Supplementary Information

Materials and methods

Regents and antibodies

Antibodies for CTPS (15914-1-AP) and ubiquitin (10201-2-AP) were purchased from ProteinTech. Antibody for GFP (11 814 460 001) was purchased from Roche. Antibodies for β-actin (ab6276) was purchased from Abcam. Antibody for HA (sc-7392) and Protein A/G-Agarose (sc-2003) was from Santa Cruz Biotechnology. Doxycycline (ab141091) was purchased from Abcam. 6-Diazo-5-oxo-L-norleucine crystalline (D2141-5MG) was purchased from Sigma. MG132 (S2619) was from Selleck Chemicals.

Purification of hCTPS1 and activity assay

The human CTPS1 was cloned as C-terminal His-tagged fusion protein in pET28 expression vector and overexpressed in

Escherichia coli BL21 DE3 strain. In brief, cells were harvested and sonicated, lysates were clarified by centrifugation at 15,000 rpm for 30 min at 4°C. Clarified lysates were incubated with the His60 Ni Superflow resin, and then washed and eluted with imidazole. The elute was collected and concentrated by centrifugation in a 3kDa cut-off centrifugal filter unite (Millipore) for subsequent activity assay. The CTPS activity assay was conducted as described by Lynch *et al*., 2017 (ref. 6).

Cell culture

HEK-293T, HeLa and SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, SH30022.01, Hyclone), supplemented with 10% fetal bovine serum (04-001, Biological Industries) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin, SV30010, Hyclone), in a humidified atmosphere containing 5% CO_2 at 37°C (normal culture conditions). Cell

transfections were carried out by using R0531 (Thermo Fisher Scientific) according to the manufacturer's instructions.

Lentivirus-mediated stable cell line construction

Wild-type or mutant mCTPS1 with a GFP tag at C-terminal were cloned into Lenti-X™ Tet-On® 3G. Plasmids were co-transfected together with psPAX2 and pMD2.G into HEK 293T cells. Virus were collected at 48 hours after transfection. Cells were infected with appropriate lentiviruses in the presence of 8 μg/ml polybrene (Millipore) for 48 hours and then cultured in normal medium containing 0.5 μg/ml puromycin for 1 week. The resulting puromycin-resistant cells were used for further analysis.

Establishment and characterisation of mutant cell colonies with CRISPR/Cas9

The plasmid pU6-(BbsI) CBh-Cas9-T2A-mCherry (Addgene plasmid #64324) was used for generating the CTPS1/R294D mutant SW480 cell line. Briefly, the plasmid was digested with BpiI (Thermo Fisher Scientific) and purified by PCR clean-up kit (AP-PCR-250G, Axygen). Subsequently, annealed sgRNA oligonucleotide was inserted into the digested plasmid with T4 DNA ligase (M0202S, New England Biolabs). Sequence of sgRNA is 5'-ttgatagatatgatcgcttgc-3', and the sequence of homology-directed repair template is 5'-tagctattttggtctcatgatagcgtgtaccttctgagtaattgggtttttcttgacgtttatttgatag atatgatgacctat tggagacctgctctattgcccttgtgggcaaataca-3'. The resulted plasmid was transfected into SW480 cells, and the cells expressing mCherry were sorted with flow cytometer (BD Biosciences) and single cells were seeded into 96-well plate. Two weeks later, the genomic DNA was extracted and the modified region was amplified with primers shown below: forward primer, 5'-

GGGCGTGAGCTCATCCTG-3'; reverse primer, 5'-CTCAAAGCCACGAAGCAACC-3'. The PCR products were sequenced for the characterization of these cell lines.

Immunoblotting

Total proteins were prepared with NP-40 lysis buffer [150 mmol/L NaCl, 1.0% NP-40, 50 mmol/L Tris (pH 8.0)], and then quantified with a BCA assay (Pierce). Lysates were separated by SDS-PAGE, and transferred to PVDF membranes (Roche). The membranes were then blocked with 5% nonfat milk in TBST for 1 hour at room temperature, followed by incubation with appropriate primary antibodies at 4°C overnight. After incubation with HRP-conjugated secondary antibody at room temperature for 1 hour, the signals of secondary antibodies were detected by an enhanced chemiluminescence system.

Immunofluorescence

Cells cultured on glass slides were fixed with 4% paraformaldehyde in PBS for 10 min, and then permeabilized with 0.2% Triton X-100 for 10 min. After blocking with 5mg/ml bovine serum albumin in PBS for 1 hour, cells were incubated with anti-CTPS antibodies overnight at 4°C. After the primary antibody reaction, samples were washed and incubated with FITC-labeled secondary antibodies for 1 hour. Finally, nuclear DNA were stained with 4, 6-diamino-2-phenylindole (DAPI) (Beyotime, Shanghai, China) for 5 min. The images were taken under a confocal laser scanning microscope (Zeiss 710).

RNA extraction and quantitative Real-time PCR

Total RNAs were extracted by Trizol (ER501-01; TransGen). The first-strand cDNA synthesis was conducted with PrimeScriptTMRT

Master Mix (RR0361; Takara). qRT-PCR reactions were performed using SYBR Green dye (B21703; Bimake) and the Applied Biosystems 7500 Fast Real-Time PCR System. Primers used for GFP (forward primer, 5'- AAGCTGACCCTGAAGTTCATCTGC -3'; reverse primer, 5'- GTCTTGTAGTTGCCGTCGTCCTTGAA -3'), CTPS1 (forward primer, 5'- CAGTGTGGGCACAATACTCAA -3'; reverse primer, 5'- CGCTCATAGTTACCCAGGTCA -3') and β-actin (forward primer, 5'-CATGTACGTTGCTATCCAGGC-3'; reverse primer, 5'- CTCCTTAATGTCACGCACGAT-3'). The resulting values were normalized to β-actin expression.

Ubiquitination assays

For mCTPS1-GFP ubiquitination assays, HEK-293T cells stably expressing Tet-On mCTPS1-GFP were transfected with HA-Ubiquitin, and then treated with the indicated concentration of DON

for 36 hours in the presence of Doxycycline (200ng/ml). MG132 (20μM) was added during the last 16 hours of treatment. Lysates prepared were subjected to immunoprecipitation by anti-GFP antibody. Immunoprecipitates were analyzed by immunoblotting using anti-HA antibody. For endogenous CTPS ubiquitination assays, Wild-type and CTPS1 R294D mutant SW480 cells were treated with MG132 for 16 hours. Lysates were prepared and subjected to immunoprecipitation by anti-CTPS1 antibody. Immunoprecipitates were analyzed by immunoblotting using anti-ubiquitin antibody.

Statistical analysis

Two-tailed unpaired Student’s t-test was used for comparisons between two groups and ordinary one-way ANOVA with Tukey's multiple comparison post-test was used to compare variables among three or more groups. $P \leq 0.05$ was considered statistically

significant. All analyses were performed using GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Supplementary figures 1-6 and figure legends

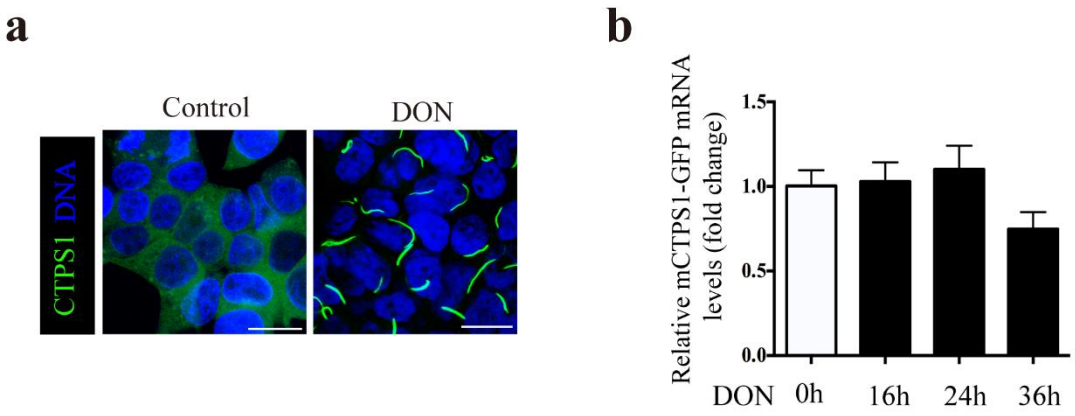

Fig. S1. DON treatment does not increase mCTPS1-GFP mRNA levels.

(a) HEK-293T cells stably expressing mCTPS1^{WT}-GFP were treated with DON (4μg/ml) for 24 hours, the cytoophidia assembly was analyzed by confocal microscope. **(b)** HEK-293T cells stably expressing mCTPS1-GFP were treated with 4μg/ml DON for the indicated time, and then subjected to qRT-PCR analysis for the mRNA expression of mCTPS1-GFP. Scale bars = 20 μm. One of three similar experiments is shown. DON, 6-diazo-5-oxo-L-norleucine.

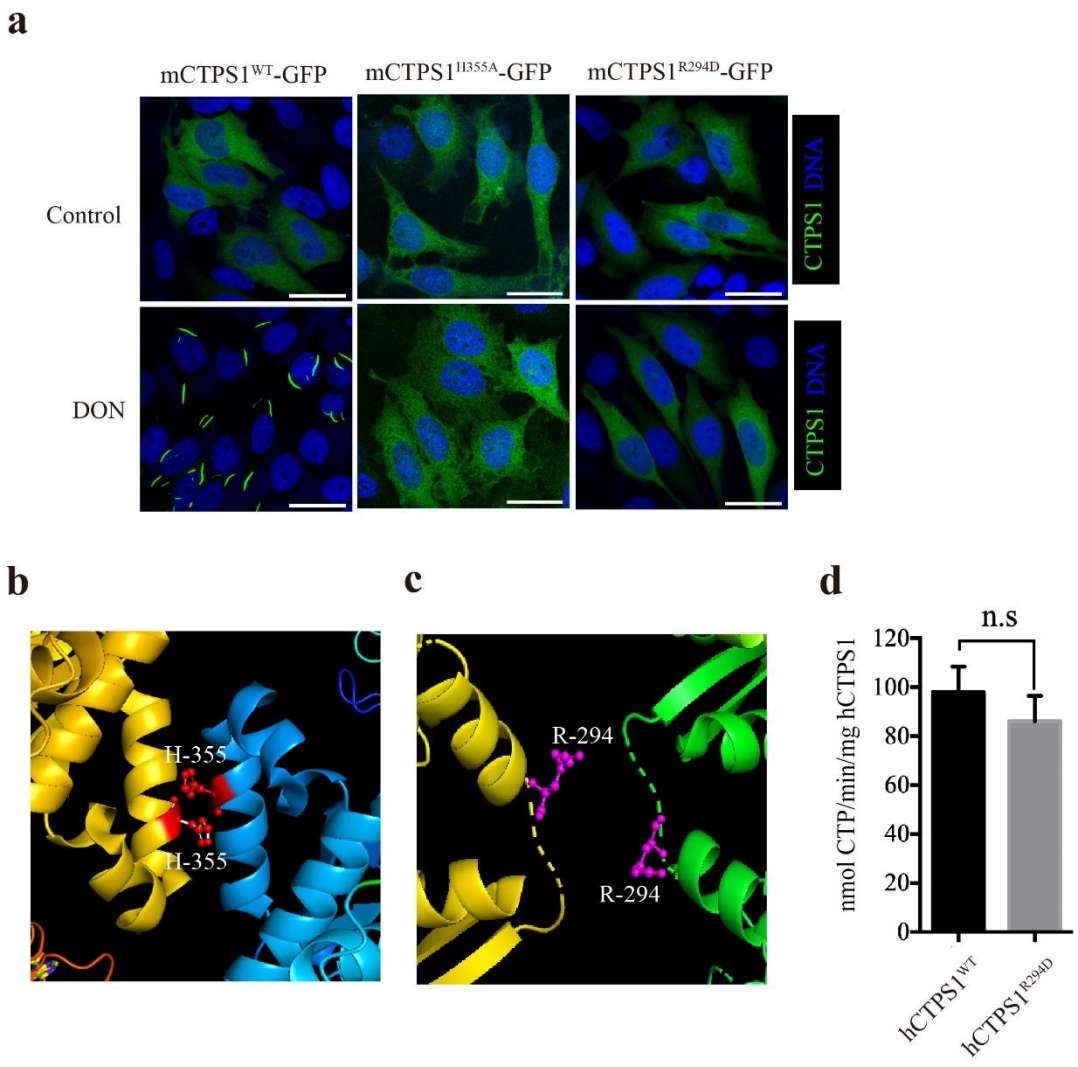

Fig. S2. mCTPS1^{H355A} and mCTPS1^{R294D} are failed to form cytoophidia under DON treatment

(a) HeLa cells transfected with mCTPS1^{WT}-GFP, mCTPS1^{H355A}-GFP or mCTPS1^{R294D}-GFP were treated with vehicle or DON (4μg/ml) for 6 hours, the cytoophidia assembly was analyzed by confocal microscope. **(b, c)** Structure modeling showed that H355 and R294

locate at the interface between two consecutive tetramers. **(d)** There is no significant difference in the enzymatic activity between wild type and R294D mutant hCTPS1. Scale bars = 20 μm. One of three similar experiments is shown. DON, 6-diazo-5-oxo-L- norleucine.

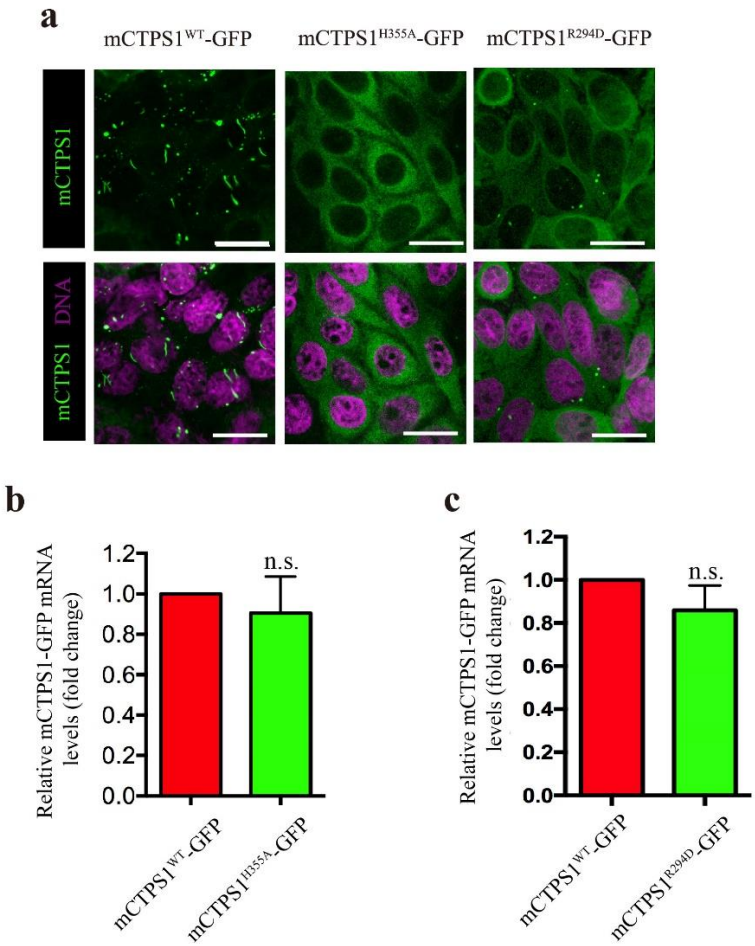

Fig. S3. Forming cytoophidia does not change CTPS mRNA levels

(a) SW480 cells stably expressing mCTPS1^{WT}-GFP, mCTPS1^{H355A}-GFP or mCTPS1^{R294D}-GFP were cultured for 3 days before fixation, cytoophidia assembly was analyzed by confocal microscope. Nuclei were labeled with DAPI (magenta). **(b, c)** SW480 cells stably expressing mCTPS1^{WT}-GFP, mCTPS1^{H355A}-GFP or mCTPS1^{R294D}-

GFP were cultured for 3 days in the presence of Doxycycline, and then subjected to qRT-PCR analysis for the mRNA expression of mCTPS1-GFP. One of three similar experiments is shown. Mean ± S.E.M, n.s. = not significant.

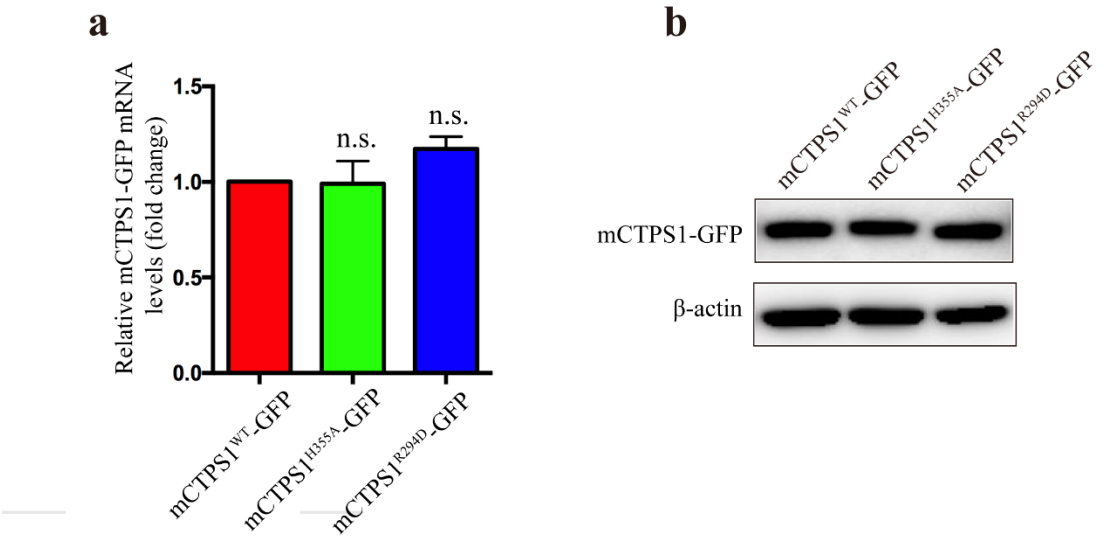

Fig. S4. mCTPS1^{H355A} and mCTPS1^{R294D} mutation do not change mCTPS1 protein levels in HeLa cells

(a, b) HeLa cells stably expressing mCTPS1^{WT}-GFP, mCTPS1^{H355A}-GFP or mCTPS1^{R294D}-GFP were cultured for 2 days, and then subjected to qRT-PCR analysis for the mRNA expression of mCTPS1-GFP **(a)**, or subjected to immunoblotting analysis with anti-GFP antibody **(b)**. One of three similar experiments is shown. Mean ± S.E.M, n.s. = not significant.

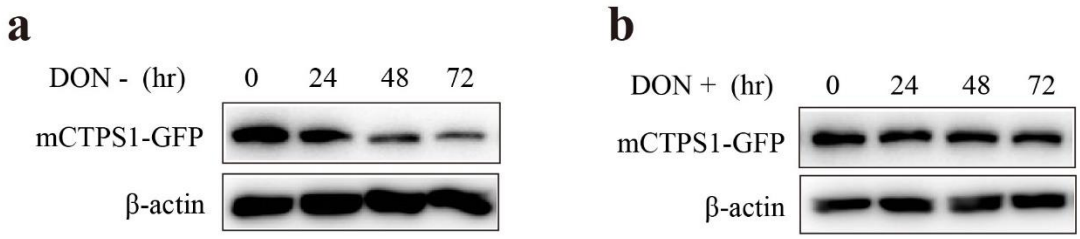

Fig. S5. DON treatment slows mCTPS1-GFP protein degradation.

(a, b) HEK-293T cells stably expressing Tet-Off mCTPS1-GFP were cultured in the medium without Doxycycline for 24 hours, followed by culturing with the medium containing Doxycycline (200ng/ml) without **(a)** or with **(b)** DON (4μg/ml) for the indicated time. Lysates were prepared and analyzed by immunoblotting with appropriate antibodies. One of three similar experiments is shown. DON, 6-diazo-5-oxo-L- norleucine.

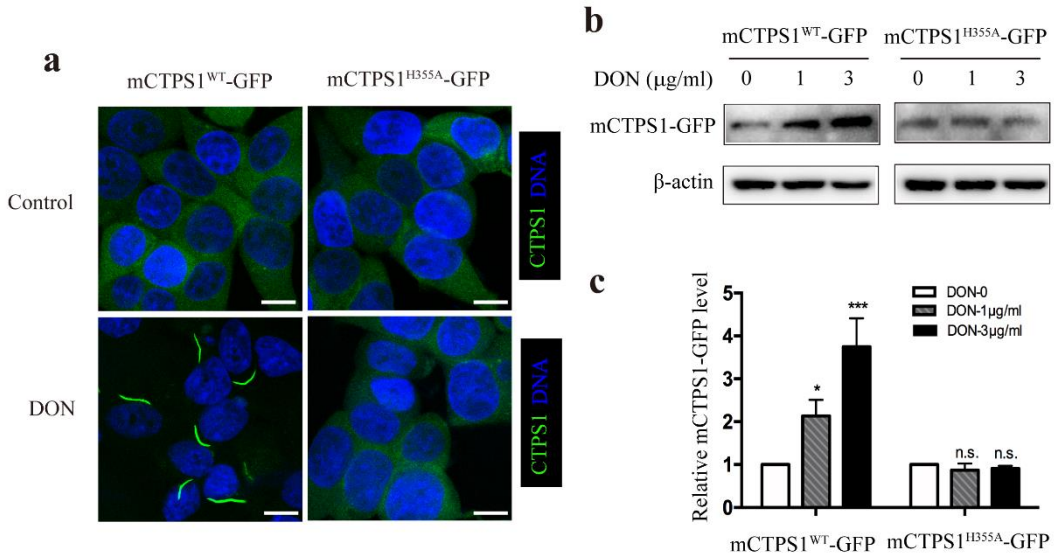

Fig. S6. DON inhibits CTPS degradation depends on the formation of cytoophidia.

(a) HEK-293T cells stably expressing Tet-On mCTPS1^{WT}-GFP or Tet-On mCTPS1^{H355A}-GFP were treated with Doxycycline (200ng/ml) and DON (4μg/ml) for 24 hours, the cytoophidia assembly was analyzed by confocal microscope. **(b)** HeLa cells stably expressing Tet-On mCTPS1^{WT}-GFP (left three lanes) or Tet-On mCTPS1^{H355A}-GFP (right three lanes) were treated with Doxycycline (200ng/ml) for 24 hours, followed by culturing with Doxycycline-free medium with the indicated concentration of DON for 48 hours. Anti-GFP antibody was used to detect mCTPS1-GFP expression. **(c)** Quantitative data

of mCTPS1-GFP protein expression in **(b).** Mean ± S.E.M, n.s. = not significant. *P<0.05; ***P<0.001; Scale bars = 10 μm One of three similar experiments is shown. DON, 6-diazo-5-oxo-L- norleucine.