

RESEARCH ARTICLE

SIRT2 inactivation reveals a subset of hyperacetylated perinuclear microtubules inaccessible to HDAC6

Renate Hvidsten Skoge and Mathias Ziegler*

ABSTRACT

Deacetylation of α -tubulin at lysine 40 is catalyzed by two enzymes, the NAD-dependent deacetylase SIRT2 and the NAD-independent deacetylase HDAC6, in apparently redundant reactions. In the present study, we tested whether these two enzymes might have distinguishable preferences for the deacetylation of different microtubule structures. Using various agents, we induced tubulin hyperacetylation and analyzed the ensuing formation of distinct microtubule structures. HDAC6 inhibition led to general hyperacetylation of the microtubule network throughout the cell, whereas hyperacetylation induced by SIRT2 inactivation was limited to perinuclear microtubules. Hyperacetylation of these perinuclear microtubules was undiminished following HDAC6 overexpression, whereas reactivation of SIRT2 restored the basal acetylation level and a normal microtubule network. By contrast, SIRT2 and HDAC6 acted similarly on the morphologically different, hyperacetylated microtubule structures induced by taxol, MAP2c overexpression or hyperosmotic stress. These results indicate overlapping and distinct functions of HDAC6 and SIRT2. We propose that the differential activity of the two deacetylases, which target the same acetylated lysine residue, might be related to the recognition of specific structural contexts.

KEY WORDS: SIRT2, HDAC6, Tubulin deacetylation, Microtubules

INTRODUCTION

A multitude of biological processes are regulated by reversible acetylation of lysine residues in proteins. Although the acetyl group is generally transferred from acetyl-CoA to the acceptor site by acetyltransferases, its removal is catalyzed by deacetylases from two families using different catalytic principles. The ‘classical’ histone deacetylases (HDACs, class I and II) perform deacetylation by hydrolytic cleavage resulting in the release of free acetate (de Ruijter et al., 2003). In the deacetylation reaction catalyzed by sirtuins (HDAC class III), nicotinamide adenine dinucleotide (NAD) is required as a co-substrate and is degraded in the process (Sanders et al., 2010) (Fig. 1A).

Interestingly, there is an overlap in the selectivity of sirtuins and the classical HDACs with regard to their acetylated target sites. A prominent example is the microtubule component α -tubulin, which is acetylated on lysine residue 40. Deacetylation of this residue is catalyzed by both the NAD-dependent SIRT2 and the NAD-independent HDAC6 (Hubbert et al., 2002; North et al., 2003). There

is also a considerable overlap in nuclear deacetylating activities. For example, the tumor suppressor p53 can be deacetylated by SIRT1, HDAC1, HDAC2 and HDAC3 (Luo et al., 2001; Vaziri et al., 2001; Juan et al., 2000; Ito et al., 2002; Wagner et al., 2014), and histone H4 lysine 16 is deacetylated by both SIRT1, SIRT2 and HDAC2 (Vaquero et al., 2004, 2006; Ma and Schultz, 2013). That is, there is a redundancy of NAD-dependent and -independent deacetylating enzymes for a variety of acetylated sites. Given that the availability of NAD is a prerequisite for the sirtuin-dependent reactions, it might be a decisive factor for the regulation of NAD-dependent deacetylations. Indeed, the enzymatic activity of SIRT1 has been linked to fluctuations in cellular NAD levels in several studies (Revollo et al., 2004; Fulco et al., 2008; Canto et al., 2009). Moreover, the acetylation state of α -tubulin depends on cellular NAD levels, as it is, in part, mediated by the NAD-dependent SIRT2 (Skoge et al., 2014). However, why NAD-dependent and -independent deacetylations take place on the same residues has remained unclear. It appears reasonable to consider that, perhaps, different deacetylases might recognize their substrates depending on the context. For example, chromatin deacetylation by SIRT1, HDAC1, HDAC2 and HDAC3 takes place in a rather complex structural environment. Therefore, it seems plausible to suggest that the different enzymes deacetylate specific histone residues depending on the structural context or the modification state of surrounding residues. Similarly, microtubules are the building blocks of a variety of stable and dynamic cellular structures with distinct morphologies that are also subjected to several different post-translational modifications. Potentially, these different microtubule environments could create distinguishable subsets of acetylated α -tubulin that could be selectively recognized and deacetylated by either HDAC6 or SIRT2. Thereby, deacetylases could exert different biological roles even though they target the same modified lysine residue in α -tubulin. It has been suggested that HDAC6 and SIRT2 act in a complex to perform the deacetylation of α -tubulin (North et al., 2003). However, at least *in vitro* both enzymes are able to deacetylate this residue independently (North et al., 2003; Hubbert et al., 2002). It has also been proposed that the occurrence of hyperacetylated microtubule structures during cell division, the mitotic spindle and the midbody, could be ascribed to the selective inhibition of SIRT2 by the human Furry homolog, Fry (Nagai et al., 2013). Simultaneous inhibition of SIRT2 and HDAC6 causes a stronger hyperacetylation of tubulin than achieved with either treatment alone (Skoge et al., 2014) further indicating that the two enzymes might target overlapping, but not identical microtubule populations.

In the present study, we addressed the hypothesis that HDAC6 and SIRT2 might act on different subsets of acetylated tubulin depending on the structural environment of the microtubules. Several different means of inducing α -tubulin hyperacetylation resulting in distinct microtubule morphologies were employed in combination with overexpression or inhibition of the two deacetylases. We found that SIRT2 and HDAC6 exhibit a substantial degree of functional overlap

Department of Molecular Biology, University of Bergen, Postbox 7803, Bergen 5020, Norway.

*Author for correspondence (mathias.ziegler@uib.no)

 M.Z., 0000-0001-6961-2396

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with regard to deacetylation of hyperacetylated microtubule structures induced by taxol, hyperosmotic stress or overexpression of the microtubule-associated protein MAP2c. However, we identified perinuclear microtubules that are hyperacetylated upon SIRT2 inhibition. HDAC6 did not influence the acetylation level of these hyperacetylated structures. These results reveal a SIRT2-specific subset of perinuclear acetylated α -tubulin that cannot be deacetylated by HDAC6.

RESULTS

Inhibition of SIRT2 leads to the formation of hyperacetylated perinuclear microtubules

The tubulin acetylation state reflects the balance of acetylating and deacetylating activities. Therefore, selective modulation of HDAC6 or SIRT2 activities could be used to reveal their contributions to the regulation of different microtubule structures. First, we made use of several pharmacological treatments that are known to induce the formation of distinct, hyperacetylated microtubule structures. As shown in Fig. 1B, the tubulin acetylation levels in HeLa cells cultivated under standard conditions were relatively low (Fig. 1B, medium). Addition of the specific HDAC6 inhibitor tubacin (Fig. 1A) increased the level of tubulin acetylation throughout the cell without affecting the morphology of the microtubule network (Fig. 1B). In contrast, exposure to the cytostatic drug taxol led to the assembly of sharp wedge-like hyperacetylated microtubule structures (Fig. 1B), whereas overexpression of the neuronal microtubule-associated protein MAP2c (an isoform of MAP2) led to a drastic reorganization of the microtubules into long hyperacetylated bundles (Fig. 1B). Incubation of the cells with a hyperosmotic NaCl solution triggered only a moderate increase in tubulin acetylation. However, this treatment caused microtubule disintegration as indicated by the rather diffuse distribution of tubulin (Fig. 1B) (Croom et al., 1986).

SIRT2 deacetylates the same lysine residue in α -tubulin as HDAC6. Therefore, inhibition of this NAD-dependent enzyme would be expected to result in a similar increase in tubulin acetylation as seen following tubacin treatment. However, treatment with the SIRT2 inhibitor AGK2 (Fig. 1A) only led to hyperacetylation of perinuclear microtubules (Fig. 1C). FK866, an inhibitor of the NAD biosynthetic enzyme NamPRT (also known as NAMPT), leads to NAD depletion in cells that rely on nicotinamide as NAD precursor. We have previously shown that FK866-induced NAD depletion results in indirect SIRT2 inactivation and gives rise to tubulin hyperacetylation (Skoge et al., 2014) (Fig. 1A). In fact, FK866-induced indirect inactivation of SIRT2 led to the formation of hyperacetylated perinuclear microtubules similar to the hyperacetylated structures observed upon treatment of cells with AGK2 (Fig. 1C).

These observations indicate that inhibition of SIRT2-dependent, but not HDAC6-dependent tubulin deacetylation evokes the formation of morphologically distinct, hyperacetylated microtubules. Consequently, despite the same molecular site of action, the two deacetylases might exert their activity in different structural contexts.

Hyperacetylated perinuclear microtubules are resistant to microtubule rearrangement

Taxol drastically affects the assembly of microtubules, whereas microtubules that are already assembled are only slightly influenced by the drug (Arnal and Wade, 1995). Hence, only dynamic microtubules are visibly rearranged upon taxol exposure. We

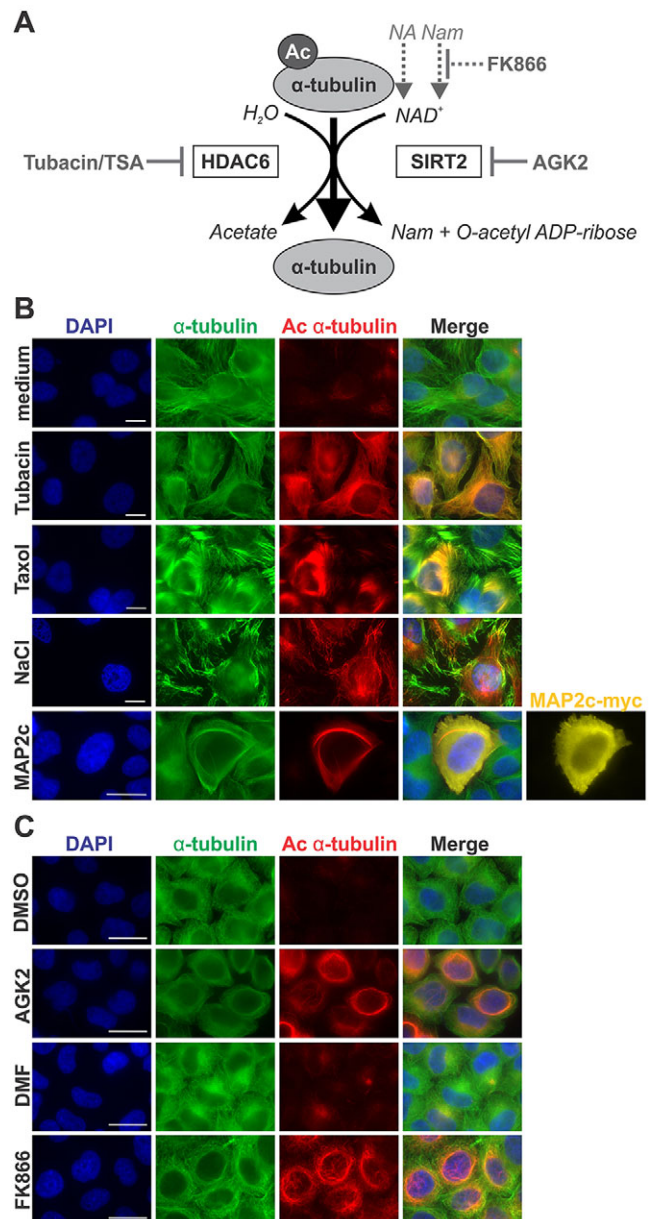


Fig. 1. Different means of inducing tubulin hyperacetylation lead to the formation of distinct microtubule structures. (A) Schematic overview of the enzymes involved in tubulin deacetylation. Tubulin is deacetylated by HDAC6 and SIRT2. HDAC6-mediated deacetylation results in the release of acetate, and the activity can be inhibited by the specific HDAC6 inhibitor tubacin, or the general HDAC class I and II inhibitor Trichostatin A (TSA). SIRT2-mediated deacetylation requires NAD⁺ as a co-substrate and results in the release of nicotinamide (Nam) and O-acetyl ADP-ribose. SIRT2 can be inhibited by the selective SIRT2 inhibitor AGK2, or inactivated through NAD⁺ depletion. NAD⁺ depletion can be achieved through inhibition of NamPRT by FK866. NamPRT is an NAD-biosynthetic enzyme that converts the NAD precursor nicotinamide into nicotinamide mononucleotide. FK866-induced NAD depletion can be bypassed through addition of the NamPRT-independent NAD precursor nicotinic acid (NA). (B) Different microtubule morphologies are detected in HeLaS3 cells cultured in either standard culture medium, or 6 h after treatment with 5 μ M tubacin or 5 μ M taxol, or 1 h after treatment with 0.25 M NaCl. MAP2c overexpression (30 h) was monitored by immunocytochemical detection of the C-terminal Myc tag. (C) SIRT2 inhibition leads to formation of hyperacetylated perinuclear microtubules. HeLaS3 cells were treated with 100 μ M AGK2 or the solvent dimethyl sulfoxide (DMSO) for 6 h, or with 2 μ M FK866 or the solvent dimethyl formamide (DMF) for 48 h. Ac α -tubulin, acetylated α -tubulin. Scale bars: 15 μ m.

combined taxol treatment with HDAC6 or SIRT2 inhibition to assess whether this drug would still cause formation and hyperacetylation of the typical microtubule structures observed in taxol-treated cells. As shown in Fig. 2A, tubacin caused a much stronger tubulin hyperacetylation than taxol. However, when taxol and tubacin were added simultaneously, the strong tubulin hyperacetylation induced by tubacin alone was prevented (Fig. 2A,B), and the microtubules were rearranged into wedge-like structures similar to those seen in cells exposed to taxol alone (Fig. 2B). When cells were pre-treated with tubacin before taxol addition, the tubulin acetylation levels increased (Fig. 2A). However, the microtubules were still rearranged into wedge-like structures (Fig. 2B). Given their rearrangement following taxol exposure, the hyperacetylated tubulin population observed upon HDAC6 inhibition appears to belong to a subset of dynamic microtubules.

In contrast, when taxol was combined with FK866-induced inactivation of SIRT2, the tubulin acetylation level was higher than for either treatment alone (Fig. 2C,D). Moreover, in FK866-treated cells, hyperacetylated tubulin was not rearranged into wedges by taxol exposure, but rather retained their perinuclear distribution, although the structures appeared somewhat sharpened (Fig. 2D). Given that the hyperacetylated perinuclear tubulin population resisted taxol-induced rearrangement, it might represent a subset of more stable rather than dynamic microtubules.

Hyperacetylated perinuclear microtubules are partially resistant to cold-induced depolymerization

Cold-induced microtubule depolymerization is accompanied by tubulin deacetylation. If the hyperacetylated perinuclear tubulin population represents a more stable subset of microtubules, it might be less prone to depolymerization upon cold exposure. In accordance with this hypothesis, we found that the microtubules in FK866-treated cells were only partially depolymerized and deacetylated when cells were incubated for 15 min on ice (Fig. 3A; green and red channel, respectively). Addition of nicotinic acid restores the NAD level in FK866-treated cells and, in turn, reactivates SIRT2 (Skoge et al., 2014) (Fig. 1A). Indeed, cells that were treated with nicotinic acid after pre-incubation with FK866 exhibited a normal arrangement of microtubules (Fig. 3A, green channel) and the microtubules were depolymerized and deacetylated when exposed to cold, similar to control-treated cells (Fig. 3A, green and red channel, respectively). Therefore, the observed stability of the hyperacetylated perinuclear microtubules in FK866-treated cells was a direct consequence of SIRT2 inactivation.

Taxol stabilizes microtubules and prevents their depolymerization and deacetylation during cold treatment (Schiff and Horwitz, 1980; Haggarty et al., 2003). Combining FK866 treatment with taxol exposure led to further stabilization of the microtubules, along with preservation of the hyperacetylation levels, similar to cells only incubated with taxol (Fig. 3B, green and red channel, respectively). HDAC6 inhibition has been reported to have no influence on microtubule dynamics (Haggarty et al., 2003). Indeed, in the presence of tubacin the microtubules were still depolymerized when exposed to cold (Fig. 3C, green channel), even though tubulin hyperacetylation was preserved (Fig. 3C, red channel).

HDAC6 does not deacetylate hyperacetylated perinuclear microtubules

Next, we tested whether increased HDAC6 activity would affect the acetylation level of the FK866-induced perinuclear microtubules.

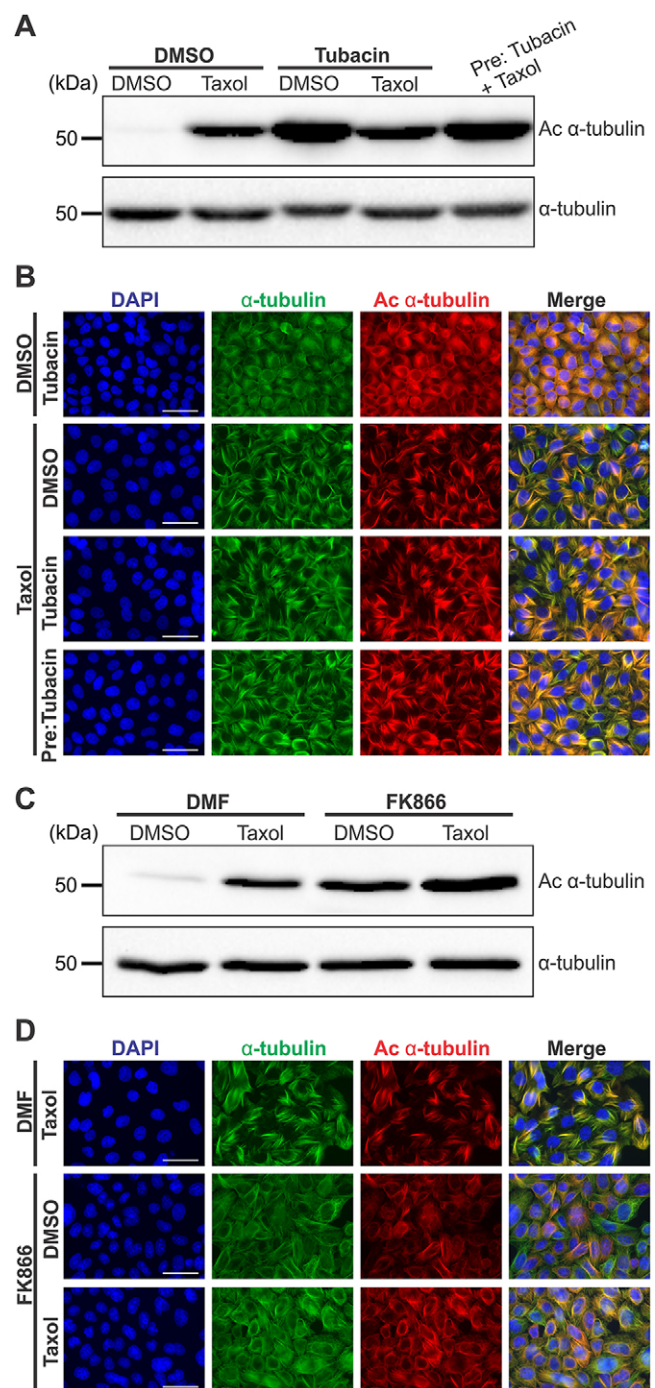


Fig. 2. Taxol differentially influences tubulin hyperacetylation induced by HDAC6 or SIRT2 inhibition. (A) Taxol attenuates tubacin-induced tubulin hyperacetylation. HeLaS3 cells were treated for 6 h with 5 μ M tubacin or DMSO, in combination with either 5 μ M taxol or DMSO. Pre, pre-treatment with 5 μ M tubacin for 2 h before addition of 5 μ M taxol for 4 h (total 6 h). Loading control, α -tubulin. (B) Taxol rearranges microtubules that are hyperacetylated as a result of HDAC6 inhibition. HeLaS3 cells were treated for 6 h with 5 μ M tubacin or DMSO, in combination with either 5 μ M taxol or DMSO. Pre, pre-treatment with 5 μ M tubacin for 2 h before addition of 5 μ M taxol for 4 h (total 6 h). (C) Taxol increases FK866-induced tubulin hyperacetylation. HeLaS3 cells were treated with 2 μ M FK866 or DMF for 48 h, or with 5 μ M taxol or DMSO, added to the cell culture medium 6 h before lysis. Loading control, α -tubulin. (D) SIRT2 inactivation precludes taxol-induced rearrangement of microtubules. HeLaS3 cells were treated with 2 μ M FK866 or DMF for 48 h, or 5 μ M Taxol or DMSO, was added to the cell culture medium 6 h before fixation. Scale bars: 30 μ m. Ac- α -tubulin, acetylated α -tubulin.

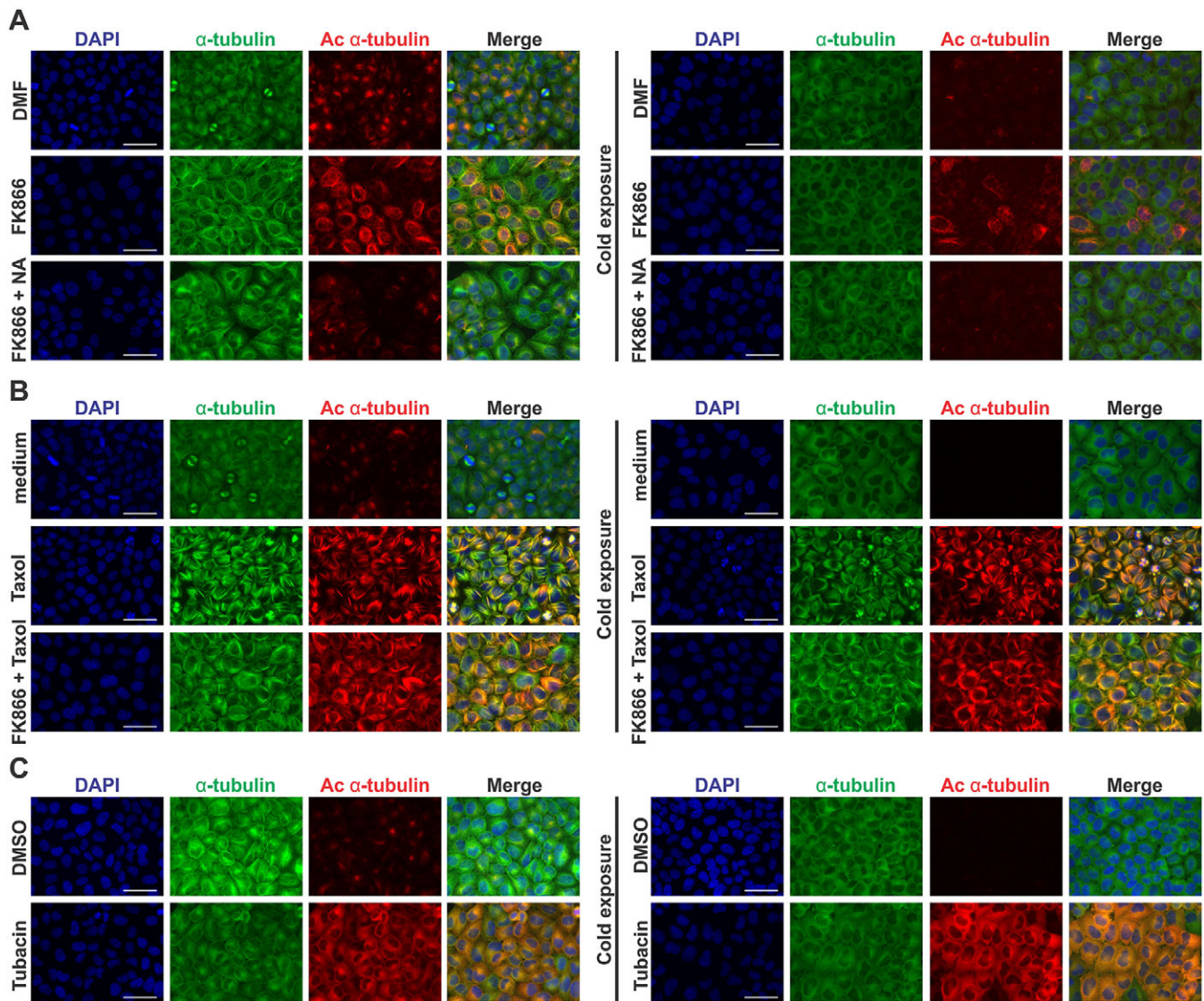


Fig. 3. Modulation of cold-induced depolymerization and deacetylation of microtubules by deacetylase inhibitors and taxol. (A) Perinuclear hyperacetylation partially remains after cold exposure of FK866-treated cells. HeLaS3 cells were subjected to the cold-induced depolymerization assay 48 h after treatment with 2 μ M FK866 or DMF. 100 μ M nicotinic acid (NA) was added to the cell culture medium 6 h before the start of the assay when indicated. The brightness is increased for acetylated α -tubulin in the panels for cold exposure. (B) Taxol stabilizes perinuclear microtubules and prevents deacetylation. HeLaS3 cells were subjected to the cold-induced depolymerization assay 6 h after treatment with 5 μ M taxol, or 48 h after treatment with 2 μ M FK866 with 5 μ M taxol added to the cell culture medium 6 h before the start of the assay. (C) General tubulin hyperacetylation does not affect the cold sensitivity of microtubules. HeLaS3 cells were subjected to the cold-induced depolymerization assay 6 h after treatment with 5 μ M tubacin or DMSO. Ac α -tubulin, acetylated α -tubulin. Scale bars: 30 μ m.

As can be inferred from Fig. 4A (right panel), cells overexpressing HDAC6 did not change the FK866-induced perinuclear hyperacetylation. To verify the tubulin deacetylase activity of overexpressed HDAC6 under these experimental conditions, we used two concentrations of the general HDAC class I and II inhibitor trichostatin A (TSA). At the lower TSA concentration, only the endogenous HDAC6 activity was inhibited, whereas at a higher TSA concentration the activity of the overexpressed HDAC6 was also inhibited (Fig. 4A, left panel). In HDAC6-overexpressing cells co-treated with both FK866 and a high concentration of TSA, both perinuclear and general hyperacetylation of tubulin was observed (Fig. 4A, right panel). However, when cells were co-treated with FK866 and a low concentration of TSA, only perinuclear hyperacetylation remained in HDAC6-overexpressing cells (Fig. 4A). These findings demonstrate that although

overexpressed HDAC6 is catalytically active under these conditions, HDAC6 is unable to reduce the acetylation level of the perinuclear microtubules induced by SIRT2 inactivation. Hence, perinuclear microtubules represent a subset of acetylated tubulin that is inaccessible to HDAC6.

Reactivated SIRT2 deacetylates and rearranges the hyperacetylated perinuclear microtubules

To verify that SIRT2 is able to deacetylate the perinuclear microtubules, we analyzed the acetylation level of these structures after reactivation of SIRT2. Indeed, when NAD levels in FK866-treated cells were recovered by addition of nicotinic acid, both the acetylation level and the morphology of the microtubule network returned to that of control cells (Fig. 4B). Interestingly, this normalization of the microtubule network also occurred when

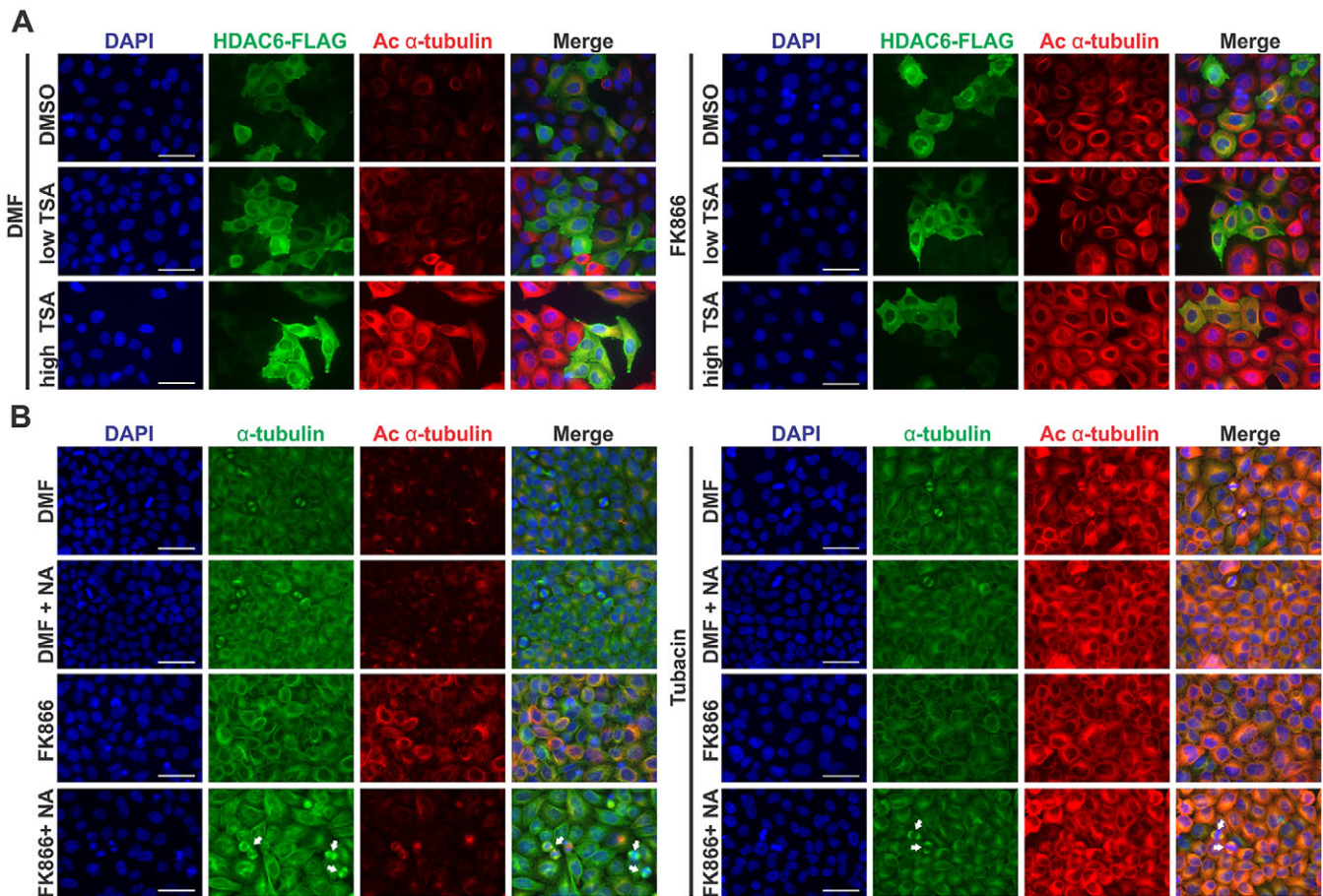


Fig. 4. HDAC6 does not deacetylate hyperacetylated perinuclear microtubules. (A) HDAC6 overexpression does not reduce the acetylation level of perinuclear microtubules. HeLaS3 cells were treated with 2 μ M FK866 or DMF for 48 h, or with 100 nM TSA (low), 4 μ M TSA (high) or DMSO, added to the cell culture medium 6 h before fixation, as indicated. HDAC6 overexpression (72 h) was monitored by immunocytochemical detection of the C-terminal Flag tag. (B) Reactivation of SIRT2 reverses the FK866-induced perinuclear tubulin hyperacetylation independently of HDAC6 activity. HeLaS3 cells were treated with 2 μ M FK866 or DMF for 54 h, in combination with 100 μ M nicotinic acid (NA) and/or 5 μ M tubacin added to the cell culture medium 6 h before fixation, as indicated. The re-appearance of mitotic structures is marked by white arrows. Scale bars: 30 μ m. Ac α -tubulin, acetylated α -tubulin.

HDAC6 was inhibited (Fig. 4B, lower right panel, green channel). These observations indicate that only (re)activation of SIRT2 can reverse the perinuclear hyperacetylation of tubulin and that this process is independent of the activity of HDAC6. In addition, we noted the re-appearance of mitotic structures for FK866-treated cells that were recovered by treatment with nicotinic acid (Fig. 4B, lower panels, white arrows).

SIRT2 depletion promotes the formation of hyperacetylated perinuclear microtubules

The occurrence of hyperacetylated perinuclear microtubules, following incubation of cells with AGK2 or FK866 (Fig. 1C), strongly suggested that this phenomenon was brought about by diminished SIRT2 activity. However, it cannot be excluded that the pharmacological inhibitors used might have affected other NAD-dependent mechanisms. To establish whether the perinuclear microtubule hyperacetylation was indeed due to diminished SIRT2 activity, we treated cells with small interfering RNA (siRNA) targeting SIRT2 mRNA. As shown in Fig. 5A, this treatment resulted in a substantial reduction of SIRT2 expression. The detection of multiple SIRT2 bands is not unexpected as it reflects the presence of two isoforms that are differentially phosphorylated (Dryden et al., 2003; North and Verdin, 2007). Knockdown of SIRT2 resulted in a general increase of α -tubulin

acetylation (Fig. 5A, upper panel). This tubulin hyperacetylation was primarily associated with perinuclear microtubule structures (Fig. 5B). Treatment with FK866 promoted the hyperacetylation of perinuclear microtubules in control cells, as expected, and it further increased the hyperacetylation of these structures in SIRT2-knockdown cells (Fig. 5C). Importantly, only in control cells was the FK866-induced perinuclear hyperacetylation reversed by nicotinic acid addition. In the SIRT2-knockdown cells, hyperacetylation of perinuclear microtubules persisted even when nicotinic acid was added (Fig. 5C). These observations demonstrate that SIRT2 downregulation (by siRNA, AGK2 or FK866 treatment) is necessary and sufficient to induce hyperacetylation of perinuclear microtubules.

Both tubulin deacetylases act similarly on other hyperacetylated microtubule structures

Finally, we addressed the question as to whether HDAC6 and SIRT2 also exhibit differences in the deacetylation of other microtubule structures. Co-treatment of cells with taxol and HDAC6 inhibitor did not lead to an additive increase in tubulin acetylation (Fig. 2A,B). Therefore, it might be that HDAC6 is not able to deacetylate tubulin in taxol-induced microtubule structures. However, both HDAC6 and SIRT2 overexpression similarly reduced the appearance of taxol-induced

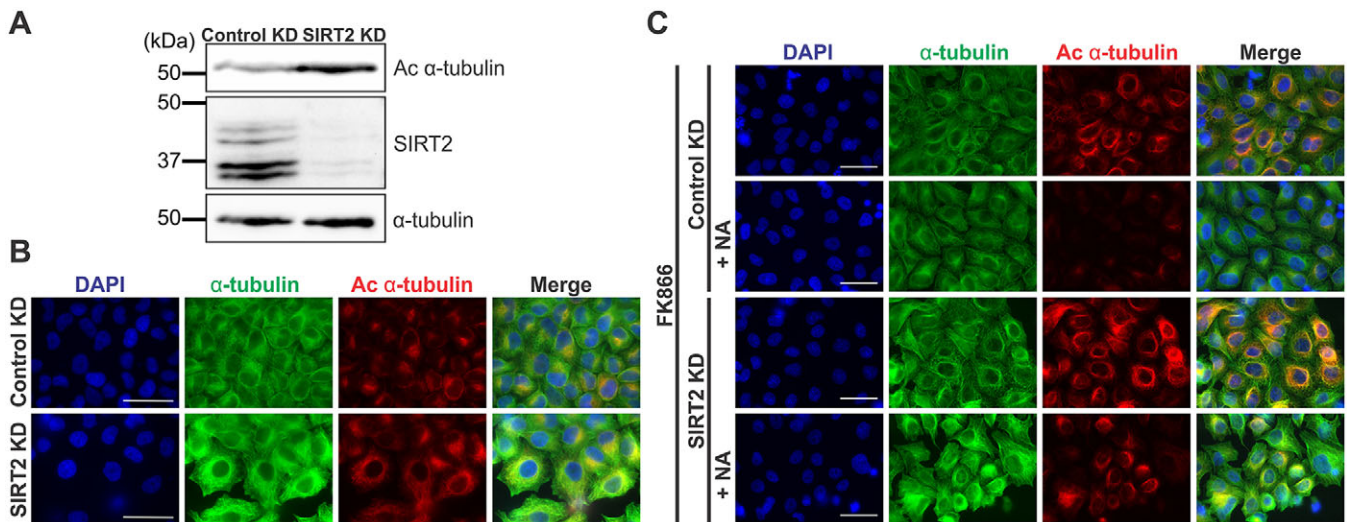


Fig. 5. SIRT2 is responsible for the deacetylation of perinuclear microtubules. (A) siRNA-mediated SIRT2 knockdown reduces the level of SIRT2 and leads to a moderate increase in tubulin acetylation. HeLaS3 cells were transiently transfected with siRNA directed against SIRT2 (SIRT2 KD) or control siRNA (control KD) and incubated for 72 h before immunoblot analysis. Loading control, α -tubulin. (B) SIRT2 knockdown results in an increase in tubulin acetylation predominantly in the perinuclear region. HeLaS3 cells were transiently transfected with siRNA directed against SIRT2 (SIRT2 KD) or control siRNA (control KD) and incubated for 72 h. (C) FK866-induced perinuclear tubulin hyperacetylation is only partially reversed by nicotinic acid (NA) upon siRNA-mediated SIRT2 knockdown. HeLaS3 cells were treated with 2 μ M FK866 24 h after transient transfection with siRNA directed against SIRT2 (SIRT2 KD) or control siRNA (control KD). At 48 h after the addition of FK866, 100 μ M nicotinic acid was added to the cell culture medium and the cells were incubated for another 6 h before fixation, as indicated. Scale bars: 30 μ m. Ac α -tubulin, acetylated α -tubulin.

hyperacetylated wedge-like microtubule structures (Fig. 6A). Likewise, overexpression of SIRT2 or HDAC6 also diminished the tubulin hyperacetylation of disintegrating microtubules induced by hyperosmotic concentrations of NaCl (Fig. 6B). Hence, both deacetylases seemed comparably capable of deacetylating wedge-like and disintegrating microtubules.

The perinuclear distribution of hyperacetylated tubulin induced by SIRT2 inactivation resembles to some extent the localization of the microtubule bundles induced by MAP2c overexpression. However, expression of neither SIRT2 nor HDAC6 affected tubulin hyperacetylation of microtubule bundles induced by MAP2c expression (Fig. 6C). Thus, it appears that the acetylated tubulin residues in these microtubule bundles are not accessible to either enzyme. Moreover, when SIRT2 or HDAC6 inhibition was combined with MAP2c overexpression, the cells exhibited the same level of microtubule bundle hyperacetylation as control-treated cells (Fig. 6D), indicating that the bundles have saturated tubulin acetylation levels. However, unlike the perinuclear microtubule structures induced by SIRT2 inactivation, MAP2c-induced bundles were still rearranged by taxol exposure into more wedge-like structures (Fig. 6D, cf. Fig. 2D). These findings demonstrate that the perinuclear hyperacetylated microtubules are also functionally different from both wedge-like microtubule structures and microtubule bundles.

DISCUSSION

The present study has revealed functional overlaps and differences between the two deacetylases that regulate the acetylation state of α -tubulin, HDAC6 and SIRT2. Both enzymes act in concert on various distinct microtubule structures (Table 1). Remarkably, inhibition of SIRT2 has revealed hyperacetylated perinuclear tubulin structures that are not a substrate for HDAC6-dependent deacetylase activity. This functional distinction regarding the substrate specificities of the two deacetylases has been corroborated by several observations. First, inactivation or

inhibition of SIRT2 leads to perinuclear hyperacetylation of microtubules. Second, overexpression of HDAC6 does not reduce the perinuclear tubulin acetylation level. Third, the perinuclear tubulin hyperacetylation induced by NAD depletion is reversed upon SIRT2 reactivation, demonstrating that only SIRT2 performs the deacetylation of this subset of microtubules. These findings imply that the formation and acetylation of these perinuclear microtubules is regulated by SIRT2 and that these structures are apparently inaccessible to HDAC6. By contrast, both SIRT2 and HDAC6 reduce the hyperacetylation of microtubules induced by other means, including taxol treatment and hyperosmotic stress. However, neither of the two deacetylases has been found to be capable of removing the modifications on hyperacetylated microtubule bundles induced by MAP2c overexpression. Consequently, perinuclear microtubule hyperacetylation induced by SIRT2 inhibition appears to be functionally distinct from tubulin hyperacetylation induced by taxol or MAP2c.

HDAC6 has been considered to be the major tubulin deacetylase (Haggarty et al., 2003). The results of the present study support this notion. Loss of tubulin acetylation observed during cold-induced microtubule depolymerization is abolished upon HDAC6 inhibition indicating that the massive deacetylation accompanying depolymerization is mainly catalyzed by HDAC6. HDAC6 activity is positively regulated by farnesyltransferase, a protein which binds to the C-terminus of α -tubulin (Zhou et al., 2009). Thus, HDAC6 activity is indirectly stimulated by the C-terminal tails of tubulin that are protruding from the outer surface of assembled microtubules, in contrast to the luminal position of α -tubulin lysine 40. This stimulation could possibly account for the strong coupling between tubulin deacetylation and microtubule depolymerization, given that the C-terminus and lysine 40 are accessible together in the same local environment in depolymerized tubulin.

In contrast, only perinuclear acetylation remains upon cold exposure when SIRT2 is inactivated. It is possible that HDAC6 acts

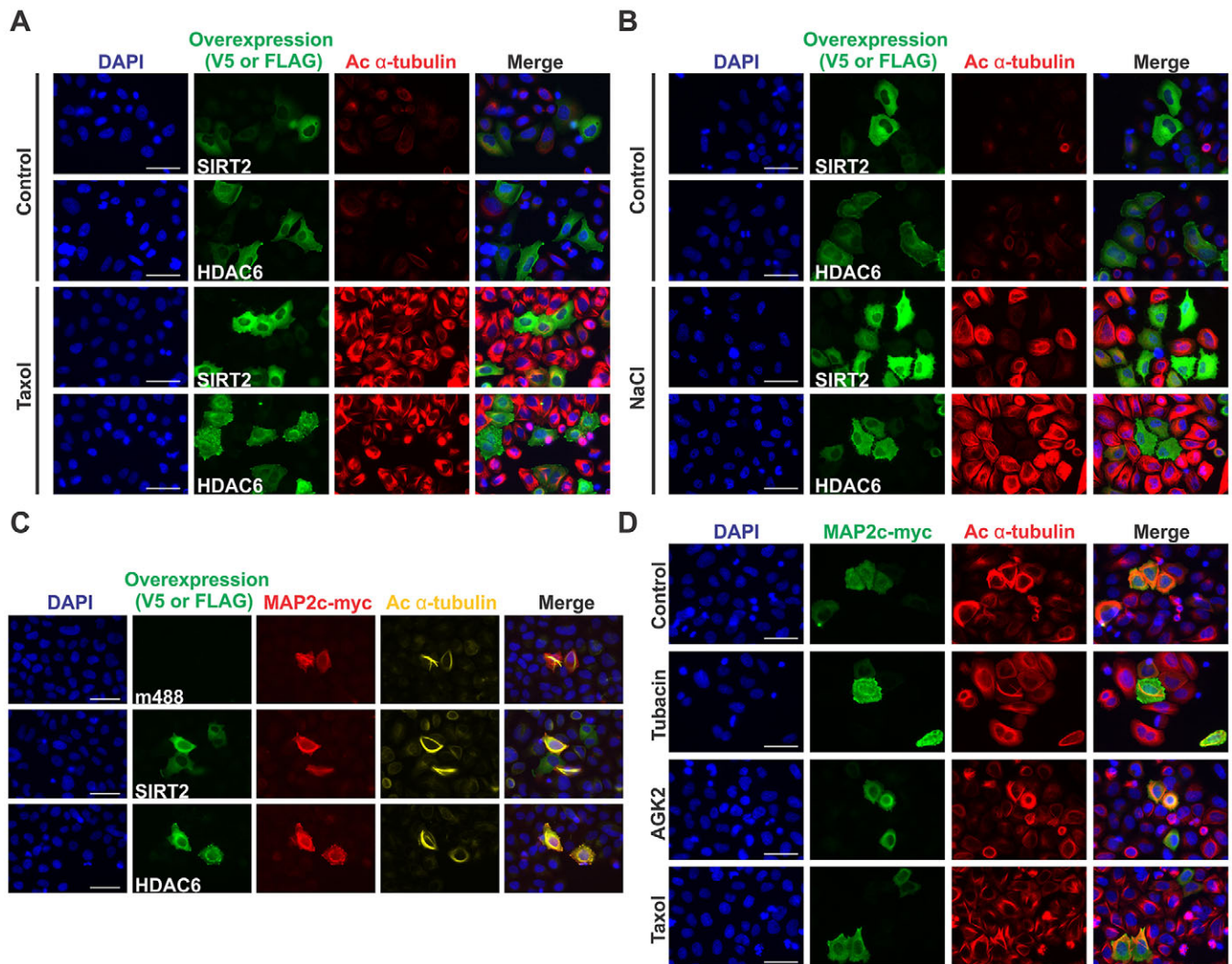


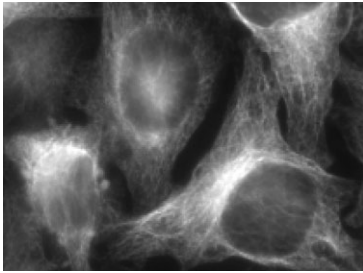
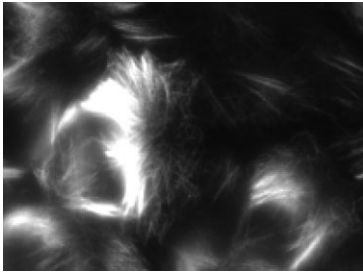
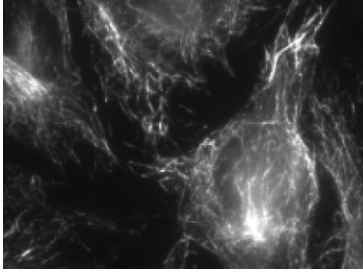
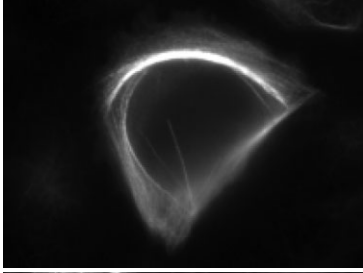
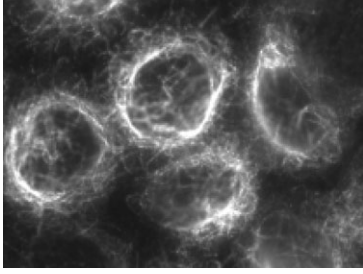
Fig. 6. SIRT2 and HDAC6 act similarly on other hyperacetylated microtubule structures. (A) Overexpression of SIRT2 or HDAC6 reduces taxol-induced hyperacetylation of tubulin. HeLaS3 cells were treated with 5 μ M taxol or DMSO as control. HDAC6 overexpression (30 h) was monitored by immunocytochemical detection of the C-terminal Flag tag and SIRT2 overexpression by the C-terminal V5 tag. (B) SIRT2 or HDAC6 overexpression reduces tubulin hyperacetylation induced by hyperosmotic stress. HeLaS3 cells were treated with 0.25 M NaCl for 1 h or H₂O as control. HDAC6 overexpression (30 h) was monitored by immunocytochemical detection of the C-terminal Flag tag and SIRT2 overexpression (30 h) by the C-terminal V5 tag. (C) Overexpression of SIRT2 and HDAC6 has no impact on the acetylation level of microtubule bundles. HDAC6 overexpression (30 h) was monitored by immunocytochemical detection of the C-terminal Flag tag, SIRT2 overexpression (24 h) by the C-terminal V5 tag and MAP2c overexpression (24 h) by the C-terminal Myc tag. m488, only secondary mouse antibody. (D) MAP2c-induced hyperacetylated microtubule bundles are subject to taxol-induced microtubule rearrangement. HeLaS3 cells were treated with 5 μ M tubacin, 100 μ M AGK2, 5 μ M taxol, or DMSO for 6 h. MAP2c overexpression (30 h) was monitored by immunocytochemical detection of the C-terminal Myc tag. Scale bars: 30 μ m. Ac α -tubulin, acetylated α -tubulin.

like a general tubulin deacetylase under normal conditions, whereas SIRT2 might have a more specialized role, possibly involving the activity of hitherto unidentified microtubule-associated proteins (MAPs). Tubulin acetylation per se does not influence the level of polymerization or the general morphology of microtubules (Howes et al., 2014). Therefore, it would appear that any local effects on hyperacetylation of microtubule structures should only be detectable at the luminal side of the microtubule. However, all MAPs studied so far bind to the outside of the microtubule wall (Nogales et al., 1999), except a short repeat motif of MAP tau which has been proposed to localize to the luminal wall (Kar et al., 2003). Interestingly, although MAP tau has been proposed to act as an inhibitor of HDAC6 in mouse neurons (Perez et al., 2009), co-overexpression of HDAC6 and MAP tau in fibroblasts resulted in less tubulin hyperacetylation compared to MAP tau overexpression

alone (Sudo and Baas, 2010). Potentially, MAPs could be indirectly regulated through the increase in tubulin acetylation. For a MAP to interact with acetylated tubulin, it would need a domain that recognizes the acetylated lysine residue. Recently, a bromodomain-containing protein from *Trypanosoma cruzi*, bromodomain factor 3 (tcBDF3), was reported to localize to the cytoplasm and to interact with acetylated α -tubulin (Alonso et al., 2014). This observation indicates that bromodomain-containing proteins that could bind to and read the ‘acetylation code’ on microtubules exist, similar to what has been shown for nuclear bromodomain-containing proteins involved in the reading of epigenetic acetylation marks (Jenuwein and Allis, 2001; Filippakopoulos et al., 2012).

Inhibition of SIRT2 increased taxol-induced microtubule hyperacetylation, whereas HDAC6 inhibition had no additional effect on the overall acetylation level. In apparent contrast,

Table 1. Hyperacetylated microtubule structures analyzed in this study

Hyperacetylated structures	Morphology	Inducing agent	Mode of tubulin hyperacetylation	Acetylation reduced by	References
Normal microtubule network		Tubacin, TSA	Tubacin is a specific HDAC6 inhibitor; TSA is a general HDAC class I and II inhibitor	SIRT2, HDAC6	Haggarty et al., 2003; Yoshida et al., 1990
Wedge-shaped microtubules		Taxol	Taxol binds β -tubulin and induces conformational changes and stabilization of the microtubules	SIRT2, HDAC6	De Brabander et al., 1981; Xiao et al., 2006
Disintegrating microtubules		NaCl	Hyperosmotic concentrations of NaCl leads to activation of the tubulin acetyltransferase α TAT1 by AMPK	SIRT2, HDAC6	Mackeh et al., 2014
Microtubule bundles		MAP2c	Overexpression of the microtubule-associated protein MAP2c rearranges the microtubules into bundles	–	Takemura et al., 1992
Perinuclear microtubules		FK866, AGK2	FK866 inhibits the NAD biosynthetic enzyme NamPRT leading to NAD depletion and inactivation of SIRT2, while AGK2 is a selective SIRT2 inhibitor	SIRT2	Hasmann and Schemainda, 2003; Khan et al., 2006; Galli et al., 2013; Outeiro et al., 2007

References for the mode of tubulin hyperacetylation are given.

overexpression of either SIRT2 or HDAC6 reduced the appearance of taxol-induced hyperacetylated microtubule wedges. It should be noted, however, that the onset of HDAC6 and SIRT2 overexpression precedes the addition of taxol by 24 h. The presence of the overexpressed proteins would enable a dynamic reduction of the tubulin acetylation state during the assembly of the taxol-induced microtubule wedges. That is, it appears possible that the overexpressed proteins do not deacetylate the structures per se, but deacetylate the dynamic tubulin oligomers before they are incorporated into the taxol-

induced wedge-like structures. In line with this notion, we found a strong increase in tubulin acetylation when cells were pre-incubated with HDAC6 inhibitor before taxol exposure. Earlier studies also support the suggestion of HDAC6 activity during, rather than after, taxol-induced assembly of microtubule structures. Namely, it was reported that recombinant HDAC6 failed to deacetylate microtubules isolated from taxol-treated cells (Matsuyama et al., 2002), whereas stable overexpression of HDAC6 reduced taxol-induced hyperacetylation (Hubbert et al., 2002). Collectively, these

and our observations indicate that HDAC6 deacetylates tubulin only before it is incorporated into microtubule wedges. Similarly, in our experiments inactivation of SIRT2 was also initiated before taxol exposure, which could have attenuated tubulin deacetylation before any conformational changes of the microtubules could occur.

In mitotic cells, taxol induces the formation of multiple microtubule asters instead of spindle formation (De Brabander et al., 1981). FK866-induced NAD depletion leads to a G₂/M phase cell cycle arrest (Muruganandham et al., 2005). As a consequence, no mitotic structures are observed in the FK866-treated cells and no multiple microtubule asters are detected following additional taxol exposure. However, when cellular NAD is restored (for example, by adding nicotinic acid), mitotic structures reappear. That is, although NAD depletion caused cell cycle arrest, the cells could be rescued by nicotinic acid addition and re-entered the cell cycle under the conditions used in this study. This indicates that, despite the rather dramatic phenotypic changes, FK866-induced NAD depletion and the ensuing SIRT2 inactivation had no permanent deleterious effects on the cells.

Reorganization of the microtubule network into perinuclear hyperacetylated microtubules was recently reported to occur upon overexpression of the N-terminal fragment of the mouse furry homolog, Fry. Fry has been shown to bind to and inactivate SIRT2 (Nagai et al., 2013). Along with the effects of direct (AGK2 and knockdown) and indirect (FK866) inhibition of SIRT2 shown here, the available evidence suggests that SIRT2 activity directly affects the morphology of the microtubule network. Namely, reduced SIRT2 activity stimulates formation of perinuclear microtubules, creating a subset of hyperacetylated tubulin that is not accessible to the activity of HDAC6. In neurons, hypoacetylated perinuclear microtubules envelop the nucleus and are involved in nuclear translocation (Rivas and Hatten, 1995; Umeshima et al., 2007). Furthermore, during Hepatitis B virus infection, mitochondria have been reported to cluster in the perinuclear region in a microtubule-dependent manner (Kim et al., 2007). Given the role of microtubules in intracellular transport, the induction of perinuclear microtubules and the regulation of their acetylation state by SIRT2 could act as a relay signal to change the localization of cellular organelles, for example, in response to cellular stress. It is not clear how the selectivity of deacetylation of perinuclear microtubules by SIRT2, but not HDAC6, is achieved. Possibly, by virtue of its smaller size compared to HDAC6, SIRT2 could be better able to diffuse inside microtubules, in the same manner as has been described for the α -tubulin acetyltransferase α TAT1 (Szyk et al., 2014), or it could have easier access through transient microtubule lattice openings between protofilaments (Howes et al., 2014; Yajima et al., 2012). However, these possibilities would also be valid for other microtubule structures. Therefore, it appears likely that other factors specifically localizing to perinuclear microtubules are involved.

In conclusion, our study has established redundant and distinct functions of the two tubulin deacetylases, HDAC6 and SIRT2. In addition to the regulation of SIRT2 activity by fluctuations in the cellular NAD levels, our observations support the suggestion that HDAC6 and SIRT2 also might be discerned by their differential recognition and deacetylation of subsets of acetylated tubulin. Thereby, this study provides evidence for the possibility that the preference for NAD-dependent or -independent deacetylation of proteins might be influenced by structural constraints.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were of analytical grade. FK866, AGK2, tubacin, trichostatin A (TSA) and taxol (paclitaxel) were from Sigma-Aldrich. The antibodies were generally diluted 1:1000, unless otherwise

stated. The following antibodies were used: rabbit anti-acetyl α -tubulin K40 (D20G3 XP) and mouse anti-V5 (clone 1H6) from Cell Signaling Technology, mouse anti- α -tubulin (clone B-5-1-2; diluted 1:10,000 for western blot analysis), mouse anti-FLAG (F3165) and rabbit anti-FLAG (clone SIG1–25) antibodies from Sigma-Aldrich, mouse anti-Myc antibody (clone 9E10) was from Abcam, rabbit anti-SIRT2 antibody (clone EP1668Y; diluted 1:200) was from Millipore, and chicken anti-Myc antibody (A-21281; diluted 1:200) was from Molecular Probes (Invitrogen). The fluorescent-conjugated secondary antibodies Alexa-Fluor-488-conjugated goat anti-mouse-IgG (A-11029), Alexa-Fluor-594-conjugated goat anti-rabbit-IgG (A-11037), Alexa-Fluor-594-conjugated goat anti-chicken-IgG (A-11042), Alexa-Fluor-647-conjugated goat anti-rabbit-IgG (A-21245) and Alexa-Fluor-647-conjugated goat anti-chicken-IgG (A-21449; diluted 1:200) were from Invitrogen (Life Technologies). Horseradish peroxidase (HRP)-conjugated goat anti-mouse-IgG and goat anti-rabbit-IgG antibodies were from Pierce (diluted 1:5000). 20 or 40 pmol of siRNA was used for transfections in 24- or 12-well plates, respectively. Flexitube siRNA directed against SIRT2 (cat. no. SI02655471) was from Qiagen whereas Ambion Silencer Select negative control #1 (cat. no. 4390843) was from ThermoFisher Scientific.

Cell culture

HeLa S3 cells (DSMZ no. ACC-161) were cultivated in high-glucose DMEM supplemented with 10% (v/v) fetal calf serum, 2 mM l-glutamine, penicillin (10,000 units/ml) and streptomycin (10 mg/ml). Transient transfection of HeLa S3 cells of vectors was performed for 24–72 h using Effectene reagent (Qiagen) and transfections with siRNAs were performed for 72–78 h using Lipofectamine 2000 (ThermoFisher Scientific), both according to the recommendations of the manufacturers.

Vector construction

The open reading frame (ORF) of human HDAC6 was amplified from cDNA from HEK293 cells and cloned into the pFLAG-CMV-5a vector through the HindIII and KpnI restriction sites. pcDNA3.1+N-Myc harboring the ORF of human MAP2C (accession number NM_031845), cloned in through the BamHI and EcoRV restriction sites, was ordered from Genscript. pcDNA3.1/V5-His harboring the ORF of human SIRT2 isoform 1 is described in Rack et al. (2014). The vectors were used for transient transfection.

Immunocytochemistry

Cells grown on cover slips were fixed with ice-cold 4% (v/v) formaldehyde in PBS for 30–45 min, permeabilized for 15 min using 0.5% (v/v) Triton X-100 in PBS, and subsequently blocked using supplemented cell culture medium for 1 h at room temperature. Cells were incubated with primary antibodies diluted in supplemented medium for 2 h at room temperature or overnight at 4°C. The cells were then washed twice with PBS and once with PBS containing 0.1% (v/v) Triton X-100 before secondary antibodies were added. After incubation for 1 h at room temperature, chromatin was stained with 4',6-diamidino-2-phenylindole (DAPI). Finally, the cells were washed once with PBS containing 0.1% (v/v) Triton X-100 and twice with PBS before the cells were subjected to fluorescence microscopy. Images were taken using a Leica DMI 6000B fluorescence microscope with a 40 \times dry objective and a 100 \times oil immersion objective.

Protein determination, SDS-PAGE and western blot analysis

Cell lysates were prepared in 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% (w/v) SDS, and collected using a cell scraper. Genomic DNA was sheared by passing the lysate through a syringe four or five times with a 23-gauge needle. The lysates were then centrifuged to remove cell debris. Protein concentration was determined using a BCA protein assay kit (Pierce), and 20 μ g protein was assessed by immunoblot analysis. SDS polyacrylamide gel electrophoresis (10% gels) and immunoblotting were carried out according to standard procedures using 5% (w/v) milk powder in TBS for blocking of the nitrocellulose membrane after protein transfer, and 5% (w/v) milk powder in 0.05% (v/v) Tween in TBS for dilution of antibodies. Enhanced chemiluminescence (SuperSignal, Pierce) was used for immunodetection.

Cold-induced depolymerization assay

The cold-induced depolymerization assay was initiated by exchanging the cell culture medium with ice-cold medium of the same composition. After incubation on ice for 15 min, the cells were fixed with formaldehyde.

All images are representative of at least three independent experiments.

Competing interests

The authors declare no competing or financial interests.

Author contributions

R.H.S. and M.Z. conceived the study, analyzed data and wrote the paper. R.H.S. performed all experiments and generated the figures.

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