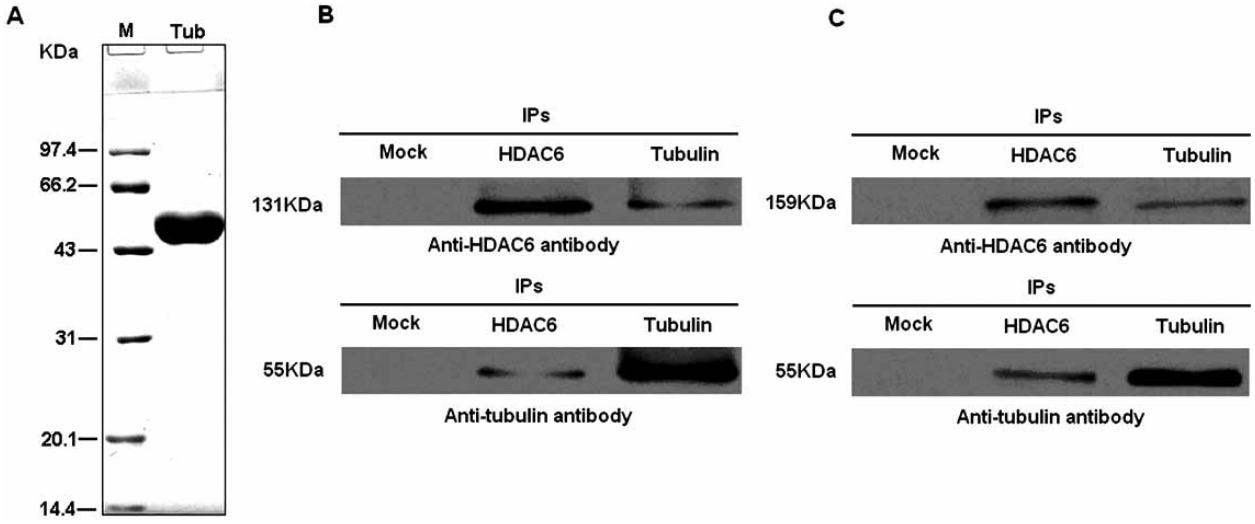


**Figure 1. HDAC6 binds to tubulin. (A)** Tubulin purity is assessed by SDS-gel electrophoresis. **(B)** Co-immunoprecipitation experiments in HEK-293T cell extracts. HDAC6 and tubulin were detected by rabbit anti-HDAC6 antibody (upper) and mouse anti-α-tubulin antibody (lower). **(C)** Co-immunoprecipitation experiments in recombinant HDAC6 and purified α/β tubulin dimer. Recombinant HDAC6 and rat α/β tubulin were detected as in **(B)**.

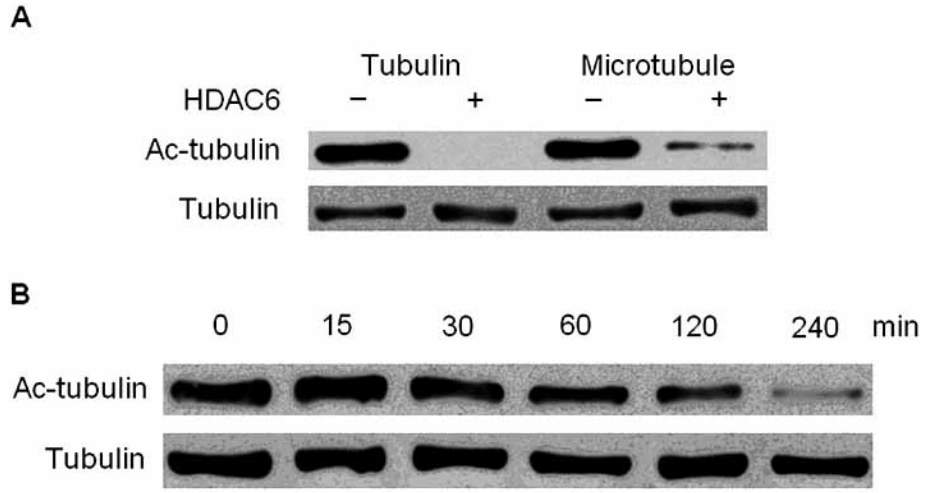


**Figure 2. Deacetylation of purified rat brain α/β tubulin dimer by recombinant human HDAC6. (A)** Recombinant HDAC6 deacetylates both purified α/β tubulin dimer and assembled microtubule. The amounts of acetylated and total α/β tubulin were determined by immunoblotting with anti-acetylated α-tubulin (upper) and anti-α-tubulin(lower) antibodies. **(B)** Time course experiment of deacetylation of depolymerized α/β tubulin dimer. The amounts of acetylated and total α/β tubulin were determined as in **(A)**.

duced efficiency of HDAC6 when deacetylation of tubulin and microtubule was performed in the presence of cell lysate [6]. 2. Different source and preparation of tubulin could acquire significant change in its acetylation levels and might affect the following deacetylation efficiency. 3. In both previous studies, deacetylation of tubulin by HDAC6 was examined only at 2 hours (120 minutes) instead of a time course. Due to different enzyme and substrate concentrations, the length of the lag phase might vary significantly so that opposite results in deacetylation have been observed.

It is also worth noticing that the deacetylation of unpolymerized tubulin is more complete (Fig. **2A**), while the initial rate could be relatively slower when compared to MAP-stabilized microtubule [4]. Whether polymerization of tubulin or MAP proteins are responsible for promoting initial deacetylation still needs further investigation, while such promotion is most likely attributed to better interactions between HDAC6 and its polymerized substrate. This hypothesis is supported by the behavior of tubulin dimer, which showed a lag phase in deacetylation most likely due to relatively poorer binding to HDAC6. However once bound to HDAC6, it is more prone to deacetylation as steric hindrance is not likely a problem in the unpolymerized form (Fig. **2A**).

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