Supplemental Materials and Methods

Plasmids. The HA-ubiquitin plasmid was a gift from Dr. Hal Moses (Vanderbilt University, Nashville, TN) and the HA_3-PP2Ac construct was a gift from Dr. David Brautigan (University of Virginia, Charlottesville, VA). The mammalian expression constructs encoding FLAG-MID1 and Myc-MID1 were described previously (20). Construction of the FLAG-tagged wild type and ΔUIM α4 mammalian expression constructs were also described previously (9). The N-terminal fragment of FLAG-α4 containing a stop codon at G256 (G256*) and the double point mutant of FLAG-α4 containing Leu residues in place of Ala52 and Ala53 (A52L/A53L) were generated using FLAG-α4/pcDNA5TO as a template and the Quick Change site-directed mutagenesis kit (Stratagene, LaJolla, CA) with the following primers: G256* forward 5'-AAC ATG GCT CAA GCC AAA GTA TTT TGA GCT GGT TAT CC-3'; G256* reverse 5'-GGA TAA CCA GCT CAA AAT ACT TTG GCT TGA GCC ATG TT-3'; A52L/A53L forward 5'-GCT TGG ACC TCC TTG AGA AGC TGC TGG AAA TGT TAT CGC AGC TCG ACT TGT TCA GCCG-3'; A52L/A53L reverse 5'-CGG CTG AAC AAG TCG AGC TGC GAT AAC ATT TCC AGC AGC TTC TCA AGG AGG TCC AAG C-3'. Proper construction of all plasmids was verified by automated sequencing (Vanderbilt University DNA core facility).

Antibodies and reagents. The mouse monoclonal PP2Ac antibody was from BD Biosciences (San Diego, CA). The rabbit polyclonal α4 antibodies and the rabbit polyclonal MID1 antibody were from Bethyl Laboratories (Montgomery, TX). Unless otherwise noted, Westerns were performed using an antibody directed against the N-terminus of α4; this antibody recognizes both full-length and cleaved α4. The mouse monoclonal ubiquitin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal Myc antibody and the mouse monoclonal FLAG antibody were from Cell Signaling (Billerica, MA) and Sigma-Aldrich (St. Louis, MO), respectively. The rabbit polyclonal phospho-tau and total tau antibodies were from Invitrogen (Carlsbad, CA). The E1 enzyme inhibitor PYR-41 was from EMD Chemicals (Gibbstown, NJ). The calpain inhibitors Z-Leu-Leu-CHO and calpeptin were from Enzo Life Sciences (Plymouth Meeting, PA) and EMD Chemicals (Gibbstown NJ), respectively. The siGENOME SMART pool human MID1-targeted siRNA was from Thermo Scientific (Lafayette, CO).

Human tissue. After receiving human subjects approval from the University of Washington human subjects division (HSD# 06-0492-E/A 01: Molecular Regulators of Tauopathy; Kraemer, Principal Investigator), we obtained de-identified samples of post-mortem temporal cortex tissue from AD and age-matched control cases from the University of Washington Alzheimer's Disease Research Center (ADRC) Neuropathology Core (Core Leader, Dr. Thomas Montine). The tissues were lysed by sonication in high salt reassembly buffer, RAB-High Salt (100 mM MES, pH 7.0, 1 mM EGTA, 0.5 mM $MgSO_4$, 750 mM NaCl, 20 mM NaF, 0.5 mM PMSF, 0.1% protease inhibitor cocktail). Protein samples were adjusted to 10 mM Tris-HCl, pH 6.8, 1 mM EDTA, 40 mM DTT, 1% SDS, and 10% sucrose by addition of 5X sample

buffer and boiled 5 min. Thirty µg of protein were loaded per lane onto 4-15% pre-cast criterion SDS-PAGE gradient gels (Bio-Rad) and immunoblotted per the manufacturer's instructions.

Cell culture and transfections. HEK293FT cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2 mM L-glutamine. HEK/tau stable cells (11) were grown under the same conditions as the HEK293FT cells except that the media was supplemented with 100 µg/ml zeocin to maintain tau selection. Human embryonic fibroblasts (HEFs) derived from a fetus with Opitz syndrome and from an age-matched control fetus were previously described (20), and grown in DMEM with 10% fetal calf serum and 2 mM L-glutamine. HEK293FT and HEK/tau cells were transfected with mammalian expression constructs using Fugene6 (Roche; Indianapolis, IN) according to the manufacturer's protocol. MID1-targeted siRNA was introduced into HEK293FT cells using Dharmafect (Thermo Scientific; Rockford, IL) according to the manufacturer's protocol.

MID1/E2 activity assay. The ability of MID1·E2 pairs to form polyubiquitin chains was determined using the E3LITE Customizable Ubiquitin Ligase Kit from LifeSensors (Malvern, PA). Briefly, increasing concentrations of immunopurified FLAG-MID1 were incubated with 20 nM E1 enzyme, 400 nM of the indicated E2 enzyme (LifeSensors, Malvern, PA), 4 µM ubiquitin, and 0.4 mM ATP for 60 min at room temperature in separate wells of a 96-well microtiter plate. The cells were subsequently washed and incubated for 60 min with a 1:1,000 dilution of the detection reagent followed by 60 min incubation with a 1:10,000 dilution of streptavadin-HRP. Polyubiquitin chain formation was measured using SuperSignal West Dura Extended Duration Substrate from Pierce (Rockford, IL) and a 96-well plate reader.

***In vitro* ubiquitination assays.** Some *in vitro* ubiquitination assays were performed using a ubiquitin-protein conjugation kit (BostonBiochem, Cambridge, MA) as described previously (9). For these experiments, purified FLAG-α4 or PP2Ac was incubated in ubiquitination assay buffer containing a mixture of E1/E2 enzymes (Fraction A) and either a mixture of E3 ligases (Fraction B) or FLAG-MID1. Ubiquitination assays were also performed using purified conjugation enzymes. These reactions consisted of 2.5 µM ubiquitin (BostonBiochem, Cambridge, MA), 100 nM E1 enzyme (Enzo Life Sciences, Plymouth Meeting, PA), 2.5 µM of the indicated E2 enzyme (LifeSensors, Malvern, MA), 100 nM FLAG-MID1, Mg-ATP solution (Enzo Life Sciences; Plymouth Meeting, PA), 50 mM DTT, 100 U/ml inorganic pyrophosphatase (Sigma, St. Louis, MO), and 1 µM FLAG-α4 and/or 50 ng PP2Ac. All reactions were incubated at 37°C for 1 h and terminated by the addition of SDS sample buffer.

Mass spectrometry (MS). FLAG-α4 was immunopurified from HEK293FT cells and subjected to SDS-PAGE. Full-length FLAG-α4 and the proteolytic fragment of FLAG-α4 were visualized by Colloidal Blue staining and excised from the gel. The gel pieces were cut into 1 mm cubes and equilibrated in 100 mM

NH_4HCO_3. Proteins in the gel pieces were reduced with DTT (1/10 volume of 45 mM DTT for 20 min at 50°C) followed by alkylation with iodoacetamide (1/10 volume of 100 mM iodoacetamide for 20 min in the dark at room temperature). The gel pieces were then dehydrated with acetonitrile and rehydrated with 15 µl of 12.5 mM NH_4HCO_3 containing 0.01 µg/µl trypsin (Trypsin Gold from Promega; Madison, WI); the trypsin digestions were carried out for >2 h at 37°C. Peptides were extracted with 60% acetonitrile and 0.1% formic acid, dried by vacuum centrifugation, and reconstituted in 15 µl 0.1% formic acid. Five µL of peptide hydrosylate were analyzed by C18 reverse-phase LC-MS/MS using a Thermo LTQ-XL Orbitrap ion trap tandem mass spectrometer equipped with an Eksigent autosampler and nanoLC ultra 1D-plus HPLC pump system, nanospray source, and Xcalibur 2.0 instrument control using standard data-dependent methods. Tandem MS data were analyzed with the Sequest algorithm.

Cycloheximide chase experiments. HEK293FT cells were seeded in six-well tissue culture plates (350,000 cells per well) and transfected with either HA_3-PP2Ac alone or together with FLAG-α4 WT, FLAG-α4 ΔUIM, or FLAG-α4 A52L/A53L. At 48 h post-transfection, cells were treated with 100 ug/ml cyclohexamide (Sigma; St. Louis, MO) for the indicated times. Cell lysates were then prepared and subjected to Western analysis.

Immunoprecipitations. HEK293FT cells expressing FLAG- or HA-tagged proteins were lysed in IP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Igepal, 5 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM PMSF, and 1 µg/ml leupeptin) and centrifuged at 12,000 x g for 10 min. Clarified lysates were incubated 20 µl of a 50% slurry of anti-HA agarose (Roche, Indianapolis, IN) overnight or anti-FLAG-agarose (Sigma, St. Louis, MO) for 4 h. The immune complexes were washed three times in 1 ml IP buffer, and bound proteins were eluted in SDS sample buffer and subjected to Western analysis. In some cases, the FLAG-tagged proteins were eluted from the beads by incubation for 60 min at 4°C in Tris-buffered saline (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl) containing 100 µg/ml FLAG peptide.

Standard Western analysis. SDS-solubilized protein samples were separated on 10% SDS-polyacrylamide gels (unless otherwise indicated) and transferred to 0.45 µm nylon-supported nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were blocked in Odyssey buffer (Li-COR; Lincoln, NE). All primary antibodies were used at a 1:1000 dilution in Tris-buffered saline containing 0.5% BSA and 0.1% Tween-20 (TTBS/BSA). For detection with the Odyssey Infrared Imaging system, the appropriate flouraphore-conjugated secondary antibodies were used at a 1:10,000 dilution in TTBS/BSA. Bound antibodies were visualized using the Odyssey Infrared Imaging system and Odyssey software (LI-COR; Lincoln, NE).

In cell Western analysis. HEK293/tau cells expressing FLAG-α4 wild type or FLAG-α4 G256* were fixed in a 96 well plate with 4% paraformaldehyde in 1X PBS for 20 min. The cells were permeabilized by washing 5 times with 0.1% Triton X-100 in 1X PBS (5 min washes). After the final wash, Odyssey

blocking buffer was added to wells for 90 min. The permeabilized cells were incubated with primary antibodies (diluted 1:1000 in Odyssey blocking buffer) overnight at 4°C, washed 5 times with 0.1% Triton X-100 in 1X PBS (5 min washes), and incubated with secondary antibodies (diluted 1:500 in Odyssey blocking buffer containing 0.2% Tween-20) for 60 min at room temperature. The cells were then washed 5 times with 0.1% Tween-20 in 1X PBS (5 min washes), and bound antibodies were visualized and quantified using the Odyssey Infrared Imaging system and Odyssey software.

Supplemental Figure Legends

Fig. S1. Multiple E2 enzymes do not allow for MID1-dependent polyubiquitination of PP2Ac. Purified PP2Ac was incubated in a ubiquitination assay solution containing a mixture of E1 and E2 enzymes and either a mixture of E3 ligases (*Fraction B*) or FLAG-MID1for 4 h at 37°C. The reactions were analyzed via Western using antibodies recognizing PP2Ac, FLAG-MID1 and ubiquitin.

Fig. S2. Identification of the *α*4 cleavage site by mass spectrometry. Tandem mass spectrum of the doubly-charged NMAQAKVF peptide (m/z 454.73). Labeled b-ions and y-ions are denoted by cleavage brackets above and below the sequence, respectively.

Fig. S3. *Drosophila* TAP42 is subject to both monoubiquitination and cleavage. (A) *Drosophila* TAP42 is subject to monoubiquitination. Ubiquitinated proteins (*HA-IPs*) were isolated from extracts of *Drosophila* S2 cells expressing either Myc-TAP42 or FLAG-TAP42 alone or together with HA-Ub. The HA-IPs and cell lysates were analyzed by Western using antibodies recognizing the FLAG and Myc epitopes. (B) *Drosophila* TAP42 undergoes C-terminal cleavage. *Drosophila* S2 cells expressing FLAG-TAP42 were treated with vehicle or 100 µg/ml cycloheximide and lysed at the indicated time points. Lysates were analyzed by Western using antibodies recognizing FLAG-TAP42.

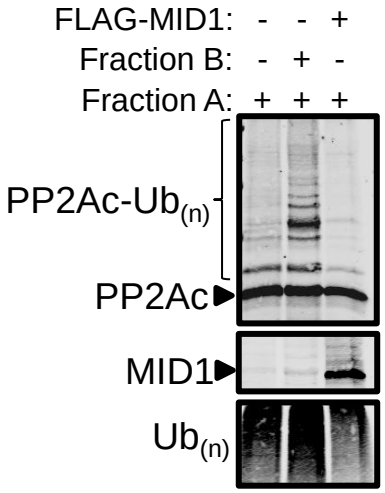

Fig. S1

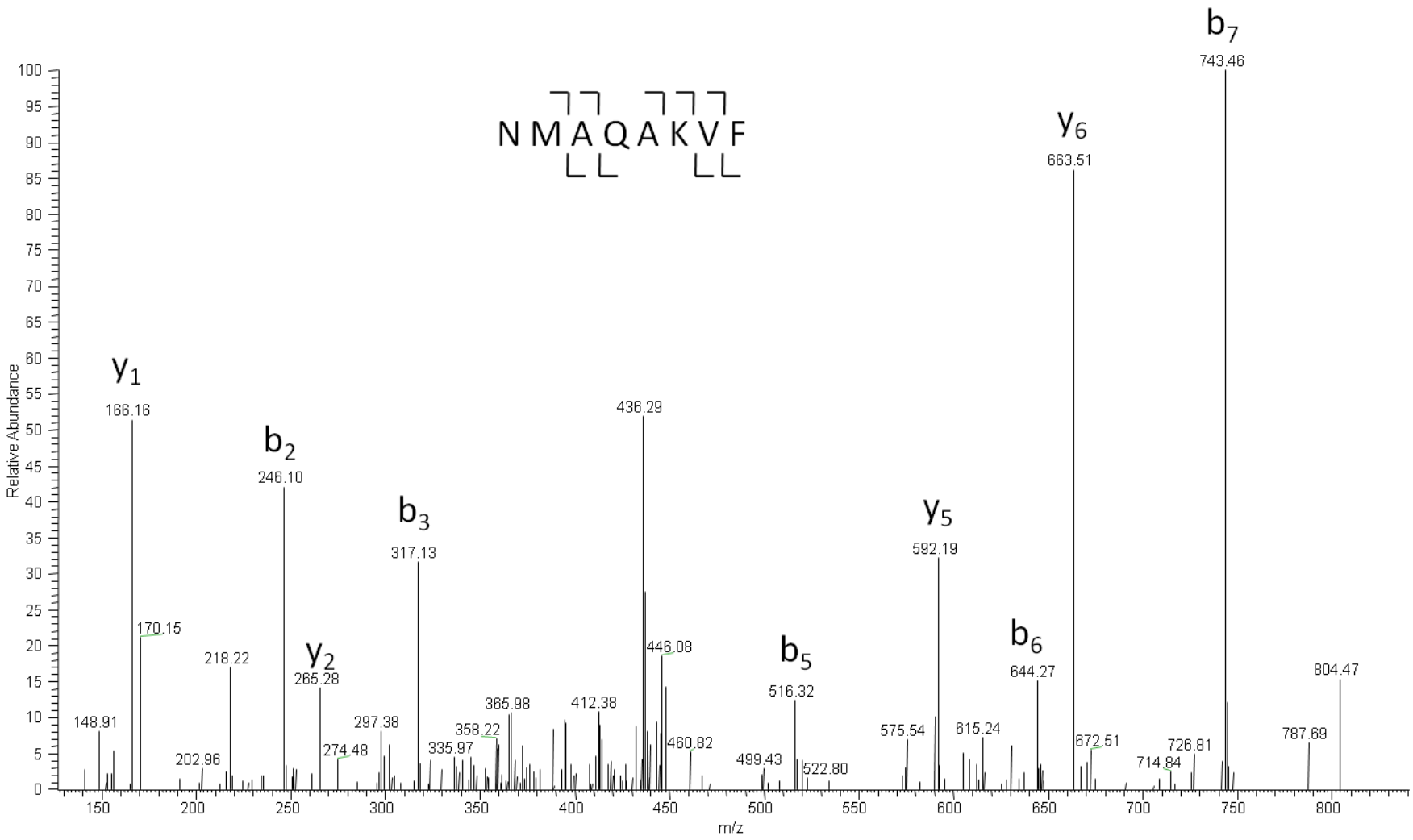
NMAQAKVF
y1
166.16
b2
246.10
b3
317.13
y2
265.28
b5
516.32
y5
592.19
b6
644.27
y6
663.51
b7
743.46
436.29
446.08
804.47
Relative Abundance
m/z

Fig. S2

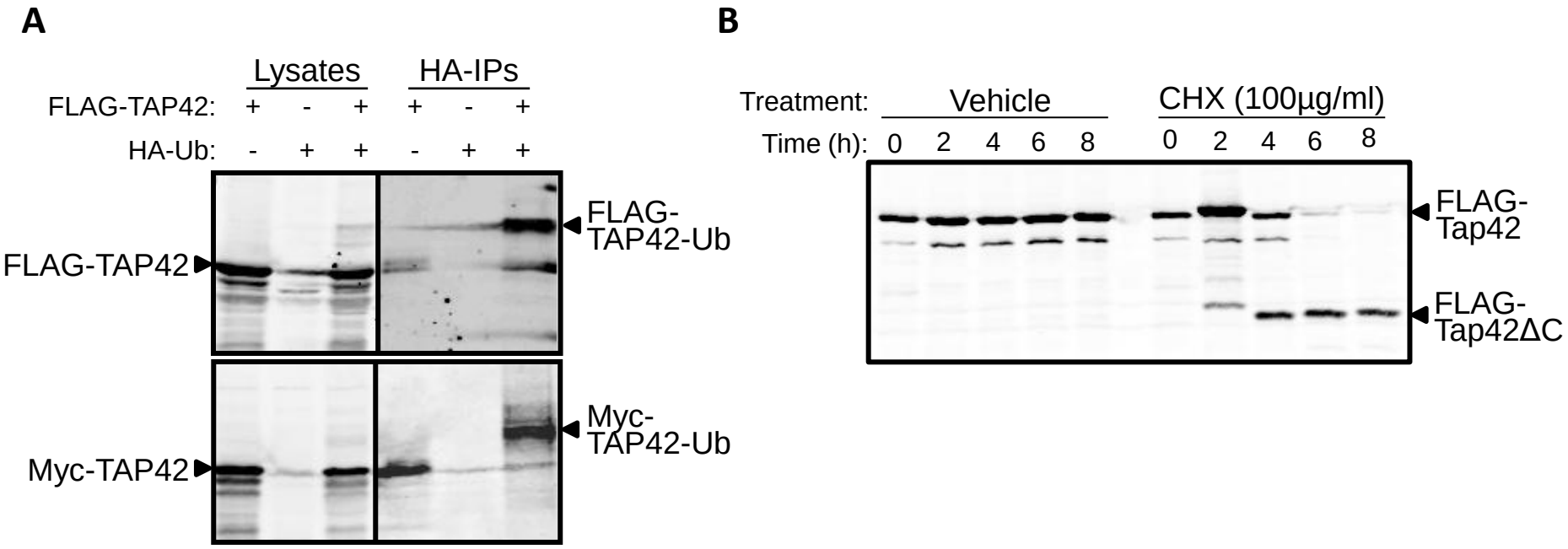
A
Lysates
HA-IPs
FLAG-TAP42: + - + + - +
HA-Ub: - + + - + +
FLAG-TAP42
Myc-TAP42
FLAG-TAP42-Ub
Myc-TAP42-Ub
B
Treatment: Vehicle CHX (100µg/ml)
Time (h): 0 2 4 6 8 0 2 4 6 8
FLAG-Tap42
FLAG-Tap42ΔC

Fig. S3