Reactive Oxygen Species, AMP-activated Protein Kinase, and the Transcription Cofactor p300 Regulate α-Tubulin Acetyltransferase-1 (αTAT-1/MEC-17)-dependent Microtubule Hyperacetylation during Cell Stress*

Rafah Mackeh†, Séverine Lorin†, Ameetha Ratier†, Najet Mejdoubi-Charef‡, Anita Baille†, Arnaud Bruneel§, Ahmed Hamaï†, Patrice Codogno†, Christian Poüs†¶ and Daniel Perdzik†

From the †Université Paris Sud, EA4530, Faculté de Pharmacie, Châtenay-Malabry, France, ‡Assistance Publique-Hôpitaux de Paris, Service de Biochimie Metabolique et Cellulaire, Hôpital Bichat, 75018 Paris, France, §INSERM U845, Université Paris Descartes, 75014 Paris, France, and the ¶Biochimie-Hormonologie, Hôpital Antoine Béclère, Assistance Publique-Hôpitaux de Paris, 92141 Clamart, France

Background: Tubulin acetylation is a hallmark of microtubule stabilization, which may modulate the binding of microtubule-associated proteins.

Results: Microtubules are hyperacetylated because of stress-induced cellular signaling upstream of the tubulin acetyltransferase MEC-17/αTAT1.

Conclusion: MEC-17/αTAT1 is regulated by p300, reactive oxygen species, and AMP-activated protein kinase.

Significance: Microtubule hyperacetylation is important for cell adaptation to stress through autophagy induction and for cell survival.

Beyond its presence in stable microtubules, tubulin acetylation can be boosted after UV exposure or after nutrient deprivation, but the mechanisms of microtubule hyperacetylation are still unknown. In this study, we show that this hyperacetylation is a common response to several cellular stresses that involves the stimulation of the major tubulin acetyltransferase MEC-17. We also demonstrate that the acetyltransferase p300 negatively regulates MEC-17 expression and is sequestered on microtubules upon stress. We further show that reactive oxygen species of mitochondrial origin are required for microtubule hyperacetylation by activating the AMP kinase, which in turn mediates MEC-17 phosphorylation upon stress. Finally, we show that preventing microtubule hyperacetylation by knocking down MEC-17 affects cell survival under stress conditions and starvation-induced autophagy, thereby pointing out the importance of this rapid modification as a broad cell response to stress.

Cellular adaptive response to various stresses involves the coordinated activation of several signaling pathways that may ultimately lead to cell survival or to cell death depending on the nature, intensity, and duration of the insult. Cells primarily try to cope with stressful stimuli by triggering responses that may modulate gene expression and/or allow protective mechanisms such as autophagy induction. In the case their defenses are overwhelmed or in response to special stimuli, cells die by apoptosis or necrosis. Cell adaptation and survival might be compromised in pathological states such as diabetes or neurodegenerative diseases. Alternatively, cell adaptation and tolerance to death signals is an important feature of cancer (for reviews see Refs. 1 and 2).

Appropriate spatial and temporal coordination of cellular signaling is required for optimal cell response. In this respect, the microtubule (MT) cytoskeleton has an important role in organizing the cytoplasm by sequestering and releasing transduction factors, allowing their assembly into complexes or supporting their vectorization by molecular motors (for reviews see Refs. 3 and 4). Depending on the signaling pathway, these properties may require MT dynamics, which consists of alternations of growth and shrinking phases termed dynamic instability (5), or they may preferentially occur on a stable MT subset, in which tubulin bears numerous post-translational modifications. Some modifications act as local structural determinants that can be readily recognized by tubulin-binding proteins. This is the case for α-tubulin detyrosination, which prevents MT binding of MCAK and CLIP-170, two proteins that regulate the transitions between growth and shrinking phases termed catastrophes (toward MT disassembly) and rescues (toward a new growth phase), respectively (6, 7). Other modifications like the acetylation of the Lys-40 residue of α-tubulin are thought to modulate MT conformation by triggering subtle changes in the way tubulin subunits interact with each other (8, 9). Although the exact molecular effect of tubulin acetylation remain largely obscure because of its localization in the MT lumen, important progress has been accomplished during the past few years in our understanding of the biological function of

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† To whom correspondence should be addressed: EA4530, Université Paris-Sud, UFR de Pharmacie, 5 Rue J. B. Clément, 92296 Châtenay-Malabry, France. Tel.: 33-146835477; E-mail: christian.pous@u-psud.fr.

‡ The abbreviations used are: MT, microtubule; ROS, reactive oxygen species; EBSS, Earle’s balanced salt solution; TAT, tubulin acetyltransferases; NAC, N-acetyl-L-cysteine.
this modification. Even though it was recently challenged in in vitro experiments (10, 11), tubulin acetylation was found to stimulate the binding of dynein and of kinesin-1 to the surface of cellular MTs (12–15). Tubulin acetylation was also found to modulate the binding and function of signaling factors involved in cell survival in response to stress. For example, the activation of endothelial nitric-oxide synthase required stable, acetylated MTs for optimal activation by phosphorylation (16). Furthermore Akt activation and p53 transport into the perinuclear area require tubulin acetylation (17), and this binding to MTs occurs via the chaperone Hsp90. In starvation-induced autophagy, the stress-induced MAP-kinase JNK is activated via a kinesin-1-mediated recruitment on MTs that also depends on tubulin acetylation (18). Interestingly, in these studies, tubulin acetylation is not a static process that may only occur on stable MTs, but it is also highly inducible, including on the dynamic MT subset, in response to genotoxic stress or to nutrient deprivation (17, 18). Tubulin acetylation levels result from a balance between the activities of the cytoplasmic deacetylases HDAC6 and SIRT2 and that of various tubulin acetyltransferases including ARD1/NAT1, NAT10, Gcn5, ELP3, and αTAT1/MEC-17 (reviewed in Ref. 19). Among these enzymes, MEC-17 appears as a major acetyltransferase, which can account for most of the tubulin acetylation in stable MTs (20, 21).

The level of tubulin acetylation in MTs is thus an important factor to allow cells to organize and possibly coordinate several signaling pathways along MTs. It functions in static conditions on stable MTs, but also in a dynamic manner that is probably tightly regulated. However, the cellular and molecular mechanisms that enhance tubulin acetylation in MTs (hereafter referred to as MT hyperacetylation) have not been explored yet. In this study, we characterized MT hyperacetylation and addressed the question of its induction in response to cell stress. We show that it is a rapid and reversible process that results from an acetyltransferase induction triggered by the release of mitochondrial reactive oxygen species (ROS) and by AMPK. We further show that MEC-17 is the sole acetyltransferase responsible for MT hyperacetylation and that AMPK stimulates its phosphorylation in response to cell stress. We also provide evidence that MT hyperacetylation is required for cell survival in response to oxidative stress and for starvation-induced autophagy stimulation.

**EXPERIMENTAL PROCEDURES**

**Chemical Products and Antibodies**—All chemicals were purchased from Sigma-Aldrich. Mouse monoclonal anti-α-tubulin (DM1-A), β-tubulin I (T7816), anti-acetylated α-tubulin (6-11B-1), and rabbit anti-β-tubulin (L7816) were from Sigma-Aldrich. β-Actin HRP-conjugated antibody (C4) was from Santa Cruz Biotechnology (sc-47778). p300 antibody (RW-105) was from Pierce Biotechnology (MA1–16622). Rabbit anti-phospho-AMPKα (Thr-172), anti-AMPKα1/2, and anti-GFP were from Cell Signaling Technology (reference nos. 2535, 2532, and 2555, respectively). Poly(ADP-ribose) polymerase mAb antibody was from Clontech (C-2-10, reference no. 630210). Alexa Fluor 488-conjugated goat anti-mouse was purchased from Invitrogen.

**Cell Culture and Stress Conditions**—Human HeLa cells, mouse embryonic fibroblast cells, and the human telomerase reverse transcriptase-immortalized retinal pigment epithelial cell line (RPE1) were cultured in DMEM (ATCC, France) supplemented with 1% sodium pyruvate, 10% FBS, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) in 5% CO₂ at 37 °C. PtK2 (porcine tridactylis kidney cells) were purchased from ATCC and cultured in Eagle’s minimum essential medium (ATCC 30-2003™) supplemented with 10% FBS. Cells were always used at less than 80% confluence and before passage 12. For amino acid deprivation, DMEM was replaced by 25 mM HEPES-buffered Earle’s balanced salt solution (EBSS) after three washes. For stress induction, culture medium (or PBS for H₂O₂) was replaced by freshly prepared medium to which the indicated concentrations of chemical reagent were added. NaCl concentration in DMEM is 150 mM (116 mM for Eagle’s minimum essential medium); thus the addition of 125, 250, or 500 mM NaCl (as indicated in the figures) yields final NaCl concentrations of 275, 400, or 650 mM for DMEM (241, 366, and 616 mM for Eagle’s minimum essential medium).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay—The cells were cultured in 12-well plates and transfected with MEC-17 siRNA. Stress was induced with NaCl for 24 h. Cells were then incubated for 2 h with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (Sigma-Aldrich) at 37 °C. Then medium was removed, formazan crystals were dissolved with DMSO, and optical density measurements were read on a microplate reader at 540 nm.

**Immunofluorescence and Microscopy**—Cells grown on glass coverslips were washed with calcium- and magnesium-free PBS and fixed with −20 °C methanol for 5 min. Cells were then washed three times before staining with monoclonal anti-acetyl-α-tubulin for 1 h at 37 °C. After three washes with PBS, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse (45 min, 37 °C). Images were acquired using a Scion CFW1312M CCD camera, on a Leica DMLB microscope (100 × 1.3 NA objective).

**Plasmid and siRNA Transfections**—jetPEI™ (Polyplus™) was used for plasmid transfection of HeLa cells according to the manufacturer’s instructions. GFP-MEC-17WT and GFP-MEC17D157N were from Dr. M. Nachury (Department of Molecular and Cellular Physiology, Stanford University School of Medicine; Addgene references 27099 and 27100, respectively). mCherry-tubulin vector was kindly provided by Dr. R. Y. Tsien (Department of Pharmacology, Howard Hughes Medical Center, University of San Diego). The mCherry tubulin K40A mutated vector was a kind gift from Dr. F. Saudou (Institut Curie, Orsay, France). For all siRNA transfections, we used HiPerFect transfection reagent (Qiagen; 301705) according to the manufacturer’s instructions. To deplete p300 from HeLa cells, we used a duplex of p300 siRNA (Sigma; SASI_Hs01_00052818, 5’-CUAGGAGACACCUCUUGUAU[dt][dt][dt]-3’ and 5’-UACGU-ACAAGGUCUCUGAG[dt][dt][dt]-3’). Cells were transfected after 24 h with p300 siRNA (200 nM/well of a 6-well plate). To deplete MEC-17 in HeLa and RPE1 cells, we used ON-TARGETplus siRNAs from Dharmacon as a pool of four siRNAs 5’-GUA-GCUAGGUGCAGUAAU-3’; 5’-GAGAUAAGCUAGAUCC-
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CUU-3’; 5’GGGAAACUCACCAGAACGA-3’; and 5’-CUU-GUGAGAUUGUGGAGA-3’.

Transfections were repeated at days 1, 2, and 3 at a final concentration of 100 nM, and cells were used at day 4. AMPK siRNA was purchased from Santa Cruz Technology (AMPKa1/2 siRNA, sc-45312). Transfections were repeated at days 1 and 2 at a final concentration of 100 nM.

Total Cell Lysate Preparation—After washing with PBS, cells were directly lysed in Laemmli sample buffer, boiled at 100 °C for 5 min, and sonicated. 10–20 μg of proteins were subjected to Western blot analysis using appropriate antibodies.

Preparation of Soluble (Cytosolic) and Polymerized Microtubule Fractions—80% confluent cells cultured in a 75 cm² flask were washed twice with 37 °C prewarmed PEM buffer. The cytosolic (nonmicrotubular) fractions were extracted after permeabilization using PEM supplemented with 0.075% Triton X-100, 10% glycerol, a protease inhibitor mixture (1 mM PMSF and 20 μM benzamidine/leupeptine), and phosphatase inhibitors (1 mM NaF and 0.1 mM orthovanadate) for 3 min at 37 °C. Cytosolic fractions were collected, and permeabilized cells were gently washed to remove residual Triton. Polymerized microtubules were depolymerized on ice for 1 h in PM buffer (80 mM PIPES, 1 mM MgCl₂, pH 6.9) supplemented with 5 mM CaCl₂ and the protease and phosphatase inhibitor mixtures and then collected. Both extracts (soluble and formerly polymerized fractions) were concentrated down to 200 μl using Vivaspin 6 filtration units of 10 kDa (Sartorius Stedim Biotech) and then subjected to Western blot analysis.

Cytoplasmic and Nuclear Extraction—80% confluent cells in a 75 cm² flask were trypsinized, and cell pellet was suspended in 1 ml of buffer A (10 mM KCl, 1.5 mM MgCl₂, 10 mM HEPES, 0.5 mM DTT, pH 7.9) and transferred into a Dounce homogenizer for cell disruption. After centrifugation (5 min, 220 × g, 4 °C), supernatants (containing the cytosolic fraction) were stored, and pellets (containing nuclei) were resuspended in 500 μl of buffer 1 (10 mM MgCl₂, 0.25 M sucrose) and mixed with 500 μl of buffer 2 (0.5 mM MgCl₂, 0.88 M sucrose). After centrifugation, the pellets were resuspended in 100 μl of lysis buffer (25 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) and sonicated. Finally, cytosol-containing and nuclei-containing fractions were centrifuged (3000 × g, 10 min), and supernatants were stored at −20 °C prior to Western blot analysis.

SDS-PAGE and Western Blot Analysis—After SDS-PAGE, proteins were transferred to PVDF membranes. After blocking nonspecific binding sites with 10% of nonfat dry milk, blots were probed with appropriate antibodies and revealed with HRP-conjugated secondary antibodies (Pierce ECL Plus Western blot substrate) on Kodak Biomax MR films. Densitometry analysis was performed using ImageJ software.

Two-dimensional Electrophoresis—6 × 10⁶ HeLa cells were cultured in 60-mm cell culture dishes. After appropriate treatment or transfection, cells were collected in 100 μl of lysis solution (50 mM DTT, 4% CHAPS, proteases inhibitors, PMSF, benzamidine/leupeptine), sonicated, kept on ice for 30 min, and centrifuged to eliminate cell debris. For phosphatase treatment, calf intestinal alkaline phosphatase (Takara Bio Inc., no. 2250A) was added to the lysate according to the manufacturer’s instructions and incubated for 30 min at 37 °C. Reactions were stopped by adding 50 mM of DTT. Protein mixtures were desalted on BioSpin®-6 Tris columns with Bio-Gel in Tris buffer (Bio-Rad), precipitated in cold acetone for 1 h, and centrifuged, and the pellet was solubilized in isoelectric focusing rehydration solution (6 M urea, 4% CHAPS, 50 mM DTT, 0.5% ampholytes, orange G). Isoelectric focusing was performed using linear IPG strips with pH 6–10 (ZOOM® STRIP; Invitrogen), and the second dimension was carried out using ready-made 4–12% Bis-Tris ZOOM® mini gel (Invitrogen).

In Vitro Tubulin Deacetylation Assay—Cell lysates of control cells or cells treated with NaCl were prepared using 10 mM Tris buffer, pH 8.0, supplemented with 1% Triton, 1 mM NAD⁺, and the protease and phosphatase inhibitor mixtures. Lysate aliquots containing 20 μg of proteins were mixed with 2 μg of soluble acetylated tubulin dimers purified from porcine brain (22) and adjusted to 50 μl with PEM buffer. After 1 h at 37 °C, reactions were stopped by adding 4× SDS-PAGE sample buffer and immediately subjected to SDS-PAGE and Western blotting of acetylated and total α-tubulin.

Reverse Transcription and Quantitative PCR—After siRNA transfection, total cellular RNAs were extracted with 200 μl of Extract-All reagent (Eurobio) according to the manufacturer’s instructions. First strand cDNA were generated by reverse transcription of 1 μg of total RNAs using oligo(dT) 12–15 primer and SuperScriptIII® reverse transcriptase kit (Invitrogen) according to the manufacturer’s instructions in a total reaction volume of 20 μl. Real time PCR was performed in a lightCycler thermal cycler (Roche Diagnostics). The amplification of each cDNA (50 ng/μl) was performed in a 10-μl volume, using the Fast Start DNA MasterPLUS SYBER GREEN 1 master mix (Roche Diagnostics) with a 500 nM final concentration of each primer. The primers used for MEC-17 were the following: MEC-17(f), 5’GGCGGAAACTCTTCCAGTAT-3’, and MEC-17(r), 5’-TTGTTCCACCTGTTGGACT-3’ (21). The data were normalized using the GAPDH cDNA content.

Statistical Analysis—Quantitative data are the means ± S.E. of at least three independent experiments. The data ± S.E. were compared using the nonparametric Mann-Whitney U test for dose-dependent hyperacetylation, cell survival, and autophagy induction experiments. Two-way analysis of variance was used to evaluate the effect of chemicals on stress-induced MT hyperacetylation.

RESULTS

Microtubule Hyperacetylation Is a General and Reversible Cell Stress Response—We found earlier that MT acetylation is induced during amino acid starvation (18) and after exposure to UVC radiation (17). To examine whether this hyperacetylation is a general stress response, HeLa cells were starved of amino acids for 2 h or treated with 10⁻⁴ μM of the synthetic hormone ethinyl estradiol (EE2), 10⁻⁴ μM methoxychlore, which is a synthetic organochlorine used as an insecticide, 0.5 μM staurosporine or with the addition of 0.25 mM NaCl (Fig. 1, A and B). All these stimuli increased tubulin acetylation with an amplitude that ranged between 1.5-fold for amino acid starvation (EBSS) and 12.5-fold for NaCl. Particularly, exposure to NaCl caused rapid hyperacetylation of the MT network (as soluble tubulin...
was not affected; data not shown) in a time- and dose-dependent manner (Fig. 1B). This NaCl-induced hyperacetylation is clearly visible in immunofluorescence experiments where control cells show a few discontinuously labeled acetylated MT, whereas stressed cells exhibit long hyperacetylated MT all over the network (Fig. 1C). Interestingly, MT hyperacetylation looks as a general cell response to stress, because we also observed it in other cell lines like RPE-1 and mouse embryonic fibroblast (data not shown). We chose to use NaCl in priority to further identify the mechanisms that stimulate MT acetylation, because of its potent inductive effect. To explore the reversibility of this hyperacetylation, cells were subjected to a recovery experiment after a 1-h induction with 0.25 M NaCl. As expected, tubulin acetylation level increased after 1 h of treatment and then regularly decreased upon NaCl washout until reaching basal level after 30 min (Fig. 1D).

**MEC-17 Is Required for Stress-induced Microtubule Hyperacetylation**—Tubulin acetylation results from the balance between tubulin acetyltransferases (TAT) and deacetylases. To examine the involvement of deacetylases in stress-induced MT hyperacetylation, we performed an *in vitro* tubulin deacetylation assay that consists in mixing brain tubulin, which is highly acetylated, with lysates of control or NaCl-treated cells. Lysates of control cells were able to deacetylate tubulin by 70%. This activity remained unchanged in lysates of NaCl-treated cells, indicating that tubulin deacetylase inhibition is not responsible for the increase in the level of tubulin acetylation (Fig. 2A), but that it rather results from an increase in TAT activity. To identify which TATs are involved, we first monitored the recruitment of putative TATs on MT fractions prepared from control and NaCl-treated cells. MEC-17 detection required the overexpression of a GFP-tagged enzyme, because all the antibodies we tested failed to recognize endogenous MEC-17 in our hands. Among the acetyltransferases we tested, only p300 increased in the MT-containing fraction in a NaCl dose-dependent manner (Fig. 2B), suggesting that it could be involved in MT hyperacetylation. MEC-17 was recently discovered as the main TAT (20, 21), but unexpectedly, GFP-MEC-17 decreased in the MT fraction upon stress (Fig. 2B) in a time-dependent manner (Fig. 2C), and this decrease was not due to degradation because GFP-MEC-17 content remained constant in total cell lysates during NaCl stress (Fig. 2E).

**FIGURE 1.** *Microtubule hyperacetylation is a broad and reversible cell stress response.* A and B, HeLa cells were treated for 15 min with 10^{-7} M methoxychloro (MTX); treated for 1 h with 10^{-10} M ethinylestradiol (EE2), 0.25 mM NaCl, or 0.5 μM staurosporine; and deprived of amino acids for 2 h (EBSS) (A) or subjected to an exposure to excess NaCl as indicated (B). C, Immunolabelling of acetyl-tubulin after 30 min of NaCl treatment. D, after 1 h of treatment with 0.25 M NaCl, the medium was removed and replaced with fresh normal medium, and tubulin acetylation level was monitored. The histograms show the mean normalized ratios ± S.E. of acetyl-α-tubulin to total α-tubulin. *, p < 0.05. Scale bar, 10 μm.
To check whether MEC-17 is involved in MT hyperacetylation, we used a siRNA approach. MEC-17 knockdown dramatically reduced tubulin acetylation both in basal and upon various stress-induced conditions (amino acid starvation, NaCl, staurosporine) (Fig. 2D). MEC-17 is also required for hyperacetylation in other cell types such as RPE-1 (Fig. 2E). Conversely, the overexpression of MEC-17\textsuperscript{WT} but not of its catalytically inactive mutant MEC-17\textsuperscript{D157N}, accentuated both basal and stress-induced tubulin acetylation as shown in Fig. 2E. MEC-17 is thus required for stress-induced MT hyperacetylation.

\textit{p300 Negatively Regulates MEC-17-dependent Tubulin Acetylation}—The high level of p300 recruitment on MT upon stress was intriguing because its main localization is expected to be nuclear. To further explore this point, we determined first in which cytoplasmic compartment p300 localizes upon stress. As shown in Fig. 2B, p300 is predominantly found in the MT fraction, suggesting that it is recruited to MT upon stress.

\textbf{FIGURE 2.} \textit{MEC-17 is required for stress-induced microtubule hyperacetylation.} A, tubulin deacetylation assay. After a 30-min treatment with NaCl as indicated, cell lysates were incubated \textit{in vitro} with porcine brain tubulin (which is naturally acetylated) and NAD\textsuperscript{+}. After Western blotting of acetylated and total \(\alpha\)-tubulin, the means \(\pm\) S.E. ratios of acetyl to total \(\alpha\)-tubulin were represented in the histogram shown. B, Western blot of different acetyltransferases detected with a variety of specific antibodies in the MT-containing fractions of HeLa cells treated for 30 min with NaCl as indicated. C, Western blot analysis of GFP-MEC-17 in MT-containing fraction of cells transiently overexpressing GFP-MEC-17 and treated for the indicated times with 0.25 M NaCl. D, after knockdown of MEC-17 by siRNA, HeLa cells were treated with NaCl (top panels), deprived of amino acids (EBSS, bottom left panel) or treated with staurosporine (ST, bottom right panel) and analyzed for tubulin acetylation. E, cells transiently overexpressing GFP-MEC-17\textsuperscript{WT} or the catalytically inactive mutant GFP-MEC17\textsuperscript{D157N} were stressed with NaCl for 30 min and analyzed for tubulin acetylation.
shown in Fig. 3A, p300 dramatically increased in the MT fraction upon NaCl addition in a dose-dependent manner but remained undetectable in the cytosol. Also, the global p300 level remained unchanged upon stress. Together, these two results suggest that p300 found in the MT fraction would originate from a fast and effective nuclear export. This hypothesis was readily confirmed by both a Western blot analysis of whole cytoplasmic (thus containing MTs) and nuclear extracts from NaCl-treated cells (left) or by immunofluorescence (right). Poly(ADP-ribose) polymerase (PARP) and β-tubulin were used as controls for nuclear and cytoplasmic purification, respectively. Scale bar, 10 μm. C and D, analysis of tubulin acetylation after p300 siRNA alone or combined with that of MEC-17. The histogram indicates the means ± S.E. normalized acetyl-to-α-tubulin ratio. *, p < 0.05. E, HeLa cells were subjected or not to siRNA as indicated, and MEC-17 mRNA levels were measured by quantitative RT-PCR. The values are the means ± S.E. of the relative MEC-17 mRNA content normalized to the level measured in untreated cells. *, p < 0.05. F, loss of p300 from the MT-containing fraction prepared from cells subjected to MEC-17 RNAi (left panel) or from cells transiently overexpressing (72 h) a nonacetylatable tubulin mutant (K40A) (middle panel). Left panel, cytosol and MT fractions prepared from PtK2 cells treated with NaCl.
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To examine whether these regulatory effects of p300 involved MEC-17, we inhibited both enzymes by RNAi. As shown in Fig. 3D, when MEC-17 was knocked down, p300 inhibition could enhance neither basal nor stress-induced tubulin acetylation. This result indicates that increases in tubulin acetylation observed upon p300 knockdown require MEC-17 and suggests that p300 may down-regulate MEC-17. To further understand how such regulation takes place and taking into consideration that p300 is a regulator of transcription, we tested whether it is involved in the modulation of MEC-17 mRNA levels. Quantitative RT-PCR experiments showed that endogenous MEC-17 mRNA level increased by 90% when p300 was knocked down compared with control (Fig. 3E). These results show that p300 negatively modulates tubulin acetylation by a mechanism that involves a control of MEC-17 mRNA levels.

Because of its indirect role on MT hyperacetylation through regulation of MEC-17 expression, we further asked whether p300 recruitment to MT could be a consequence of hyperacetylation. Monitoring the association p300 on MTs in cells transfected with MEC-17 siRNA or with a cDNA encoding K40A tubulin, (which is readily incorporated into MTs (17, 18) and is nonacetylatable) showed that upon NaCl-mediated stress, p300 was recruited to MT in controls but not in MEC-17 siRNA or in tubulin K40A transfected cells (Fig. 3F). Moreover, in NaCl-treated PtK2 cells, which naturally lack MEC-17 and tubulin acetylation, cytoplasmic p300 could not bind to MT-containing fraction upon stress (Fig. 3F, right panel). Taken together, these results indicate that p300 recruitment to MT is a consequence of hyperacetylation.

Mitochondrial ROS Production Participates in the Induction of Stress-induced Microtubule Hyperacetylation—An increase in ROS production is a common feature of the cellular stresses used in this project (23–25). To examine whether ROS could trigger MT hyperacetylation, HeLa cells were treated with H$_2$O$_2$, which is known to generate the hydroxyl radical OH$. H_2$O$_2$ indeed induced MT hyperacetylation in a time- and dose-dependent manner (Fig. 4, A and B), and pretreatment of cells with the radical scavenger N-acetyl-l-cysteine (NAC) blocked this effect (Fig. 4C).

H$_2$O$_2$ being able to induce MT hyperacetylation, we further tested whether an oxidative component could participate in the induction of acetylation by other stresses. We found that NAC prevented MT hyperacetylation to a variable extent that ranged from complete inhibition in response to EE2 (data not shown) to a lesser (~50%) inhibition early after induction by NaCl as shown in Fig. 4D. ROS production may thus contribute to MT hyperacetylation. However, other mechanism(s) may also be involved in cell response to NaCl because NAC only partially reduced MT hyperacetylation (Fig. 4D).

To further explore the mechanisms of NaCl-mediated hyperacetylation involving ROS, we examined the contribution of superoxide anions O$_2^-$.

To finally determine which source of ROS formation mainly contributes to MT hyperacetylation early upon a stress triggered by the addition of 0.125 M NaCl, cells were pretreated with 0.5 mM of the specific mitochondria-targeted antioxidant MitoQ (26) for 24 h. MT hyperacetylation significantly decreased under MitoQ conditions compared with control (Fig. 4F), suggesting that mitochondria are the main organelles involved in stress-induced MT hyperacetylation, early after exposure to NaCl. Altogether, our results indicate that ROS generation, at least from mitochondria, participates in inducing MT hyperacetylation.

Phosphorylation of MEC-17 Increases upon Stress in an AMPK-dependent Manner—Because ROS production stimulates AMP-kinase (AMPK) activity (27), we first examined the level of phosphorylation of AMPK on Thr-172 and found it was increased after 5 min of NaCl induction and lasted 15 min after stress (Fig. 5A, top left panel). This stress-induced AMPK activation was also detected in H$_2$O$_2$-stressed cells. Interestingly, we found that AMPK is present and activated in MT-containing fractions (Fig. 5A, top right panel) and that overall AMPK activation depended on ROS production. Indeed, pretreatment of cells with NAC before stress induction effectively prevented AMPK phosphorylation (Fig. 5A, bottom panel). To determine whether such AMPK stimulation is actually involved in stress-induced MT hyperacetylation, we knocked down AMPKα1/2 by RNAi. In this condition, both basal and stress-induced MT acetylations were decreased by ~50% compared with controls (Fig. 5B, left panels). MT hyperacetylation was also inhibited when cells were treated with the chemical inhibitor STO609 (Fig. 5B, right panels). STO609 is widely used as an inhibitor of AMPK through the inhibition of the upstream activating Ca$^{2+}$/calmodulin-dependent protein kinase (28).

To finally examine whether AMPK could mediate MEC-17 phosphorylation, we explored the overall level of GFP-MEC-17 post-translational modifications by performing two-dimen- sional electrophoresis. The analysis revealed the presence of five major MEC-17 spots in basal conditions (Fig. 5C, top panel). Interestingly, two more acidic species appeared after 15 min of NaCl treatment (Fig. 5C, black arrows). The treatment of cell lysates with alkaline phosphatase prior to two-dimensional electrophoresis prevented the occurrence of these two forms on the benefit of two more basic spots (Fig. 5C, white arrows), indicating that they corresponded to phosphorylated MEC-17. A similar two-dimensional analysis of the MEC-17 modifications performed after AMPK RNAi showed that AMPK knockdown prevented the occurrence of stress-induced phosphorylated MEC-17 spots. These results indicate that, in response to ROS-mediated activation, AMPK is involved in MEC-17 phosphorylation upon stress.

MEC-17-dependent Microtubule Hyperacetylation Promotes Cell Survival and Starvation-induced Autophagy—The reversibility of MT hyperacetylation shown in Fig. 1 suggests that it is rather an adaptive cell response to various stresses than a signal that would activate a death pathway. Among the adaptive mechanisms that occur rapidly after exposure to stress,
autophagy induction is essential to favor cell survival (29). In the case of amino acid starvation, the breakdown of cellular components that results from autophagy induction can ensure survival by maintaining cellular energy levels. During autophagy induction, targeted cytoplasmic constituents are isolated within autophagosomes, which then fuse with lysosomes, allowing their contents to be degraded and recycled. We have previously shown that starvation-induced MT hyperacetylation is required for autophagy using the nonacetylatable K40A mutant of tubulin (18). Here, we quantified autophagy induction in control and MEC-17 siRNA conditions by measuring the amount of cytosolic microtubule-associated protein light chain 3-I (LC3-I) that is converted to a lipid-conjugated form (termed LC3-II), which associates with autophagosome membranes. The amount of LC3-II being dependent on the balance between autophagosome formation and their clearance by lysosomes, its accumulation was measured in the presence of bafilomycin A1, which prevents autophagosome fusion with lysosomes. As expected, the autophagic flux (i.e. the difference between the levels of LC3-II measured in the presence and absence of bafilomycin A1) was reduced by ~60% in MEC-17 siRNA conditions compared with controls (Fig. 6A).

Exposure to NaCl has been demonstrated previously to stimulate autophagy, and such autophagy stimulation is important to make cell survival possible (30). To demonstrate that in our conditions, autophagy induction is the process that actually allows cell survival, we measured it by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after autophagy was inhibited by knocking down Atg7, which is required for the conversion of LC3-I into the lipid conju-
Atg7 knockdown reduced cell survival upon 24 h of mild NaCl-mediated stress by $\approx 50\%$ compared with control RNAi conditions (Fig. 6B), indicating that autophagy participates in cell survival upon NaCl treatment. To confirm that MT hyperacetylation contributes to cell survival, it was also measured in MEC-17 RNAi conditions. Fig. 6A shows that cell viability was decreased by up to $\approx 20\%$ in conditions of MEC-17 inhibition relative to controls. Altogether, these data indicate that MT hyperacetylation favors cell survival through autophagy induction and point out the important physiological role of this modification and of its major effector MEC-17 in ensuring cell adaptability to stress.

**DISCUSSION**

In this study we show that, beyond the basal acetylation of stable MTs (14), the increase in the level of acetylated tubulin in the MT network, i.e., MT hyperacetylation is a general, acute, and reversible response that can be highly induced when cells are exposed to a variety of stresses. We also show that this induction involves the major acetyltransferase MEC-17.

Furthermore, in keeping with our previous findings that tubulin Lys-40 acetylation is required to allow starvation-induced autophagy stimulation (18) or the activation of protective signaling pathways (p53 translocation after genotoxic...
ACETYLATION DURING CELL STRESS

Stress-induced Microtubule Hyperacetylation

Until now, it was proposed that physiological and pathological variations in the level of tubulin acetylation would be mainly attributed to the inhibition of tubulin deacetylases and especially that of HDAC6 (19, 31). Our study shows for the first time that MT hyperacetylation, which occurs in a variety of stresses, is not due primarily to tubulin deacetylase inhibition but would rather result from enhanced tubulin acetylation. Among the multiple acetyltransferases that have been found to acetylate tubulin either in vitro or in vivo, MEC-17/αTAT-1 was shown to be the major tubulin acetyltransferase in basal conditions (20, 21). We show now that MEC-17 is also a crucial effector of stress-induced MT hyperacetylation.

An approach to understand what happens in terms of MT acetylation during cell stress was to determine whether MEC-17 could be subject to modulation of expression and/or to post-translational modifications. The first appealing point from our data were the massive recruitment of p300 on MTs in response to stress. Because of the existence of multiple nuclear localization sequences, p300 is known to be a transcriptional coactivator that mainly resides in the nucleus (32). The control of its nuclear localization is critical to ensure transcriptional regulation (for review, see Ref. 33). Here we show that p300 is redistributed to the cytoplasm and binds to acetylated MTs upon stress, which would be expected to prevent any shuttling back to the nucleus. Similar nucleocytoplasmic shuttling of p300 was previously observed in response to the HDAC inhibitors valproic acid or butyrate (34). In these conditions, cytoplasmic p300 is targeted to aggresomes (which bind to and need MTs for their formation), where it is ubiquitylated and subsequently degraded by the proteasome. In our models of cell stress, p300 nuclear export and cytoplasmic sequestration would favor the tubulin acetylation response by relieving negative regulation of MEC-17 gene transcription. Note that such down-regulation not only affects the extent of MT hyperacetylation, but also controls the basal acetylation level of tubulin. p300 is best known as an activator of gene transcription (35), but it was also reported to be required for down-regulation. For example, the DNA binding repressor protein PLZF (promyelocytic leukemia zinc finger gene) requires acetylation by p300 for binding to DNA, which leads to the recruitment of deacetylases and repression of PLZF-dependent HoxB2 reporter gene activity (36). In addition, recent studies show that p300 mRNA and protein levels decrease in metastatic cells (37). Also, p300/CBP mutations in some cancer cells decrease its acetylase activity (38). Given our results, such inhibition of p300 in cancer cells might presumably increase MEC-17 gene expression, which could explain why cancer cell lines like HeLa display higher levels of tubulin acetylation than non-cancer cell lines like RPE-1 (17). p300 is thus a potent regulator of MEC-17 levels and function in basal conditions and in stressed cells. However, it is expected to exert a medium or long term effect to sustain tubulin acetylation in stressed cells.

The burst in MT acetylation we observed very early after the onset of stress thus ought to involve other effectors. ROS are the major reactive atomic or molecular species produced in living organisms (39) and have been incriminated in numerous physiological adaptive reactions and diseases such as cancers, neurological disorders, or cardiovascular impairments. The superoxide anion $O_2^-$ is generated early during ROS production. It is produced mainly in mitochondria, at the plasma membrane by NADPH oxidase, at the endoplasmic reticulum by cytochromes...
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P450, but also in the cytoplasm by xanthine oxidase (40). Either spontaneously or after SOD-mediated catalysis, $\text{O}_2^-$ dismutates into $\text{H}_2\text{O}_2$, which can subsequently yield hydroxyl radicals $\cdot\text{OH}$ known to be highly reactive against organic compounds. Our results suggest that ROS and especially $\cdot\text{OH}$ and $\text{O}_2^-$ anions both participate in the induction of MT hyperacetylation but may be differently involved according to stress duration and/or strength. This variability may reflect the heterogeneity of ROS generation in response to NaCl treatment (41). Our experiments using MitoQ also suggest an interesting link between the release of ROS by mitochondria and the induction of MT acetylation.

ROS are effective second messengers, which may be quickly released in conditions of stress. Downstream of ROS, we focused our attention on AMPK activation. In agreement with previous studies (25, 42–45), we found that AMPK was rapidly activated by NaCl and $\text{H}_2\text{O}_2$.

Upon NaCl treatment, STO609 inhibited MT hyperacetylation, indicating that AMPK is at least in part activated by $\text{Ca}^{2+}$/calmodulin-dependent protein kinase kinase (28). $\text{Ca}^{2+}$/calmodulin-dependent protein kinase kinase activation is triggered both by ROS and by an increase in cytoplasmic $\text{Ca}^{2+}$ (46), which both participate in cell response to NaCl treatment (40). Thus, $\text{Ca}^{2+}$-mediated signaling could explain why (i) hyperacetylation was higher with NaCl than with $\text{H}_2\text{O}_2$, and (ii) NAC was only partly effective to prevent MT hyperacetylation in response to NaCl.

AMPK activation is involved in controlling MT hyperacetylation, most likely via the phosphorylation of MEC-17. To determine whether AMPK-dependent phosphorylation of MEC-17 actually increases the activity of the enzyme, target Ser or Thr should be identified and mutated. AMPK-mediated phosphorylation of MEC-17 could also play another role in controlling the affinity of MEC-17 for MTs. Recently, it has been shown that MEC-17 binding to MTs occurs via an interaction with the C terminus of $\alpha$-tubulin, thus on the outer surface of the MT lattice (47). However, the affinity of MEC-17 for MTs is not affected by MT acetylation (47), suggesting that the decrease of MEC-17 we observed in MT-containing fractions upon stress would be stimulated, perhaps by phosphorylation.

Interestingly, two-dimensional gel electrophoresis of GFP-MEC-17 reveals many spots that resist phosphatase treatment, indicating that MEC-17 presents other post-translational modifications that change the pl of the enzyme and that could modulate its activity. Recently, Kalebic et al. (48) showed that MEC-17 autoacetylation increases its catalytic activity toward $\alpha$-tubulin.

We found that part of cytoplasmic AMPK binds to MTs and is likely to be activated in situ. This localization, together with the subcellular organization of mitochondria by MTs, suggests that MT hyperacetylation could be controlled and organized locally on MTs. Such integration of the organelles and molecules that control the level of tubulin acetylation in MTs may thus provide cells with an effective adaptive mechanism in case of exposure to stress. The differences we measured in cell survival in response to NaCl exposure show that MT hyperacetylation is not only an important hallmark of stress but also actively participates in cell adaptation and survival.

In the future, it will be important to distinguish which function(s) involved in the control of cell survival actually require MTs being hyperacetylated and which involve only the presence of MEC-17 on/inside MTs. Interestingly, the wide range of tubulin acetylation that can be covered by MTs between basal conditions and stress situations also suggests that hyperacetylation can affect highly dynamic and stable MTs to similar extents. It will thus be important to determine precisely how boosting tubulin acetylation may affect MT dynamic instability. For example, NaCl-mediated stress seems to affect MT stability and organization at least transiently, because EBI1 comets are lost during the first minutes of stress (30).

That most of the cellular MTs can be hyperacetylated very early after stress induction suggests that increased acetylated tubulin level in MTs may occur faster than the time required to break down and rebuild the whole dynamic MT network. Such rapid kinetics of acetylation virtually excludes that hyperacetylation would happen only upon the elongation of new MTs. In addition, our counterintuitive result that exogenous MEC-17

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decreases upon stress from MT-containing fractions reveals that the enzyme is not statically present on MTs but can rather undergo rapid exchange from the MT. In our observations, we only see GFP-MEC-17 loss from MTs. This suggests that all the MT-bound MEC-17 is released from binding to tubulin once the enzyme reaction is complete. Alternatively, the GFP-tagged MEC-17 isoform we followed could behave differently from the endogenous enzymes, which could be recruited to MTs and exchanged against GFP-MEC-17 during stress. Given the impossibility of detecting MEC-17 using almost all the commercially available antibodies against C6ORF134/αTAT-1/MEC-17, we failed to test this hypothesis. Also, because we failed to render the MEC-17 cDNA resistant to RNAi despite all the mutations we successfully introduced in the sequences targeted by the siRNA, we could not follow the dynamics of GFP-MEC-17 alone in stressed cells. Tubulin structure shows that the Lys-40 of α-tubulin belongs to a loop that is exposed at the opposite of the surface helices, suggesting it is located in the MT lumen (52). The Lys-40 loop is also readily accessible from the inner of the MT as shown by the binding of anti-acetyl tubulin antibodies (10). MEC-17 was shown to directly interact with α-tubulin (48), most likely through a direct recognition of the Lys-40 loop (53). Acetylation of tubulin in MT, i.e. in polymerized rather than in a soluble form (21, 48), thus raises the question as to how MEC-17 accesses the Lys-40 loop to bind to its substrate. MEC-17 may access the lumen through the apertures at the plus and minus ends of MTs. Alternatively, the acetylase may bind the MT surface between protofilaments and access the Lys-40 loop through the MT wall and/or through defects of the MT lattice. Such modes of interaction between MEC-17 and tubulin would more easily account for the dynamics of MEC-17 and of MT acetylation we observed during cell stress.

Although it has only been uncovered for a few years, MT hyperacetylation thus appears as a core adaptive event in cell response to a wealth of stresses. It also reinforces the notion that the MT cytoskeleton is a central structure that coordinates and organizes signaling in the cytoplasm, in relation with membrane-bound organelles like mitochondria. Our findings demonstrate that acetylation of Lys-40 is a highly plastic post-translational modification of tubulin, which may rapidly alter MT function to promote cell survival. Other well known tubulin modifications like detyrosination are not affected by cell stress like nutrient deprivation (18), but we could not exclude that other modifications (unidentified yet) could be triggered by stress and could also participate in the control of MT functions.

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