Chorein interacts with α-tubulin and histone deacetylase 6, and overexpression preserves cell viability during nutrient deprivation in human embryonic kidney 293 cells

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ABSTRACT: The autophagy pathway has recently been implicated in several neurodegenerative diseases. Recently, it was reported that chorein-depleted cells showed accumulation of autophagic markers and impaired autophagic flux. Here, we demonstrate that chorein overexpression preserves cell viability from starvation-induced cell death in human embryonic kidney 293 (HEK293) cells. Subsequent coimmunoprecipitation and reverse coimmunoprecipitation assays using extracts from chorein that stably overexpressed HEK293 cells revealed that chorein interacts with α-tubulin and histone deacetylase 6, a known α-tubulin deacetylater and central component of basal autophagy. Indeed, acetylated α-tubulin immunoreactivity was significantly decreased in chorein that stably overexpressed HEK293 cells. These results suggest that chorein/histone deacetylase 6/α-tubulin interactions may play an important role in starvation-induced cell stress, and their disruption may be one of the molecular pathogenic mechanisms of chorea-acanthocytosis.—Sasaki, N., Nakamura, M., Kodama, A., Urata, Y., Shiokawa, N., Hayashi, T., Sano, A. Chorein interacts with α-tubulin and histone deacetylase 6, and overexpression preserves cell viability during nutrient deprivation in human embryonic kidney 293 cells. FASEB J. 30, 3726–3732 (2016). www.fasebj.org

KEY WORDS: chorea-acanthocytosis · starvation · autophagy · acetylated tubulin · *VPS13A*

Chorea-acanthocytosis (ChAc; Online Mendelian Inheritance in Man database ID: 200150; National Institutes of Health, Bethesda, MD, USA; *http://www.omim.org*) is a rare autosomal recessive neurodegenerative disorder that is characterized by Huntington disease–like progressive hyperkinetic movement disorder and erythrocyte acanthocytosis. The main pathologic feature of ChAc is striatal neurodegeneration (1). ChAc is inherited in an autosomal recessive pattern and is caused by loss-of-function mutations in the vacuolar protein sorting 13 homolog A (*VPS13A*) gene that encodes a 360-kDa protein named chorein (2, 3). The *VPS13A* gene is located on human chromosome 9q21 and spans an approximately 250-kb region. Pathogenic mutations are widely distributed throughout the *VPS13A* gene, and chorein is absent or markedly reduced in patients with ChAc with these mutations (4, 5).

Little is known about the function of chorein at the molecular level. Chorein is highly expressed in mouse testis, kidney, spleen, and brain (6). Subcellular distribution studies indicate that chorein localizes to the Golgi apparatus in microsomal fractions and to dense-core vesicles in synaptosomes (6, 7). The *Saccharomyces cerevisiae* homolog, *VPS13p*, is required for proper trafficking between the *trans*-Golgi network and the prevacuolar compartment (8). The mutant *TipC* gene in *Dictyostelium discoideum*, an ortholog of *Vps13p*, displays abnormal cell-sorting behavior (9). Recently, *Dictyostelium discoideum* cells that lacked TipC displayed a reduced number of autophagosomes and an impaired autophagic degradation (10). VPS13A protein in *Tetrahymena thermophila* (TtVPS13A) is required for phagocytosis (11). Furthermore, in the PC12 pheochromocytoma cell line, chorein is involved in dopamine release (7). Recently, we found that chorein interacts with β-adducin and β-actin, both of which are components of the cytoskeleton (12). Altogether, these findings suggest that chorein interacts with cytoskeletal proteins and is involved in intracellular transport and vesicle-mediated sorting. In the ChAc model mouse,

ABBREVIATIONS: ChAc, chorea-acanthocytosis; co-IP, coimmunoprecipitation; HDAC6, histone deacetylase 6; HEK293, human embryonic kidney 293; LDS, lithium dodecyl sulfate; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS-T, PBS with 0.1% Tween-20; SQSTM1, sequestome-1; VPS13A, vacuolar protein sorting 13 homolog A

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deficiency in chorein function leads to apoptosis of striatal neurons (13). Although there have been a few reports on the biologic function of chorein, its detailed physiologic mechanism remains unclear.

Autophagy is reported to play a protective role in several neurodegenerative diseases, such as Alzheimer's disease, Huntington's disease, and Parkinson's disease (14). Autophagy is up-regulated in response to stress and signals as a result of nutrient deprivation, growth factor deprivation, ER stress, and pathogen infection. Recently, the importance of histone deacetylase 6 (HDAC6)–regulated tubulin acetylation in autophagy dynamics has been reported (15–17).

In this study, we demonstrate that chorein overexpression preserves cell viability after nutrient deprivation in human embryonic kidney 293 (HEK293) cells. To determine whether chorein interacts with related autophagic machinery proteins, we performed immunoprecipitation and immunoblot analysis by using chorein that stably overexpressed cells and we identified α-tubulin and HDAC6 as chorein interactors.

MATERIALS AND METHODS

Chemicals and antibodies

Rabbit polyclonal anti-chorein antibody was generated as described previously (6). Rabbit polyclonal antibody against chorein (NBP1-85641) was obtained from Novus Biologicals (Littleton, CO, USA). Rabbit monoclonal antibodies against α-tubulin (11H10; CST#2125), β-tubulin (9F3; 2128), LC3B (D11; 3868), and acetyl-α-tubulin (Lys40; D20G3; 5335) were obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit monoclonal antibody against sequestome-1 (SQSTM1; TA307334) was obtained from OriGene (Rockville, MD, USA). Nocodazole, Krebs-ringer bicarbonate buffer, tubacin, chloroquine, mouse monoclonal anti-acetylated tubulin antibody (6-11B-1; T7451), and β-actin (A1978) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibody against HDAC6 (H-300; sc-11420) and normal mouse IgG (sc-2025) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody against LC3 (M186-3) was obtained from MBL (Nagoya, Japan), and α-tubulin (ab7291) was obtained from Abcam (Cambridge, United Kingdom). Wortmannin was obtained from Wako (Osaka, Japan). Anti-rabbit IgG, horseradish peroxidase–linked species-specific, and anti-mouse IgG, horseradish peroxidase–linked species-specific (both obtained from GE Healthcare, Little Chalfont, United Kingdom), were used as secondary antibodies.

Cell culture and generation of stably transfected cell lines

HEK293 cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan). Cell lines that stably overexpressed full-length chorein with an Myc tag were generated and cultured as previously described (12).

ChAc model mice and mouse brain preparation

ChAc model mice encoding a human disease mutation with deletion of exons 60–61 in *Vps13a* were produced by gene targeting as previously described (13). ChAc model mice were backcrossed for at least 10 generations on an FVB background (Clea Japan, Tokyo, Japan). Brain striatum tissue was obtained from FVB wild-type (+/+) and ChAc model mice with the homozygous deletion genotype (−/−). Mice were used at 24–34 wk as young mice or at 95–96 wk as old mice. Tissue was homogenized in 5 volumes of ice-cold lysis buffer (pH 7.4, PBS, 0.5% NP-40, 1× protease inhibitor cocktail, 0.5 mM PMSF) with a standard Dounce homogenizer and was centrifuged at 13,000 *g* at 4°C for 20 min. Soluble fractions were subjected to NuPAGE followed by immunoblot analysis. This study was approved by the Committee on Animal Experimentation of Kagoshima University, and was carried out in accordance with its guidelines.

Immunoprecipitation

Coimmunoprecipitation (co-IP) and reverse co-IP assays were performed by using the Pierce co-IP c-Myc-Tag IP/co-IP Kits (Thermo Fisher Scientific, Waltham, MA, USA). HEK293 cells or HEK293 cells that stably overexpressed chorein and were grown on 100-mm-diameter dishes were lysed in 500 μl of Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) with 1× protease inhibitor cocktail (M-PER; Roche Diagnostics, Indianapolis, IN, USA) and 0.5 mM PMSF. Total protein concentrations were quantified by using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Equivalent amounts of soluble fractions from cell lysates (input) were incubated overnight at 4°C with antibody-immobilized beads. Part of the soluble supernatant from cell lysates from HEK293 cells that stably overexpressed chorein was divided into 2 equal aliquots, one of which was incubated with anti–α-tubulin antibody or anti-HDAC6 antibody, and the other with IgG control, each immobilized on the AminoLink Plus Coupling Resin (Thermo Fisher Scientific). Beads were then centrifuged and washed 3 times with PBS that contained 0.1% Tween-20 (PBS-T) or lysis buffer before eluting. Protein samples eluted in NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Thermo Fisher Scientific) were heated to 99°C for 5 min. Samples of input proteins and elutes were analyzed by immunoblot analysis.

Immunoblot analysis

Cell and striatum tissue lysates were prepared as previously described. Total cell and tissue extracts were denatured in NuPAGE LDS sample buffer, separated on NuPAGE gels (Thermo Fisher Scientific), and electrophoretically transferred to PVDF membranes (GE Healthcare). Membranes were blocked overnight at 4°C or for 1 h at room temperature with nonfat dried milk in PBS-T and were incubated overnight at 4°C or 1 h at room temperature with primary antibodies for each target protein in PBS-T milk. For detection of LC3 and SQSTM1, protein lysates were prepared from HEK293 cells or HEK293 cells that stably overexpressed chorein by using LDS sample buffer that contained M-PER Mammalian Protein Extraction Reagent, 1× protease inhibitor cocktail, 0.5 mM PMSF, 4× LDS, and 2.5% 2-ME. Those lysates were separated on NuPAGE gels and were electrophoretically transferred to PVDF membranes as above. Membranes were blocked overnight at 4°C with 10% nonfat dried milk in PBS and were incubated for overnight at 4°C or 1 h at room temperature with primary antibodies for each target protein in PBS-T milk. After rinsing in PBS-T, membranes were incubated with the appropriate secondary antibodies for 1 h at room temperature. Proteins were visualized by using ECL Prime Western Blotting Detection Reagent (GE Healthcare), and images were recorded by digital analyzer (Fujifilm LAS-1000; Fujifilm, Tokyo, Japan).

Starvation assays

Each dish was washed 3 times by using Krebs-ringer bicarbonate buffer to remove the medium, then incubated in Krebs-ringer

bicarbonate buffer for the indicated time. Autophagosome maturation was blocked by using chloroquine at a final concentration of 50 μM in Krebs-ringer bicarbonate buffer. Phosphatidylinositol 3-kinase catalytic subunit type 3 was blocked by addition of 0.2 μM wortmannin in Krebs-ringer bicarbonate buffer.

Cell viability assay

Approximately 25 × 10^3 cells from the HEK293 cell line that stably overexpressed chorein and the mock-transfected control cell line were plated in 96-well plates and were allowed to attach for approximately 18 h. Relevant mixtures of additives (*e.g.*, 10 μM nocodazole, 20 μM tubacin) were added at time 0. For starvation assay, each well was washed 3 times by using Krebs-ringer bicarbonate buffer to remove the medium, then incubated in Krebs-ringer bicarbonate buffer for the indicated time. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), inner salt reagent (20 μl; Promega Cell Titer 96 Aqueous One Solution; Promega, Southampton, United Kingdom) was added directly to each well, and the plates were incubated at 37°C with 5% CO_2 atmosphere for 3 h. Absorbance of the solution was read at 490 nm by using a plate reader. Each assay was performed in triplicate. Results were expressed as a percentage of the control, and statistical significance was determined by using an independent 2-sample Student's *t* test.

Statistics

Data are presented as means ± 95% confidence interval or ± SD. Two-sample Student's *t* tests were performed accordingly on different sets of data. Differences were considered significant at $P < 0.05$.

RESULTS

Increased viability of HEK293 cells that stably overexpressed chorein during nutrient deprivation

We previously generated HEK293 cells that stably overexpressed Myc-DDK–tagged chorein (HEK293 cells stably overexpressing chorein) (12). We used the MTS assay to compare cell viability between HEK293 cells that stably overexpressed chorein and mock-transfected control cells after nutrient deprivation at 0, 6, 18, and 24 h. During nutrient deprivation, HEK293 cells that stably overexpressed chorein showed significantly increased cell viability compared with mock-transfected control cells (**Fig. 1**). We semiquantitated the autophagosome markers, microtubule-associated proteins LC3-I and LC3-II, by immunoblotting. The LC3-II/LC3-I ratio was significantly higher in cells that stably overexpressed chorein than in mock-transfected control cells after nutrient deprivation for 6 h (**Fig. 2*A*, *C***). To confirm autophagy, we treated cells that stably overexpressed chorein and mock-transfected control cells with chloroquine and wortmannin. Chloroquine is a lysosomal inhibitor that blocks autophagic degradation and accumulates LC3-II. The LC3-II/LC3-I ratio expression level was significantly increased in chloroquine-treated cells (Fig. 2*B*). Wortmannin is a phosphatidylinositol 3-kinase inhibitor that blocks the formation of autophagosomes. LC3-II/LC3-I ratio was not increased in wortmannin-treated cells (Fig. 2*B*). Moreover, to confirm autophagic induction, we performed immunoblot analysis of SQSTM1 (also known as p62), which is commonly used as an indicator of autophagic flux. Immunoreactivity of SQSTM1 was significantly decreased after nutrient deprivation in cells that stably overexpressed chorein (Fig. 2*D*, *E*).

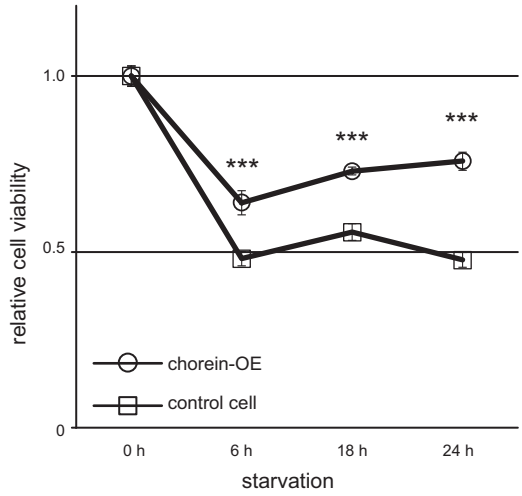

Figure 1. Chorein overexpression preserves cell viability during nutrient deprivation. Cell viability during nutrient deprivation in HEK293 cells that stably overexpressed chorein (chorein-OE) and mock-transfected control cells (control cell). Each well was washed 3 times by using Krebs-ringer bicarbonate buffer to remove the medium, then incubated in Krebs-ringer bicarbonate buffer for 0, 6, 18, and 24 h before performing MTS assays in triplicate. Values are means ± SD. ***$P < 0.001$ (2-sample Student's *t* test), HEK293 cells that stably overexpressed chorein compared with mock-transfected control cells.

Chorein interacts with α-tubulin and is involved in microtubule stabilization

To examine the association between tubulin and chorein, we performed co-IP and reverse co-IP assays by using anti–c-Myc and anti–α-tubulin antibodies. Cell lysates extracted from HEK293 cells that stably overexpressed chorein and mock-transfected control cells after nutrient deprivation at 0, 1, 6, and 24 h were immunoprecipitated with anti–c-Myc antibody and immunoblotted with anti–α-tubulin and anti–β-tubulin antibody. Distinctive α-tubulin bands were observed in chorein immunoprecipitates, but no positive β-tubulin bands were detected (**Fig. 3*A***). Unequivocal chorein bands were also observed in α-tubulin immunoprecipitates with or without starvation (Fig. 3*B*). These results suggest that chorein interacts with α-tubulin.

We compared cell viability between HEK293 cells that stably overexpressed chorein and mock-transfected control cells treated with or without nocodazole. After nocodazole treatment, cell viability significantly increased in cells that stably overexpressed chorein compared with mock-transfected control cells (**Fig. 4**).

Chorein interacts with HDAC6 and promotes tubulin deacetylation

We determined whether chorein interacts with the major α-tubulin deacetylase, HDAC6, in HEK293 cells.

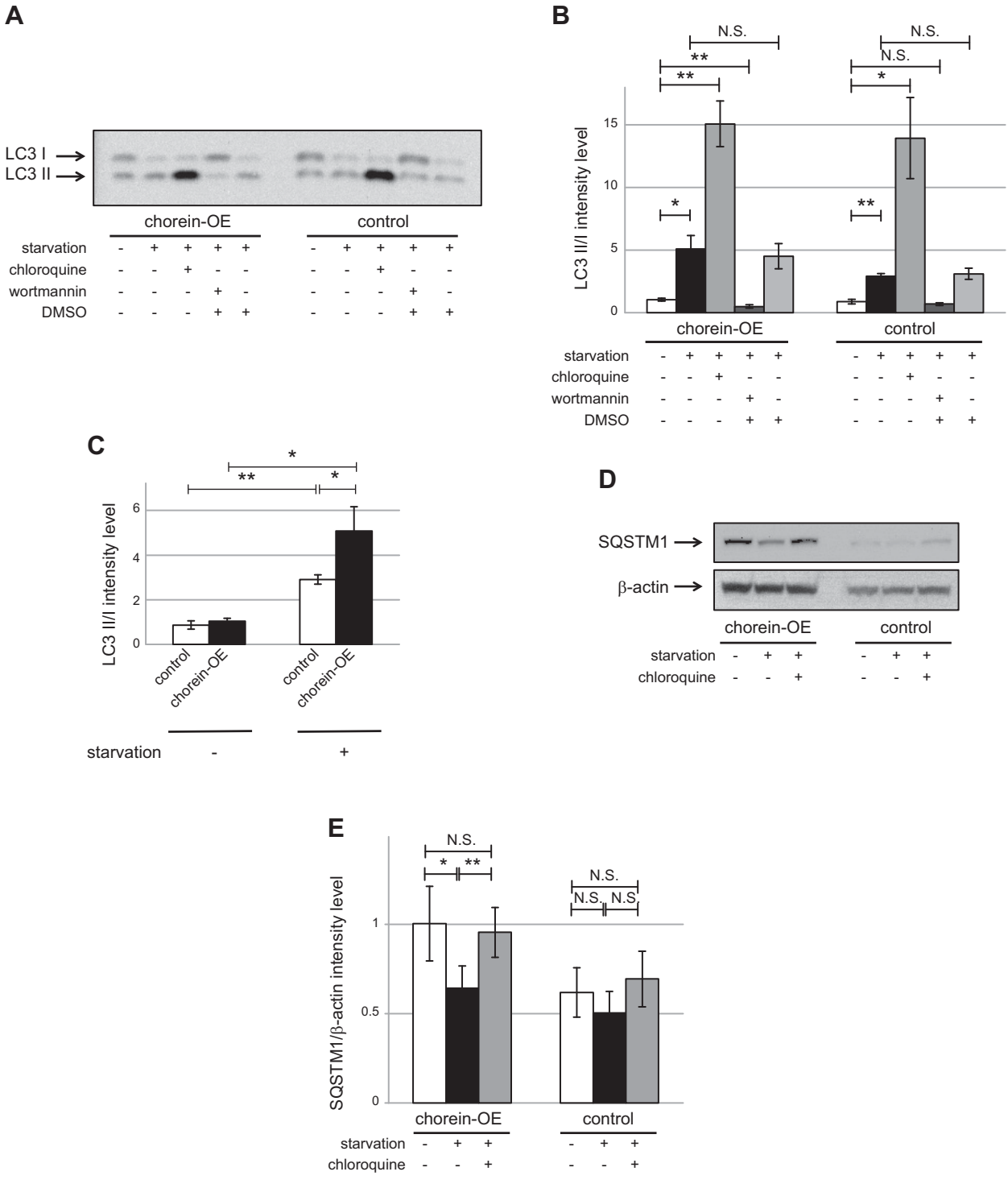

Figure 2. Chorein overexpression induces autophagy. *A*) LC3-I and -II were analyzed by immunoblot analysis in cell lysates prepared from HEK293 cells that stably overexpressed chorein (chorein-OE) and mock-transfected control cells after nutrient deprivation for 6 h, with or without chloroquine and wortmannin. Of cell lysate, 10 μg was loaded. *B*) For LC3-II/LC3-I ratio, densitometric analysis was performed ($n = 3$). *C*) Densitometric analysis for comparison of LC3-II/LC3-I ratio between chorein-OE and control cells before/after nutrient deprivation ($n = 3$). *D*) SQSTM1 was analyzed by immunoblot analysis in cell lysates prepared from HEK293 cells the stably overexpressed chorein and mock-transfected control cells after nutrient deprivation for 6 h, with or without chloroquine. Of cell lysate, 10 μg was loaded. *E*) For SQSTM1/β-actin ratio analysis, densitometric analysis was performed ($n = 9$). N.S., not significant. Values are means ± 95% confidence interval. $^*P < 0.05$; $^{**}P < 0.01$ (2-sample Student's t test).

Cell lysates extracted from HEK293 cells that stably overexpressed chorein and mock-transfected control cells after nutrient deprivation at 0, 1, 6, and 24 h were immunoprecipitated with anti–c-Myc antibody and immunoblotted with anti-HDAC6 antibody. Positive HDAC6 signal was observed in chorein immunoprecipitates (**Fig. 5*A***). Similarly, positive chorein signal was observed in HDAC6 immunoprecipitates with or without starvation (Fig. 5*B*).

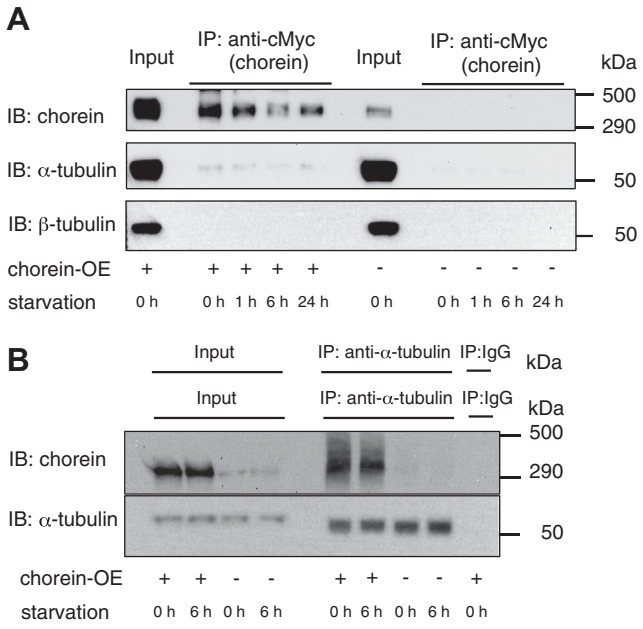

Figure 3. Chorein coimmunoprecipitates with α-tubulin. *A*) co-IP assay using HEK293 cells that stably overexpressed Myc-tagged chorein (chorein-OE) after nutrient deprivation at 0, 1, 6, and 24 h were performed by using anti-c-Myc antibody. Immunoblot (IB) analysis used anti-chorein, anti–α-tubulin, and anti–β-tubulin antibodies. Chorein coimmunoprecipitated with α-tubulin but not β-tubulin. *B*) Reverse co-IP assay with anti–α-tubulin antibody after nutrient deprivation at 0 and 6 h confirmed the interaction between chorein and α-tubulin.

Moreover, acetylated tubulin immunoreactivity was significantly lower in HEK293 cells that stably overexpressed chorein (**Fig. 6**). These results suggest that the chorein/HDAC6 interaction may promote α-tubulin deacetylation in HEK293 cells that stably overexpressed chorein.

Increased viability of HEK293 cells that stably overexpressed chorein during nutrient deprivation in the presence of HDAC6 inhibitor

To determine whether α-tubulin deacetylation by HDAC6 affects cell viability of HEK293 cells that stably overexpressed chorein under starvation conditions, we measured cell viability by MTS assay with or without tubacin, a selective HDAC6 inhibitor that directly inhibits HDAC6-mediated α-tubulin deacetylation. During nutrient deprivation, tubacin-treated HEK293 cells that stably overexpressed chorein showed significantly increased cell viability compared with mock-transfected control cells (Supplemental Fig. S1*A*). Treatment with tubacin increased the immunoreactivity of acetylated tubulin by immunoblot analysis in both HEK293 cells that stably overexpressed chorein and mock-transfected control cells (Supplemental Fig. S1*B*).

Autophagic activity and acetylated tubulin in the striatum of ChAc mouse model

To examine autophagic activity and α-tubulin acetylation, we performed LC3, SQSTM1, and acetylated tubulin immunoblot analyses in the striatum of ChAc mouse model. Striatum tissue lysates were extracted from ChAc model mice and wild-type mice SQSTM1. There were no remarkable differences in the immunoreactivity of SQSTM1 and LC3 between ChAc model mice and wild-type mice (data not shown). There was no difference in the acetylated tubulin immunoreactivity in ChAc model mice and in wild-type mice; however, the acetylated tubulin immunoreactivity of young ChAc model mice was significantly lower than that of old ChAc model mice (Supplemental Fig. S2).

DISCUSSION

Autophagy is a cellular self-degradation pathway for cellular proteins and organelles that are enclosed by an isolation membrane to form an autophagosome. Autophagosomes fuse with lysosomes or vacuoles for breakdown by hydrolases in the autolysosome (18). The LC3-II/LC3-I ratio correlates with autophagosome number (19). SQSTM1 (p62) is one of the best-known selective substrates of autophagy and is widely used as an indicator of autophagic degradation (20). Recently, it was reported that chorein-depleted cells showed an accumulation of autophagic markers and impaired autophagic flux (10). Here, HEK293 cells that stably overexpressed chorein were resistant to cell death induced by nutrient deprivation *via* up-regulation of autophagy. This suggests that chorein may play a protective role in cell death induced by autophagy. We identified α-tubulin as a chorein interacter by using co-IP and reverse co-IP assays (Fig. 3). Cell viability assays revealed that cells that stably overexpressed chorein are resistant to cell death induced by microtubule depolymerization with nocodazole. Microtubules, a

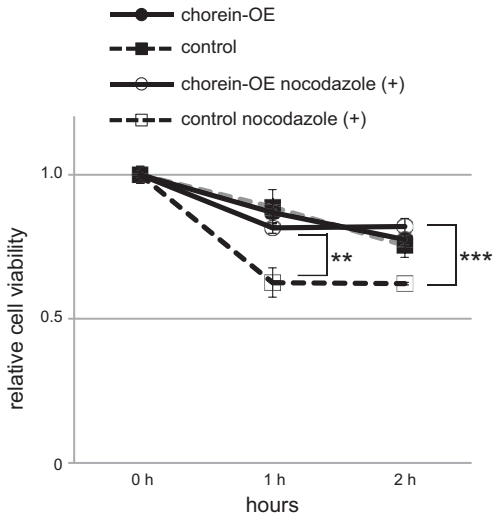

Figure 4. Chorein overexpression preserves cell viability after microtubule depolymerization. Cell viability after DSMO or 10 μM nocodazole treatment for 0, 1, and 2 h in cells that stably overexpressed chorein (chorein-OE) and mock-transfected control cells (control), estimated from MTS assays performed in triplicate. Values are means ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$ (2-sample Student's *t* test) nocodazole-treated cells that stably overexpressed chorein compared with mock-transfected control cells.

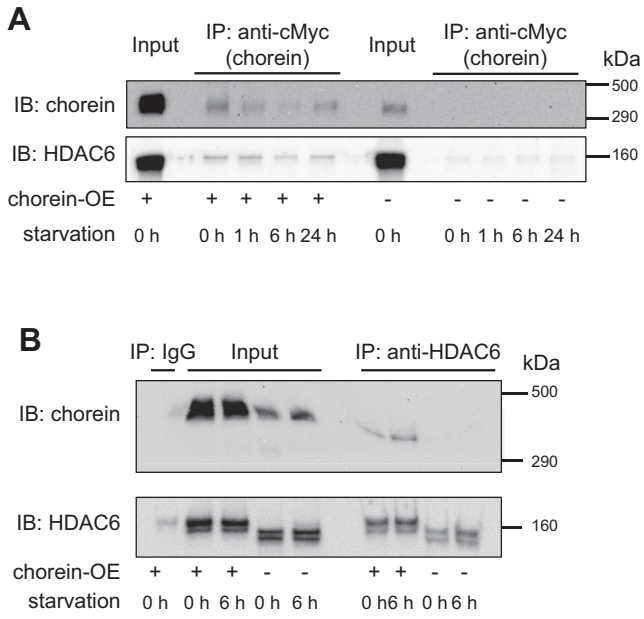

Figure 5. Chorein coimmunoprecipitates with HDAC6. *A*) co-IP assay using HEK293 cells that stably overexpressed Myc-tagged chorein (chorein-OE) after nutrient deprivation at 0, 1, 6, and 24 h was performed with anti–c-Myc antibody. Immunoblot (IB) analysis used anti-chorein and anti-HDAC6 antibodies. Chorein was coimmunoprecipitated with HDAC6. *B*) Reverse co-IP assay using the same cell after nutrient deprivation at 0 and 6 h with anti-HDAC6 antibody confirmed the interaction between chorein and HDAC6. As a negative control, cells that stably overexpressed chorein were immunoprecipitated by using control beads without antibody.

platform for intracellular transport, are involved in autophagosome formation and motility (15). Nocodazole acts by binding to tubulin subunits and preventing microtubule polymerization. Recent studies have shown that disassembling microtubules with nocodazole prevents autophagosome formation (16). These observations suggest that the chorein–α-tubulin interaction is involved in microtubule polymerization and/or depolymerization.

We show a chorein–HDAC6 interaction and increased tubulin deacetylation in cells that stably overexpressed chorein. HDAC6, which also binds ubiquitin and interacts directly with microtubules, is involved in autophagic degradation (21, 22). HDAC6 has been shown to modulate α-tubulin acetylation, which has an important impact on the microtubule network (17). Acetylation of α-tubulin by HDAC6 may contribute to microtubule stability (23). In addition, impaired mitochondrial transport and protein aggregate elimination are common features in various neurodegenerative diseases and are linked to both deacetylase and ubiquitin ligase activities of HDAC6 (23). Taken together, this suggests that chorein is involved in the promotion of tubulin deacetylation mediated by HDAC6.

Specific HDAC6 inhibitors are reported to be neuroprotective by increasing α-tubulin acetylation levels, with subsequent improvement in axonal transport (23); however, currently, treating neurodegenerative diseases by using HDAC6 inhibitors has not been established. In contrast, HDAC6 induction theoretically contributes to protein aggregate degradation (23). For example, tubulin acetylation is increased in the brains of patients with Alzheimer's disease. Tau binds to HDAC6 and is a HDAC6 inhibitor, which prevents autophagy induction by inhibiting proteasome function (24). Septins provide a physical scaffold for HDAC6 to achieve efficient microtubule deacetylation, thereby negatively regulating microtubule stability to an optimal level for neuritogenesis (25). From these findings, a balance between acetylation and deacetylation is important to maintain quality control in neuronal cells. In this study, ChAc model mouse, which harbors loss of chorein function mutations, demonstrated age-dependent variable acetylation of α-tubulin, which indicates that chorein may adjust α-tubulin acetylation to maintain striatal neuron. Tubacin, a selective HDAC6 inhibitor, directly inhibits tubulin deacetylation activity of HDAC6 (26). Unexpectedly, treatment of cells that stably overexpressed chorein with tubacin significantly increased cell viability compared with tubacin-treated mock-transfected control cells during nutrient deprivation by unknown mechanism. This study revealed that chorein interacts with HDAC6 and α-tubulin and is involved in acetylation of α-tubulin. As it is well known that these molecules have important roles in autophagy, chorein may be involved in autophagy. These results suggest that chorein is involved in clearance of subcellular organelles *via* autophagy to starvation-induced cell death resistance, which may involve chorein interactions with α-tubulin and HDAC6. Although further studies are needed to reveal chorein function in neuronal cells, chorein may play an important

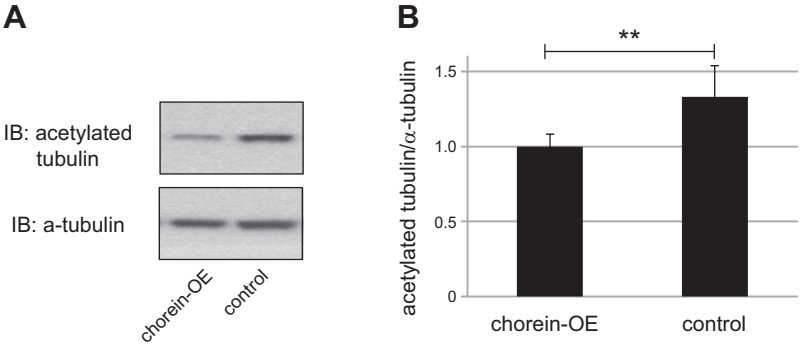

Figure 6. Chorein overexpression decreases acetylated tubulin. *A*) Acetylated tubulin was analyzed by immunoblot (IB) analysis in cell lysates prepared from HEK293 cells that stably overexpressed chorein (chorein-OE) and mock-transfected control cells. *B*) For the acetylated tubulin/α-tubulin ratio, densitometric analysis was performed ($n = 6$). Values are means ± SD. $^{**}P < 0.01$ (2-sample Student's t test).

role in the maintenance of neuronal cellular quality. Disrupting these molecular events by loss of chorein function may be a molecular pathogenic mechanism of ChAc. FJ

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AUTHOR CONTRIBUTIONS

N. Sasaki, M. Nakamura, and A. Sano designed research; N. Sasaki, M. Nakamura, Y. Urata, N. Shiokawa, T. Hayashi, and A. Sano analyzed data; N. Sasaki, A. Kodama, Y. Urata, and N. Shiokawa performed research; and N. Sasaki and M. Nakamura wrote the paper.

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Chorein interacts with α-tubulin and histone deacetylase 6, and overexpression preserves cell viability during nutrient deprivation in human embryonic kidney 293 cells

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Figure S1

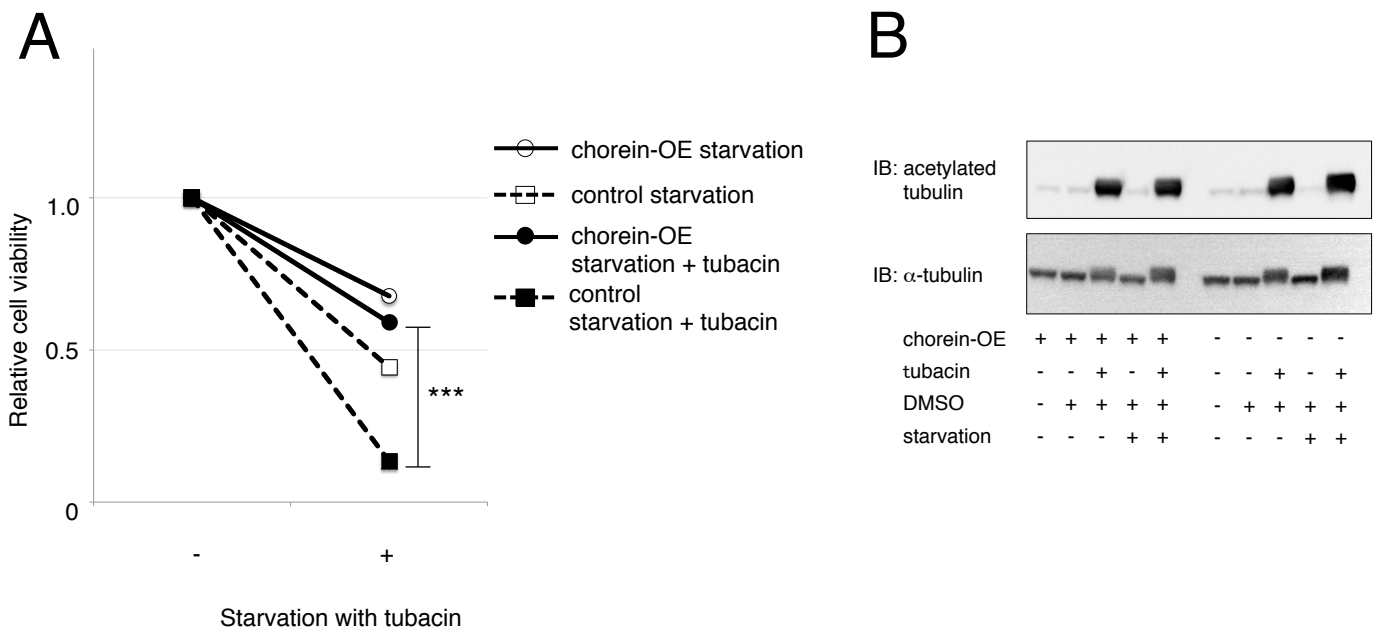

Figure S1. Increased viability of HEK293 cells stably overexpressing chorein during nutrient deprivation in the presence of HDAC6 inhibitor

A) Cell viability after nutrient deprivation for 6 h and treatment with or without 20μM tubacin was estimated from MTS assays performed in triplicate. Values are mean ± S.D. ***$P < 0.001$ (two-sample *t*-test). B) Acetylated tubulin with or without tubacin treatment were analyzed by immunoblot analysis in cell lysates prepared from HEK293 cells stably overexpressing chorein (chorein-OE) and mock-transfected control cells (control) after nutrient deprivation at 0h and 6h.

Figure S2

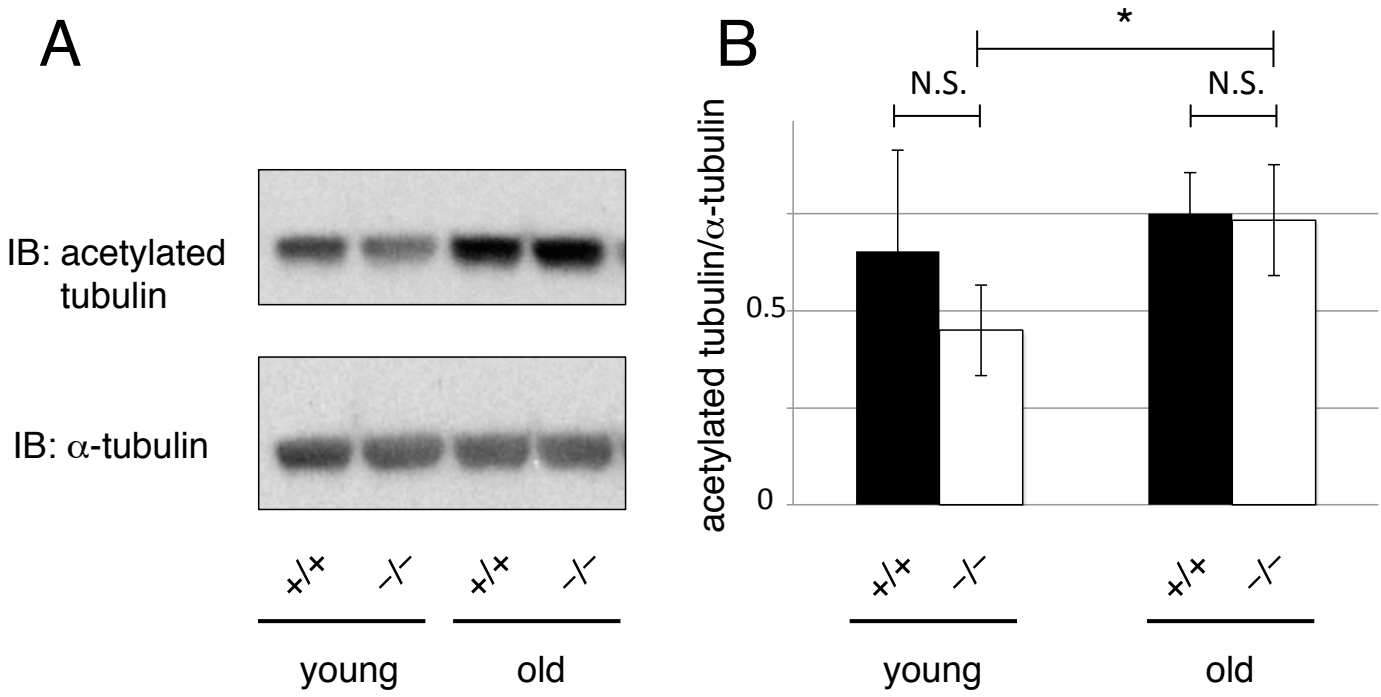

Figure S2. The immunoreactivity of acetylated tubulin in ChAc model mice striatum
A) Acetylated tubulin was analyzed by immunoblot analysis in striatum tissue lysates prepared from young- and old- ChAc model mice (-/-) and young- and old- wild-type mice (+/+). B) For analysis of the acetylated tubulin/a-tubulin ratio, densitometric analysis was performed (each n = 5). Values are mean ± 95%CI. **P* < 0.05 (two-sample *t*-test). N.S. indicates not significant.