Supporting Information

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SI Materials and Methods

Plasmid Constructs and siRNA. Mitochondrial ubiquitin ligase (MITOL) expression vectors were described previously (1). Microtubule-associated protein 1B-light chain 1 (MAP1B-LC1) expression vectors were obtained by PCR using cDNA clone (Image clone no. 5502353). Point mutations of LC1 and MITOL were generated by site-directed mutagenesis kit (Stratagene). MITOL-specific siRNA and MITOL-sh were described previously (2, 3). The sequences of LC1-sh and gigaxonin-sh were shown below, with an order of sense, loop (underlined), and antisense: LC1-sh for human (5′-gatccGGACACAAACCTGATTGAATGCGAACATTCAATCAGGTTTGTGTCCtttttta-3′), LC-1-sh for mouse (5′-gatccGCTTCACTCTCCAGTACTTAACGAATTAAGTACTGGAGAGTGAAGCtttttta -3′), Gigaxonin-sh (5′-gatccGGAACAGATGCTGAATGAACCCGAAGGTTCATTCAGCATCTGTTCCtttttta-3′).

Yeast Two-Hybrid. Yeast two-hybrid was performed by Matchmarker Yeast Two-hybrid system (Clontech) according to the manufacturer's instructions. Mouse adult heart cDNA library was purchased from Clontech.

Cell Culture and Transfection. Human neuroblastoma SH-SY5Y cells and HeLa cells were cultured in DMEM supplemented with 10% FBS at 5% CO_2 at 37 °C. Transfection of expression vectors in SH-SY5Y cells were performed by lipofectamine 2000 reagents (Invitrogen) according to the manufacturer's instructions. For immunofluorescence, cells were seeded on coverslips coated with poly-D-lysine in six-well plates. Mouse primary cortical neurons were cultured in Neurobasal medium supplemented with B27. NMDA neurotoxicity was determined by exposing the 7-d-old cultures to Mg-free Hepes buffer containing 300 μM NMDA (Sigma) and 5 μM glycine (Wako) for 10 min. Three days before NMDA exposure, cells were transfected with indicated vectors using Lipofectamine 2000. Neuronal cell death was assessed by measuring nuclear condensation 24 h after the NMDA exposure.

Reagents. For ubiquitination assay, transfected cells were treated with 10 μM MG132 for 3 h. SH-SY5Y cells were treated with 7 μM calcimycin (Sigma) with or without 300 μM *N*-nitro-L-arginine methyl ester (L-NAME; Sigma) for 3 h. Next, 100 μM S-nitroso-*N*-acetylpenicillamine (SNAP; Calbiochem) was used for NO-donor. Hoechst 33258 was used for nuclear condensation assay. CM-H_2DCFDA (Molecular Probes) was used for reactive oxygen species (ROS) generation assay.

Antibodies. Anti-MITOL rabbit polyclonal Ab was described previously (1). Anti-FLAG (M2), FLAG rabbit, tubulin, acetylated tubulin, actin and gigaxonin Abs were from Sigma. Anti-myc mouse (Roche), myc rabbit (Cell Signaling), HA (Babco), V5 (MBL), Tom20 and Tim23 (BD Biosiences) were purchased from indicated companies. Anti-LC1 (H-130 and C-20) and anti-ubiquitin (P4D1) Abs were purchased from Santa Cruz.

Immunoprecipitation and Immunoblotting. Preparation of cell lysates, immunoprecipitation (IP) and immunoblotting (IB) were performed as described previously (1). In brief, to investigate the ubiquitinated proteins, cells were solubilized in RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 2 mM *N*-ethylmaleimide) to dissociate protein complexes. IP and cell lysates were separated by SDS/PAGE and transferred to the PVDF membrane (Millipore). The blots were probed with the indicated Abs, and protein bands were visualized by the chemiluminescence reagent (Millipore).

Flow Cytometry. At 24 h after transfection, cells were treated with Rhodamine-123 for 30 min at 37 °C, and cells were washed with PBS(−). After gating on DsRed-Mito–positive cells, the fluorescence of Rhodamine-123 was analyzed by flow cytometer (Beckman Dickinson).

In Vitro Ubiquitination Assay. GST-LC1 prepared from DH5α and immunoprecipitated MITOL prepared from lysates of SH-SY5Y cells transfected with MITOL-FLAG were incubated with the reaction buffer (50 mM Tris, pH 7.4, 2 mM $MgCl_2$, 4 mM ATP, 100 ng E1, 300 ng UbcH5b, and 2 μg ubiquitin) for 2 h at 30 °C and then terminated with 2× sample buffer. For auto-ubiquitination assay of MITOL, immunoprecipitated MITOL were treated with SNAP for 30 min at room temperature and then washed four times with ubiquitination buffer to avoid the generation of S-nitrosylation of E1 and E2 enzymes followed by in vitro ubiquitination assay.

Subcellular Fractionation. Isolation of mitochondria was performed using a mitochondria isolation kit (Active Motif). Isolated mitochondria were lysed in 1% Triton X-100 lysis buffer or RIPA buffer supplemented with protease inhibitors.

Carbonate Extraction and Trypsin Protection Assay. Carbonate extraction was performed with isolated mitochondria. Mitochondrial suspensions in isotonic buffer (250 mM sucrose, 2.5 mM Hepes pH 7.5) were added with an equal volume of 200 mM Na_2CO_3 (pH 11.5) and incubated on ice for 30 min. The suspensions were centrifuged at 144,000 × *g* at 4 °C for 1 h. The supernatant and the pellets were analyzed by Western blot. For trypsin treatment, trypsin was added to the mitochondrial fraction at final concentration of 1% and incubated on ice. After addition of protease inhibitor and centrifugation at 10,000 × *g* at 4 °C, each pellet was suspended with sample buffer.

Immunofluorescence Microscopy. Transfected cells were fixed with 4% paraformaldehyde in PBS(−) for 15 min at room temperature, then washed twice with 0.2% Tween20 in PBS(−), permiabilzed with 0.2% Triton X-100 in PBS(−) for 10 min, washed four times with PBS(−), and blocked with 3% BSA in PBS(−). For double-staining, the cells were incubated with appropriate primary Ab for 1 h at room temperature, washed three times with 0.5% Triton X-100 in PBS(−), and then appropriate secondary Ab for 30 min. The samples were analyzed using OLMPUS IX71.

In Utero Electroporation. In utero electroporation to dorsal neocortex was performed by injecting the DNA plasmid solution (5 mg/mL) plus 1% Fast Green using a glass capillary into the E14.5 ICR mouse ventricle, as previously described (4). DNA mixture was two- to threefold higher than that of the EGFP plasmid, which was the electroporation marker. Electroporation was performed using a CUY-21 electroporator (NEPA GENE) and the following parameters: four 50-ms long pulse separated by 950-ms long intervals at 33 V. TUNEL assay was performed with postnatal day (P) 2 mouse brain sections by in situ cell death detection kit, TMR red (Roche).

1. Yonashiro R, et al. (2006) A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. *EMBO J* 25:3618–3626.
2. Sugiura A, et al. (2011) A mitochondrial ubiquitin ligase MITOL controls cell toxicity of polyglutamine-expanded protein. *Mitochondrion* 11:139–146.
3. Yonashiro R, et al. (2009) Mitochondrial ubiquitin ligase MITOL ubiquitinates mutant SOD1 and attenuates mutant SOD1-induced ROS generation. *Mol Biol Cell* 20:4254–4530.
4. Fukuda T, Sugita S, Inatome R, Yanagi S (2010) CAMDI, a novel disrupted in schizophrenia 1 (DISC1)-binding protein, is required for radial migration. *J Biol Chem* 285:40554–40561.

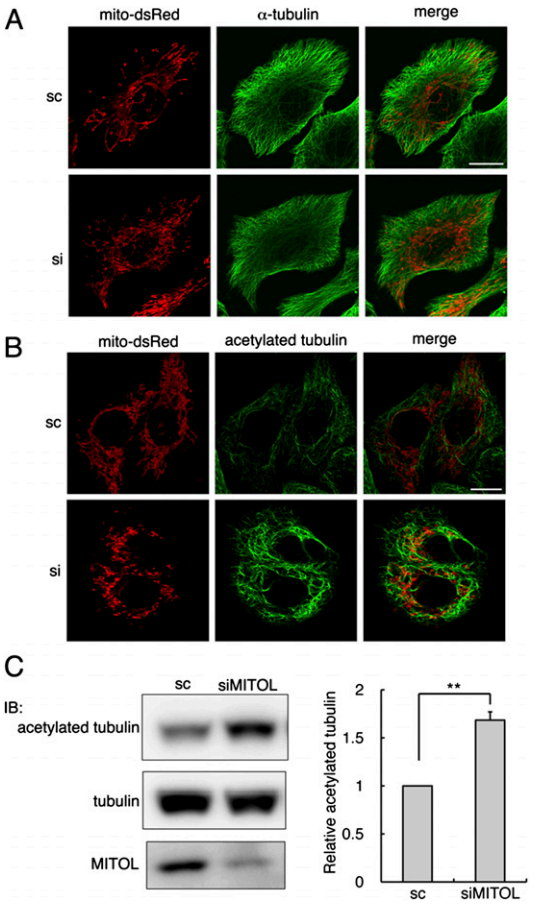

Fig. S1. MITOL knockdown induces microtubule stabilization. (*A* and *B*) Effect of siRNA-mediated MITOL knockdown on microtubule stabilization. HeLa cells cotransfected with scramble (sc) or siMITOL (si) with MITO-dsRed were immunostained with anti–α-tubulin (*A*) and anti-acetylated α-tubulin (*B*) Abs. Note that acetylated α-tubulin signal is significantly enhanced in MITOL-knockdown cells. (Scale bar, 10 μm.) (*C*) Increased acetylated α-tubulin by MITOL knockdown. Lysates of HeLa cells as described above were immunoblotted with antiacetylated α-tubulin Ab. Statistical data are shown in the right panel. $^{**}P < 0.01$, $n = 3$, Student *t* test.

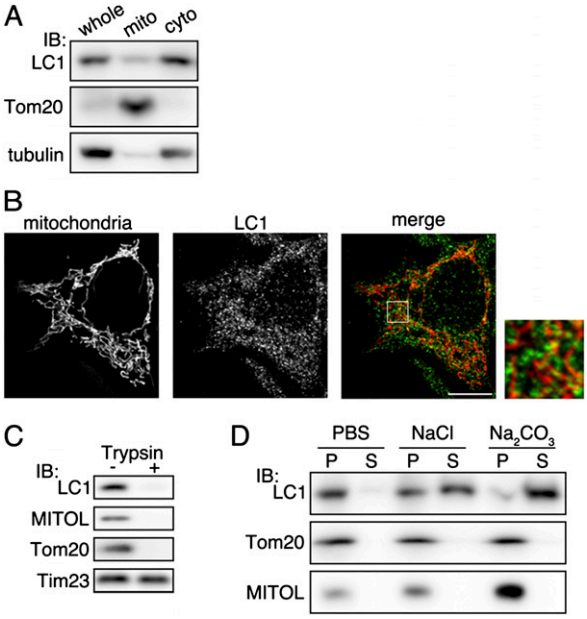

Fig. S2. Localization of LC1 in mitochondria. (*A*) Existence of LC1 in mitochondrial fraction. IB assay was performed on lysates of whole, mitochondrial, and cytosolic fractions isolated from SH-SY5Y cells. Anti-Tom20 and anti-tubulin antibodies (Abs) were used as a mitochondrial marker and a cytosolic marker, respectively. (*B*) Partial colocalization of LC1 with mitochondria. SH-SY5Y cells were immunostained with anti-LC1 Ab and MitoTracker. A square inset was magnified in the merged panel. (Scale bar, 10 μm.) (*C* and *D*) Localization of LC1 on the mitochondrial outer membrane. Trypsin protection assay (*C*) and carbonate extraction assay (*D*), followed by IB assay, was performed on mitochondrial fractions of SH-SY5Y cells. Anti-MITOL and Tom20 Abs were used as outer membrane markers and anti-Tim23 Ab was used as an inner membrane marker. P, pellet; S, supernatant.

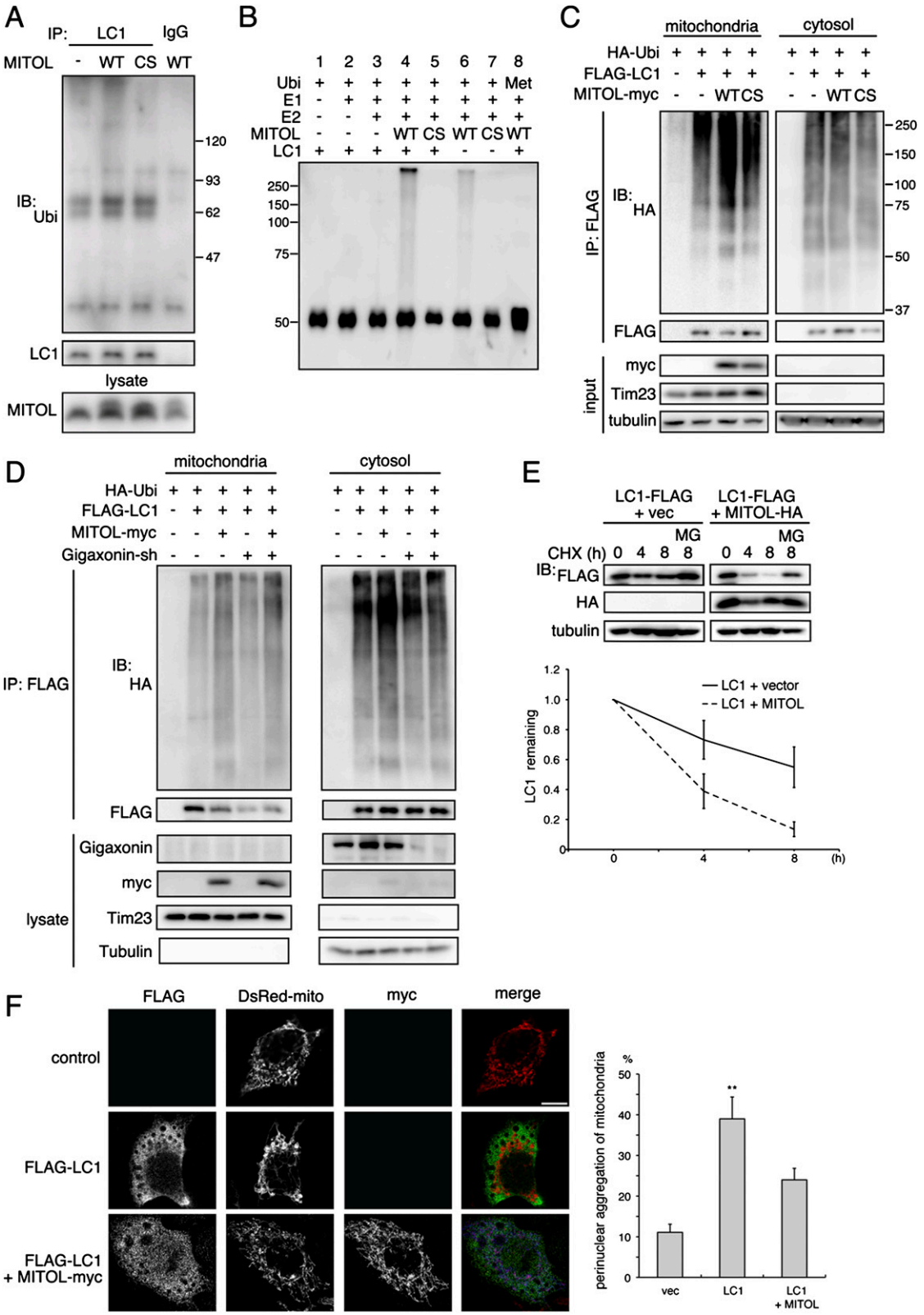

Fig. S3. Ubiquitination and degradation of mitochondrial LC1 by MITOL. (*A*) MITOL ubiquitinates endogenous LC1. IP-IB assay was performed on lysates of SH-SY5Y cells cotransfected with indicated vectors. WT, MITOL wild-type; CS, MITOL CS mutant lacking ubiquitin ligase activity. (*B*) In vitro ubiquitination of LC1 by MITOL. In vitro ubiquitination assay was performed as described in *SI Materials and Methods*. Purified GST-LC1 was incubated with or without indicated materials including MITOL WT and MITOL CS, E1, E2, ubiquitin, and methylated ubiquitin. MITOL is immunopurified from SH-SY5Y cell. E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; Met, ubiquitin mutant methylated on all lysine residues, which prevents formation of polyubiquitin chains; Ubi, ubiquitin. (*C*) MITOL ubiquitinates mitochondrial LC1. IP-IB assay was performed on mitochondrial and cytosolic fractions separated from SH-SY5Y cells co-transfected with indicated vectors. (*D*) Effect of gigaxonin knockdown on LC1 ubiquitination. IP-IB assay was performed on mitochondrial and cytosolic

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fractions separated from SH-SY5Y cells cotransfected with indicated vectors. Note that by gigaxonin knockdown, ubiquitination of cytosolic LC1 was reduced by gigaxonin knockdown but ubiquitination of mitochondrial LC1 was not affected. In addition, gigaxonin is dominantly localized in the cytosolic fraction. (*E*) MITOL facilitates a rapid degradation of LC1 via ubiquitin-proteasome pathway. Cycloheximide (CHX)-chase assay was performed as described previously (1). The amount of FLAG-LC1 was monitored in SH-SY5Y cells cotransfected with indicated vectors. MG; a proteasome inhibitor MG132. Statistic data are shown in the bottom. Error bars represent SD. (*F*) MITOL blocks LC1-induced mitochondrial aggregation. SH-SY5Y cells cotransfected with indicated vectors were immunostained with anti-FLAG and anti-myc Abs. (Scale bar, 10 μm.) Statistical data are shown in the right panel. Error bars represent SD. $^{**}P < 0.01$, $n = 3$, one-way ANOVA.

1. Yonashiro R, et al. (2006) A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. *EMBO J* 25:3618–3626.

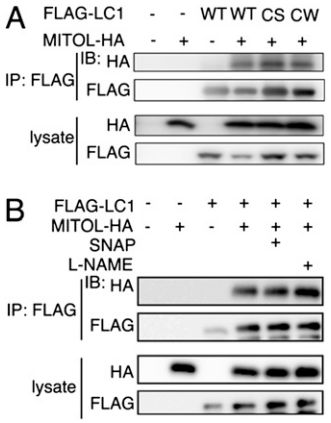

Fig. S4. S-nitrosylation of LC1 is not required for the interaction with MITOL. (*A*) Effects of LC1 mutants (C257S and C257W) on the interaction with MITOL. SH-SY5Y cells cotransfected with MITOL-myc and each FLAG-LC1 mutant (C257S and C257W) with HA-ubiquitin were IP with anti-FLAG Ab, and the immunoprecipitants or total lysates were IB with anti-HA or indicated Abs. (*B*) Effect of SNAP and L-NAME treatment on the interaction of MITOL with LC1. SH-SY5Y cells cotransfected with indicated vectors were treated with SNAP or L-NAME and cell lysyates were IP with anti-FLAG Ab, and the immunoprecipitates or total lysates were IB with indicated Abs.

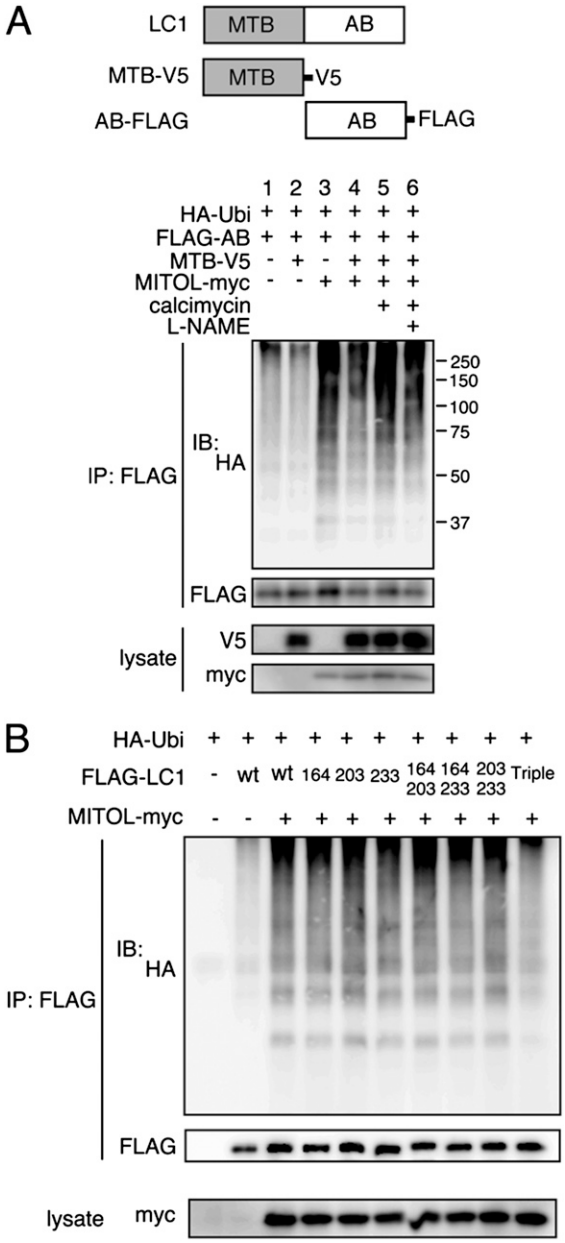

Fig. S5. Involvement of S-nitrosylation in the conformational change of LC1 and ubiquitination by MITOL. (*A*) Effect of microtubule-binding domain (MTB) fragment on the ubiquitination of actin-binding domain (AB) fragment by MITOL. The full-length LC1 is separated into the former (MTB) and latter (AB) fragments, as illustrated. SH-SY5Y cells cotransfected with indicated vectors were treated with calcimycin with or without L-NAME and then IP with anti-FLAG Ab, and the immunoprecipitates or total lysates were IB with the indicated Abs. (*B*) Identification of ubiquitin sites of LC1 by MITOL. Effects of various lysine mutants of LC1 on ubiquitination of LC1 by MITOL were examined. IP-IB assay was performed on SH-SY5Y cells transfected with indicated LC1 mutants. Note that ubiquitination by MITOL was severely attenuated in triple lysine mutant (K164, 203, 233R), suggesting that these lysine residues of LC1 are major ubiquitin sites for MITOL.

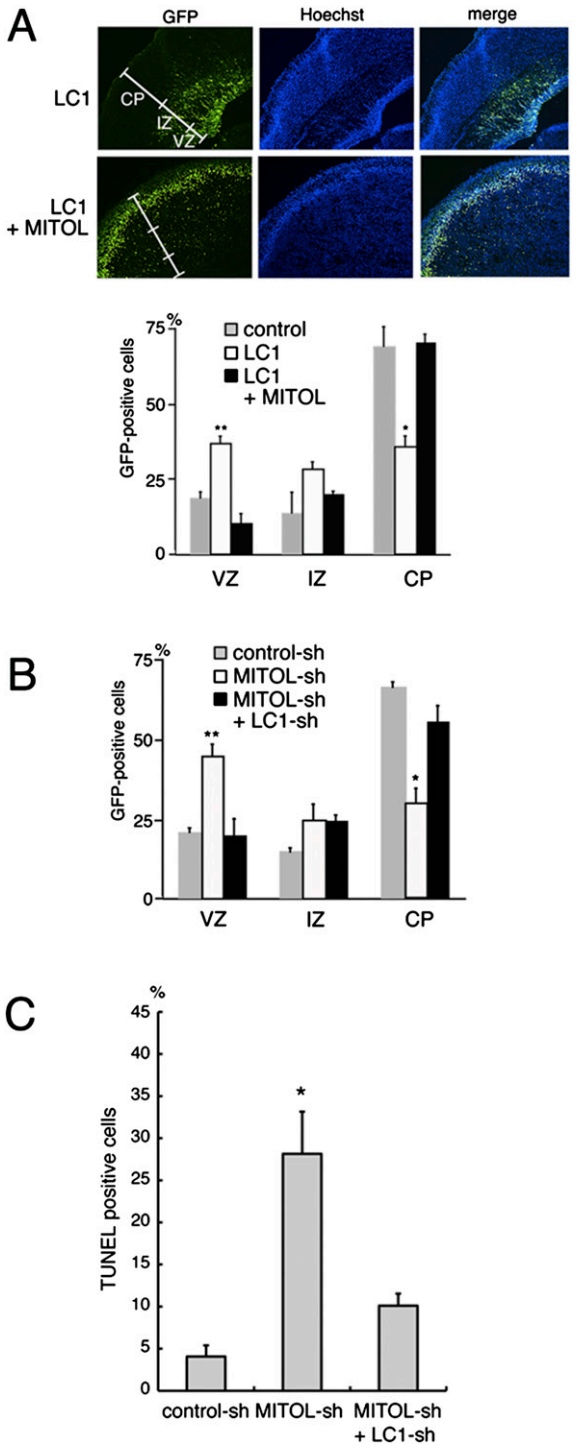

Fig. S6. Protective role of MITOL against LC1-mediated cytotoxicity in vivo. (*A*) Coexpression of MITOL rescued an impaired migration by LC1 expression. Sagittal sections of mice brains electroporated at E14 in utero with indicated vectors plus EGFP were analyzed at P2. Statistical data are shown in the right panel. (Scale bar, 100 μm.) (*B*) Impaired cortical migration by MITOL knockdown was rescued by LC1 co-knockdown. Statistical data of Fig. 4*A* are shown. Graphs in *A* and *B* represent the quantification of GFP^+ cells. CP; cortical plate, IZ; intermediate zone, VZ; ventricular zone. (*C*) Increased TUNEL-positive cells in MITOL-knockdown cortical neurons. Statistical data of Fig. 4*B* are shown. TUNEL assay was performed in the sections of mouse brain at P2. The number of TUNEL-positive cells in GFP^+ cells was counted and each percentage was indicated. Error bars represent SD. $*P < 0.05$, $**P < 0.01$, $n = 3$, one-way ANOVA.

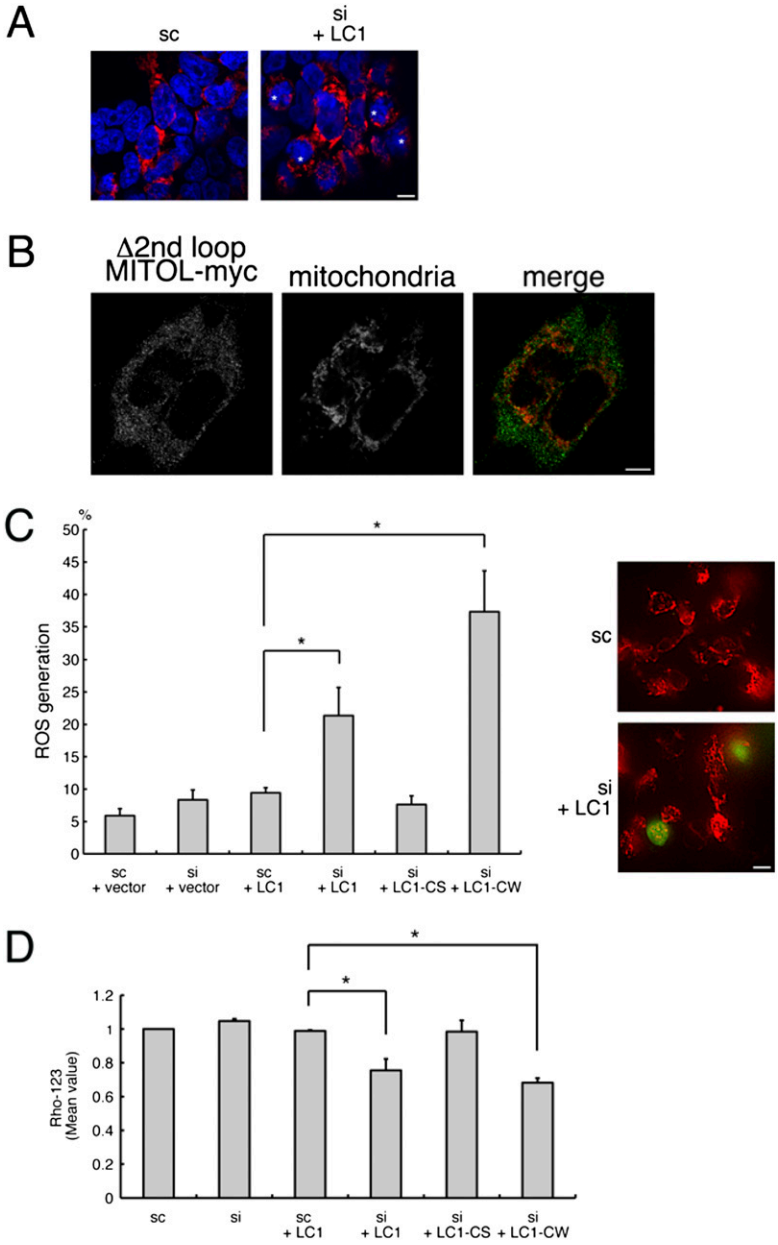

Fig. S7. LC1 induced ROS generation and reduction of mitochondrial membrane potential in MITOL-knockdown cells. (*A*) Cell death with nuclear condensation by LC1 under MITOL knockdown. SH-SY5Y cells were stained with Hoechst 33258 72 h after indicated transfection, as shown in Fig. 4*C*. The asterisks show condensed nucleus. (*B*) MITOL mutant lacking the second loop domain localizes to the cytosol. SH-SY5Y cells were transfected with the second loop-defective MITOL-myc mutant and DsRed-Mito vector. After 24 h, cells were fixed and immunostained with anti-myc Ab. (Scale bar, 10 μm.) (*C*) LC1 induced ROS generation in MITOL-knockdown cells. SH-SY5Y cells were transfected with indicated vectors and DsRed-Mito. After 24 h, cells were treated with ROS indicator CM-H_2DCFDA. (*Right*) The control cells and ROS-producing cells by LC1 under MITOL knockdown. The number of cells showing ROS generation was counted from 100 cells without nuclear condensation and each percentage was indicated. (Scale bar, 10 μm.) Error bars represent SD. *$P < 0.05$, $n = 3$, one-way ANOVA. (*D*) Reduction of mitochondrial membrane potential by LC1 in MITOL-knockdown cells. The mitochondrial membrane potential of cells shown in *C* was assessed by Rhodamine 123 (Rho-123) using flow cytometry after gating on pDsRed-Mito–positive cells. *$P < 0.05$, $n = 3$, one-way ANOVA.

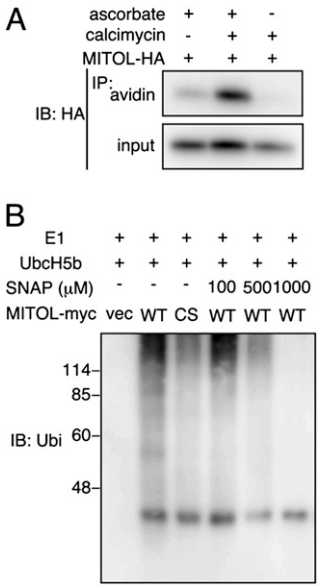

Fig. S8. Effect of S-nitrosylation of MITOL on its enzymatic activity. (*A*) S-nitrosylation of MITOL by calcimycin overstimulation. SH-SY5Y cells transfected with MITOL-HA were treated with calcimycin 10 μM for 3 h followed by biotin-switch assay. (*B*) In vitro SNAP treatment inhibits an ubiquitin ligase activity of MITOL. Immunoprecipitated MITOL were treated with 100 μM SNAP for 30 min at room temperature and then washed four times with ubiquitination buffer to avoid the generation of S-nitrosylation of E1 and E2 enzymes, followed by in vitro ubiquitination assay.