Supplementary data

Complete Material and Methods

Materials

Reagents were obtained from the following sources: antibody to ATP6V0a1 from Santa Cruz; antibodies to S6K1, p-S6K1, mTOR, RagA, RagC and p18 (LAMTOR1) from Cell Signaling; antibodies to mouse Lamp1 from DSHB (1D4B); antibody to β-catenin from BD Biosciences; antibody to Zonula Occludens-1 (ZO-1) from Invitrogen; antibody to SV40 T antigen from Oncogene; ECL anti-rabbit/mouse HRP from GE Healthcare; antibody to GFP and Complete Protease Cocktail from Roche; DMEM, DMEM/F-12, RPMI, MEM amino acids (50x liquid) and certified fetal bovine serum from Gibco; DMEM/F12 without amino acids from US Biological; μMACS anti-GFP and protein A MicroBeads from Miltenyi Biotec; disuccinimidyl suberate (DSS) from Thermo Scientific; Alexa Fluor 488-, 555- and 647-conjugated secondary antibodies and ITS Liquid Media Supplement (100X) from Life Technologies; cysteamine hydrochloride, tri-iodothyronine and dexamethasone from Sigma; epidermal growth factor from R&D Systems; mouse IFN-γ from Tebu-bio; rat tail type-I collagen from Corning.

Immunoblotting

Equal amounts of protein were loaded on a 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes. All primary antibodies were used at a final dilution of 1:1000 in PBS, 0.05% Tween and 5% milk and incubated overnight at 4°C, except for the anti-GFP incubated for 1 h at room temperature. Secondary anti-rabbit/mouse-HRP antibodies were used at a final dilution of 1:5000 in PBS, 0.05% Tween and 5% milk and incubated for 30 min.

Peptide extraction and analysis by MS

Extracted peptides were dissolved in 0.1% (v/v) trifluoroacetic acid, 10% acetonitrile, and preconcentrated on a 75 μm i.d. reversed-phase (RP) trapping column and separated with an aqueous-organic gradient on a 75 μm i.d. RP column (Acclaim PepMap RSLC 75 μm x 15 cm, 2 μm, 100Å, Dionex) at 400 $nl.min^{-1}$ flow rate. Solution A was 0.1% (v/v) formic acid in 5% (v/v) acetonitrile; solution B was 0.085% (v/v) formic acid in 80% (v/v) acetonitrile. Samples were eluted using a linear gradient from 5% to 40% solvent B in 40 min. MS instrument settings were as follows: spray voltage 1.8 kV; capillary temperature 250°C; FT full MS target 1,000,000 (maximum injection time 500 ms); IT MSn target 5,000 (maximum injection time 100 ms). 1 FTMS full scan was performed (resolution 60,000 for LTQ-Orbitrap velos; positive polarity; centroid data; scan range 400 to 2,000 m/z) and the 20 most intense signals were subjected to MS/MS in the collision induced dissociation (CID) cell (resolution 15,000; centroid data) with dynamic exclusion (repeat count 1; exclusion list size 500; repeat /exclusion duration 12 s; exclusion mass width ± 10 ppm), preview mode for FTMS master scans, charge state screening, monoisotopic precursor selection and charge state rejection (charge state 1 and 4+) enabled. Activation type was CID with default settings. LC-MS/MS raw data were extracted and peak lists were generated by Proteome Discoverer v1.2 (Thermo Scientific) and

searched against the *Canis lupus familiaris* subset of the NCBI non-curated database (31834 non curated sequences; available online on http://www.ncbi.nlm.nih.gov/protein) for MDCK samples and against the *Mus musculus* subset (16611 sequences) from UniprotKB/Swiss-Prot complete and curated proteome database (release 2013_04; 539829 sequences) for fibroblast samples. The sequence of cystinosin-EGFP fusion protein was also added to both of the databases. For protein identification, default parameters were: fixed modification (Carbamidomethyl (C)), and variable modification (Oxidation (M)) were allowed as well as one missed cleavage. Enzyme was trypsin, monoisotopic peptide mass tolerance was ± 5 ppm (after linear recalibration), fragment mass tolerance was ± 0.5 Da, false discovery rate was lower than 2%.

Assay of cystine levels

The MPT cells were trypsinised and centrifuged. The cell pellet was extensively washed with PBS and sonicated in 200 µl of 5.2 mM N-ethylmaleimide (NEM). Proteins were precipitated by the addition of 50µl of 12% sulfosalicylic acid (ASS) for 15 min at 4°C. After 20-min centrifugation at 10 000 g, the protein pellet was then diluted in 300 µl of 0.1 N NaOH, and protein content was determined using the Lowry protein assay kit (Thermo Scientific). After addition of a D,L cystine-2,2',3,3,3'3'-d6 (C/D/N isotope) internal standard, the protein-depleted supernatants were assayed for cystine content by butylation and LC/MS/MS (API 3000 LC/MS/MS System; Applied Biosystems) as already described.[34] Statistical analysis using a Student's t-test was performed with Prism Version 5 (GraphPad software). The threshold for statistical significance was set to $P < 0.05$. Each bar represents the mean ± S.E.M from 2 independent experiments carried out in triplicates.

Immunofluorescence

For MPT characterization, cells were plated on collagen-coated Transwell membrane and grown during 27 days. Membranes were rinsed with PBS and fixed for 20 min with 4% formaldehyde in PBS at room temperature. After rinsing with PBS, membranes were incubated with primary anti-β-catenin (1:50) or -ZO-1 (1:50) antibodies in PBS containing 0.075% saponin and 0.1% BSA for 1 h at room temperature, rinsed thrice with PBS and incubated with AlexaFluor555- and 488-conjugated secondary antibodies (diluted in PBS with 0.075% saponin, 0.1% BSA) respectively for 1 hr at room temperature and washed three times with PBS. Transwell membranes were mounted on microscope slides using Fluoprep (bioMatérieux®sa) and imaged on a ZEISS LSM 700 scanning laser confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

For SV40 T antigen expression, MPT cells grown on collagen-coated glass coverslips during 9 days at 33°C or 37°C, than washed twice in PBS++ (supplemented by 0.49 mM MgCl2 and 0.9 mM CaCl2) and fixed for 5 min with methanol at -20°C. After rinsing twice with PBS++, slides were incubated with SV40 T Ag antibody diluted 1:400 in PBS++ containing 1% BSA, for 1 h at room temperature. After three rinses with PBS++, samples were incubated with Alexa Fluor 555 anti-mouse diluted 1:400 in PBS++ with 1% BSA for 1 h at room temperature, washed twice with PBS++ and nuclei were stained using Hoechst stain (Life Technology). Glass coverslips were mounted on microscope slides using FLUOPREP (bioMatérieux®sa) and imaged with an epi-illumination microscope (DMR, HC, Leica).

Gamma Glutamyl Transferase (γ-GT) activity and alkaline phosphatase assay

In 7-day-old confluent cell cultures, the expression level of the brush-border membrane enzyme γ-glutamyl transferase was assessed by the method described by Glossmann *et al*.[35] Cells were incubated with 2.5 mM γ-glutamyl-p-nitroanilide and 42 mM glycyl-glycine in 0.3 M Tris-buffer, pH 8.2, at 37°C for 10 min. The reaction was stopped by cold 1.5 N acetic acid and release of γ-glutamyl-p-nitroaniline was measured at 405 nm. Enzyme activity was normalized to protein concentration as measured by the Bradford protein assay. One unit γ-glutamyl-transferase activity was defined as the amount of enzyme that released 1 µmol of p-nitroaniline per unit time at 37°C. Statistical analysis using a Student's t-test was performed with Prism Version 5 (GraphPad software). The threshold for statistical significance was set to $P < 0.05$. Each bar represents the mean ± S.E.M from 2 independent experiments carried out in triplicates.

The expression of the other proximal tubule brush border enzyme, alkaline phosphatase, was assessed by colorimetric *in situ* detection. Cells plated on collagen-coated glass coverslips were fixed for 5 min with 2% formaldehyde in PBS at room temperature. After rinsing twice with PBS, slides were incubated for 1 h with 150 µg/mL BCIP (5-bromo-4-chloro-3-indolyl-phosphate) in conjunction with 300 µg/mL NBT (nitro blue tetrazolium) in 100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, pH 9.5 buffer. Cells incubated with $2.5.10^{-5}$ M levamisole (inhibitor of alkaline phosphatase) were used as a negative control. Glass coverslips were mounted on microscope slides using Fluoprep (bioMatérieux®sa) and imaged on a Leica DMR microscope.

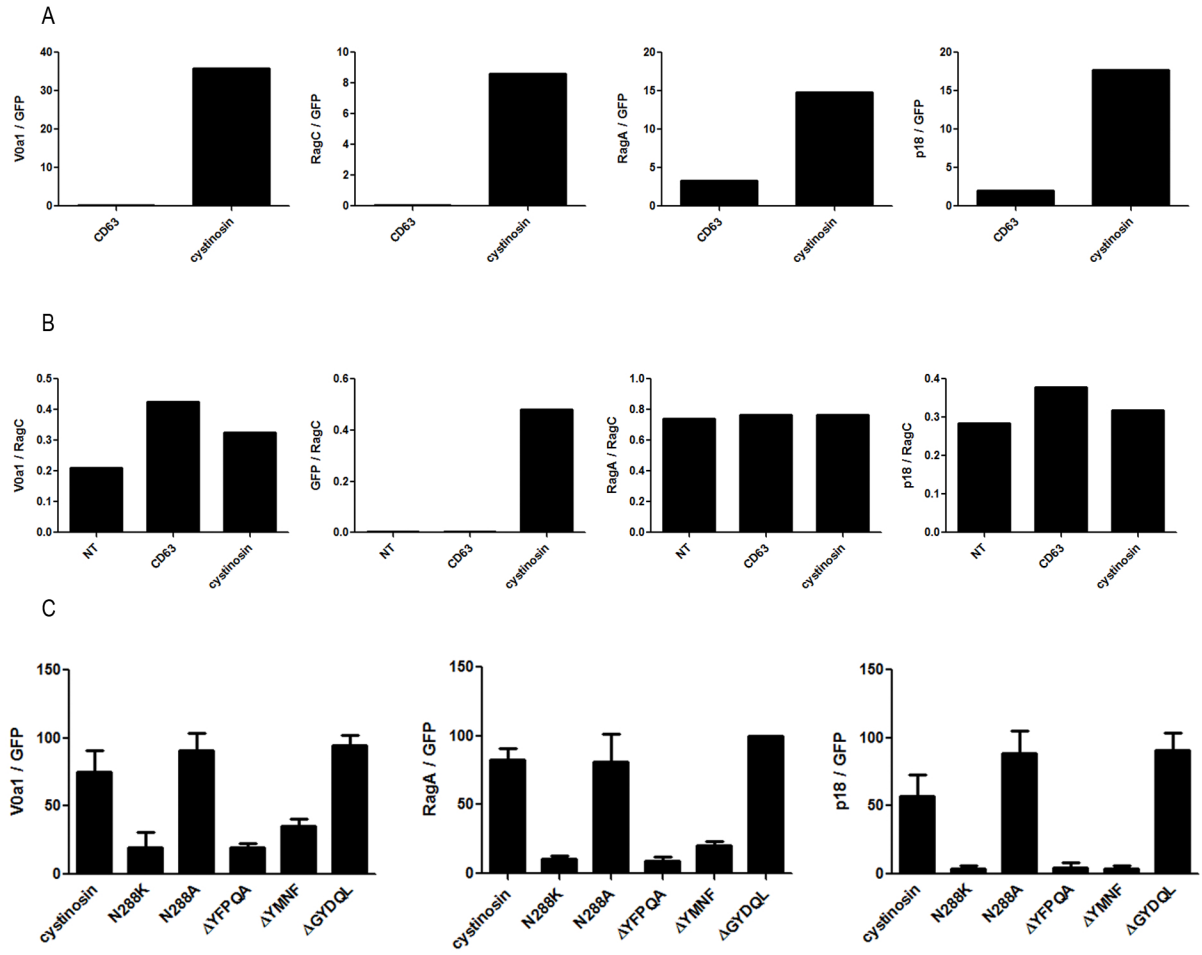


Figure S1. Quantification of the force of interactions between protein partners

(A) Lysates of 3T3 cells stably expressing cystinosin-EGFP or EGFP-CD63 were immunoprecipitated with anti-GFP, the co-immunoprecipitated proteins were analyzed by western blotting (Fig.1A) and the ratio of V0a1, RagC, RagA, p18 to GFP was quantified. **(B)** Lysates of 3T3 cells stably expressing cystinosin-EGFP or EGFP-CD63 were immunoprecipitated with anti-RagC, co-immunoprecipitated proteins were analyzed by western blotting (Fig.1B) and the ratio of V0a1, GFP, RagA, p18 to RagC was quantified (NT = non transfected cells). **(C)** Lysates of 3T3 cells stably expressing WT cystinosin-EGFP and its mutated forms (N288K, N288A, ΔYFPQA, ΔYMNF or ΔGYDQL) were immunoprecipitated with anti-GFP antibodies, co-immunoprecipitated proteins were analyzed by western blotting (Fig.2A) and the ratio of V0a1, RagA, p18 to GFP was quantified (each bar represents the mean ± S.E.M. from 2 independent experiments.

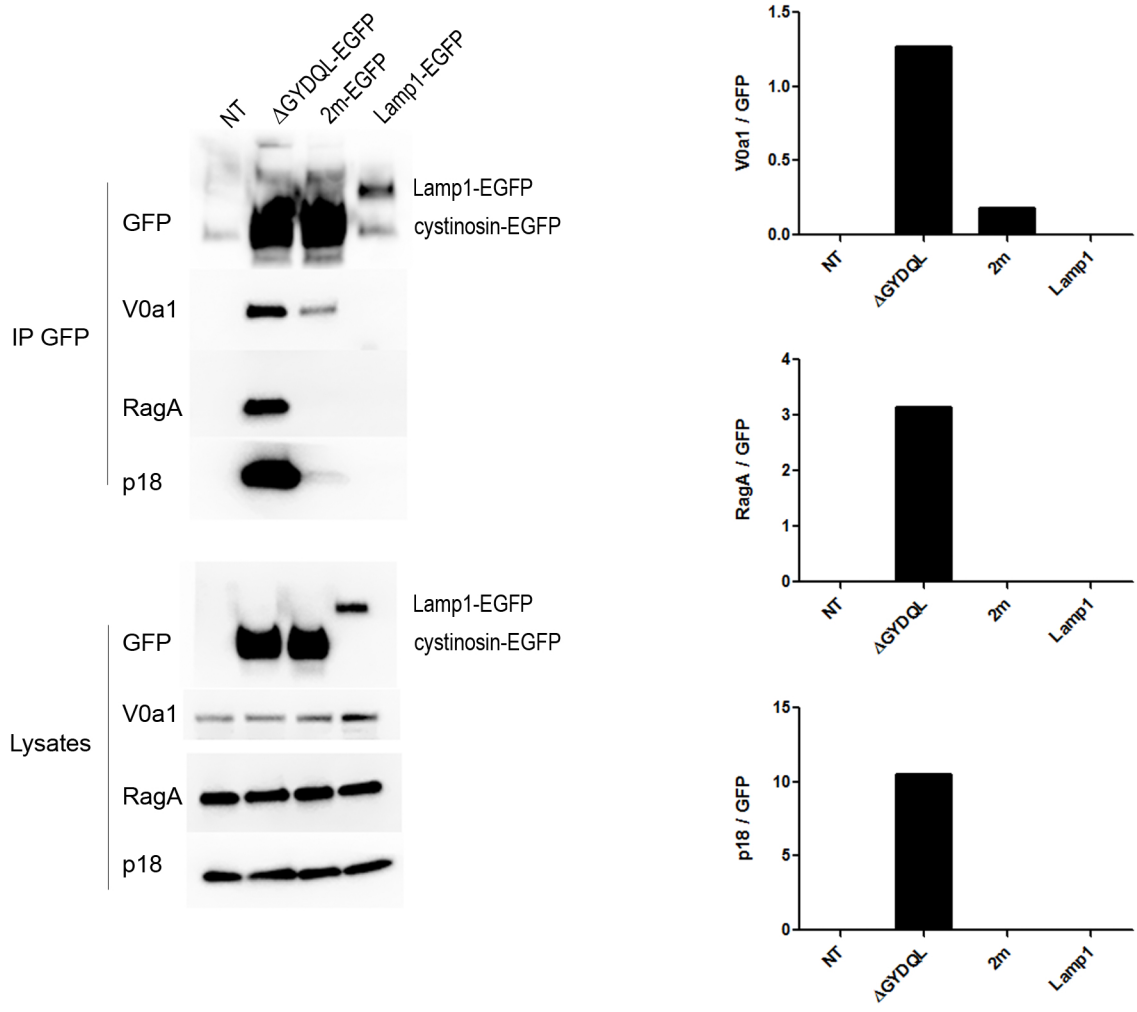


Figure S2. Disruption of the interactions of cystinosin with its partners in the absence of the lysosomal targeting motifs

Lysates of 3T3 cells stably expressing ΔGYDQL-EGFP, 2m-EGPF or Lamp1-EGFP were immunoprecipitated with anti-GFP antibodies, co-immunoprecipitated proteins were analyzed by western blotting and the ratio of V0a1, RagA, p18 to GFP was quantified (n=1; NT = non transfected cells).

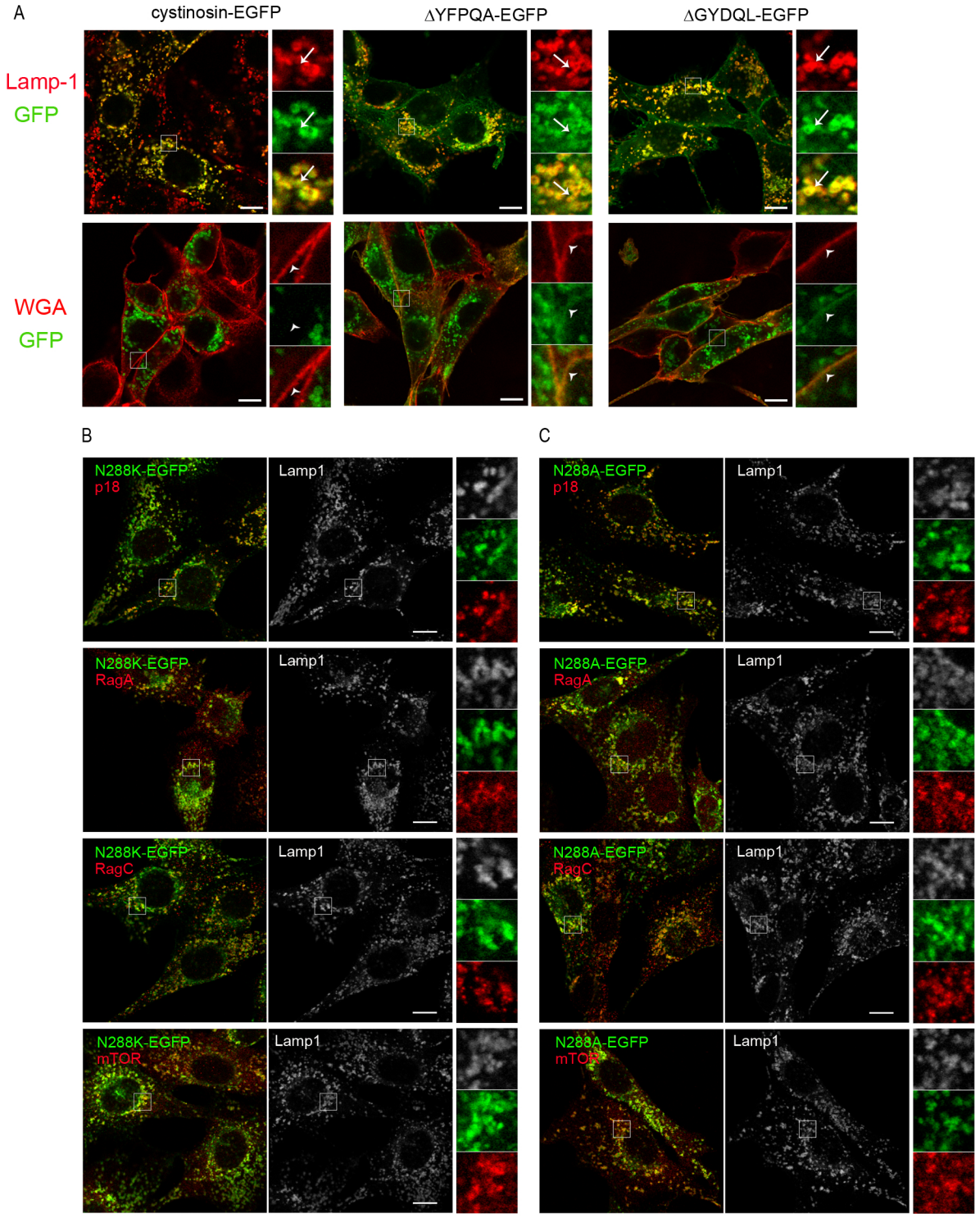


Figure S3. Cellular distribution of cystinosin and its mutated forms

(A) 3T3 cells stably expressing cystinosin-EGFP, ΔYFPQA-EGFP or ΔGYDQL-EGFP were co-immunolabeled for Lamp1 (lysosomal marker) or WGA (wheat germ agglutinin; PM marker). While WT cystinosin is found only in Lamp-1 positive vesicles, ΔYFPQA-EGFP and ΔGYDQL-EGFP are partially relocalized to the PM, but still the majority of these proteins is present on lysosomes. Arrows and arrowheads indicate lysosomal and PM localization respectively (confocal microscopy; scale bars = 10 μm). **(B-C)** 3T3 cells stably expressing N288K-EGFP (B) or N288A-EGFP (C) were co-immunolabeled for Lamp-1 and indicated mTORC1 pathway proteins: p18, RagA, RagC or mTOR (confocal microscopy; scale bars = 10 μm).

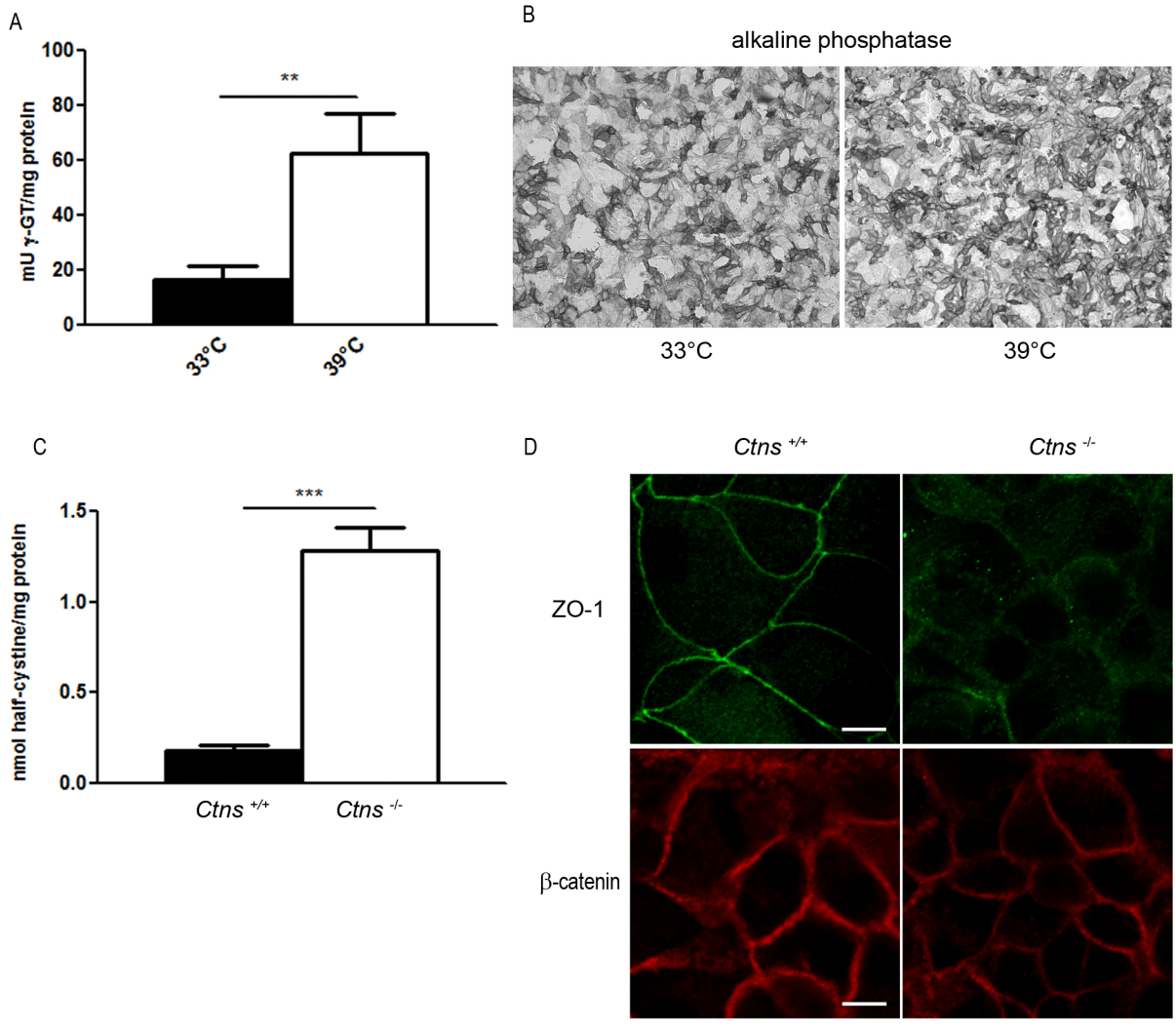


Figure S4. Characterization of MPT cells

(**A,B**). **MPT cells maintain an epithelial phenotype with characteristics of proximal tubular cells.** Gamma glutamyltransferase (γ-GT) and alkaline phosphatase, markers of the brush border in kidney proximal tubular cells, were found in all the MPT cell lines at 33°C and 39°C (graph represents results of two independent experiments with two *Ctns*$^{+/+}$ and two *Ctns*$^{-/-}$ cell lines, **P < 0.01). Enzyme activities were higher after differentiation upon shift to non-permissive (39°C) as compared to permissive growth conditions for SV40 (33°C) (78.1 ± 5.9 mU/mg protein vs 21.4 ± 2.1 mU/mg protein, respectively). Cytochemistry for alkaline phosphatase was similar in permissive and non-permissive conditions for *Ctns*$^{+/+}$ and *Ctns*$^{-/-}$ cells, the intensity depending only on the confluence of the cell monolayer (inversed microscopy). Cells treated with levamisole, as alkaline phosphatase inhibitor, showed no staining (data not shown). **(C) *Ctns*$^{-/-}$ cells accumulate cystine.** *Ctns*$^{-/-}$ MPT cell lines shows a 7.9-fold higher cystine level compared to the *Ctns*$^{+/+}$ cells (1.42 ± 0.13 nmol half-cystine/mg protein for *Ctns*$^{-/-}$ cells vs. 0.18 ± 0.02 nmol half-cystine/mg protein for *Ctns*$^{+/+}$ cells, **P < 0.01). **(D) MPT cells are polarized epithelial cells.** At day 27 (D27) after plating at 33°C, cells were immunolabeled for ZO-1 and β-catenin (confocal microscopy; scale bars = 10 µm).

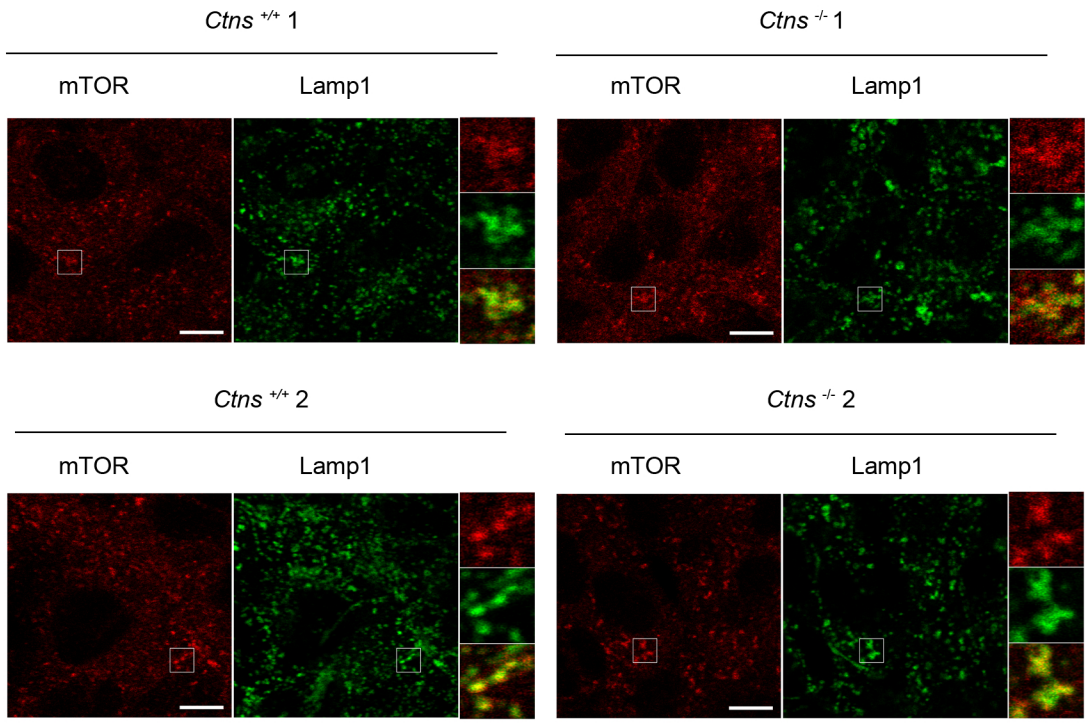


Figure S5. Lysosomal recruitment of mTOR under normal culture conditions in *Ctns* $^{+/+}$ and *Ctns* $^{-/-}$ cell lines

Cells were co-immunolabeled with mTOR and Lamp1 antibodies (confocal microscopy; scale bars 10μm); *Ctns*$^{+/+}$ 1 and 2 and *Ctns* $^{-/-}$ 1 and 2 are two cell lines derived from different *Ctns* $^{+/+}$ and *Ctns* $^{-/-}$ mice respectively used in the study.

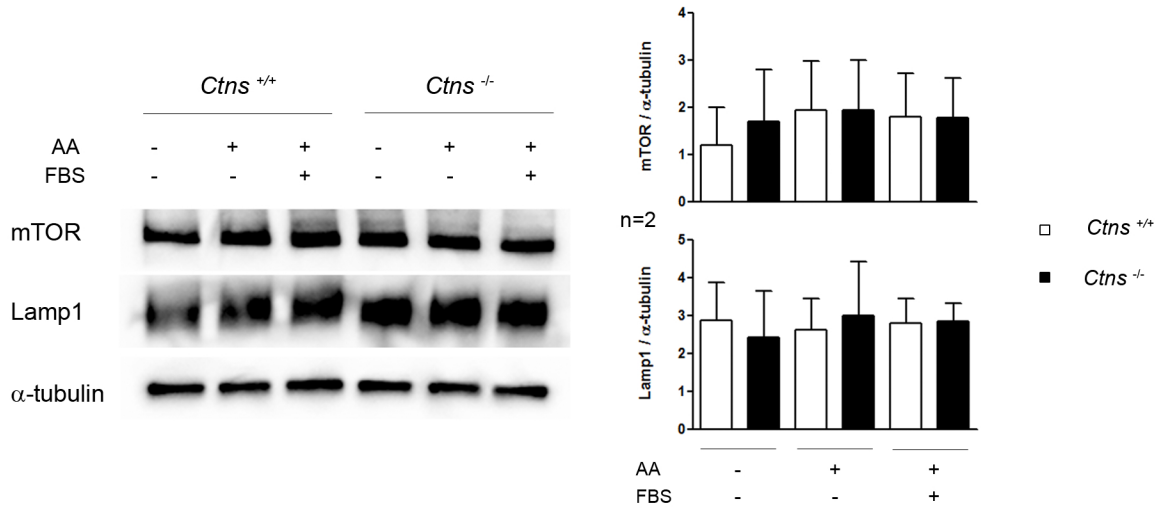
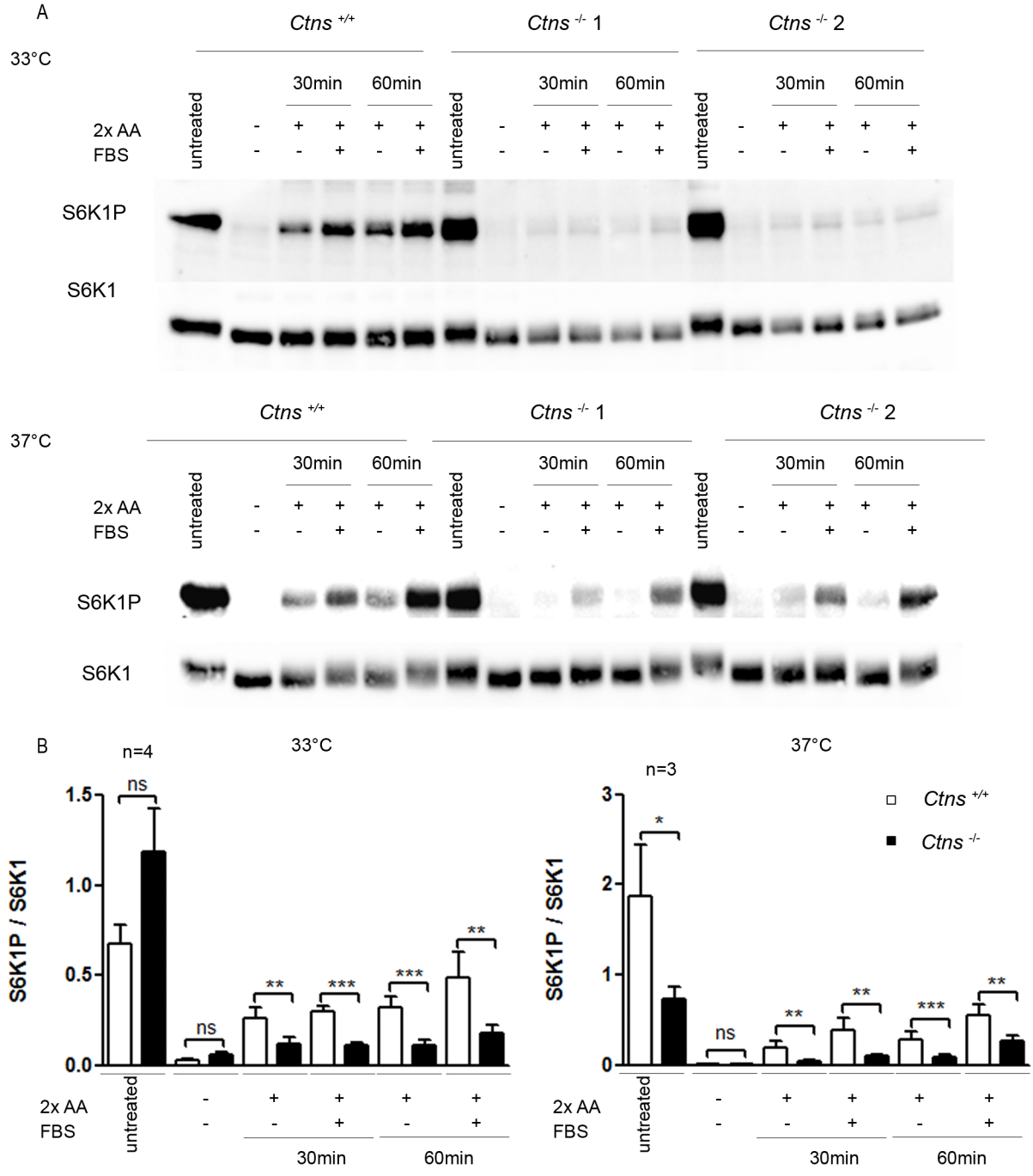


Figure S6. No effect of the nutrient level on the expression of mTOR or Lamp1

Ctns$^{+/+}$ or *Ctns*$^{-/-}$ MPT cells were either amino acid/FBS starved for 30 min, or starved and then recovered in AA or AA/FBS containing medium for 30 min. To evaluate expression levels of mTOR and Lamp1, total cell lysates were analyzed by western-blotting with anti-mTOR, anti-Lamp1 and anti-α-tubulin antibodies. Representative blots from 2 independent experiments are shown (each bar represents the mean ± S.E.M from 2 independent experiments).



Firuge S7. Delay of the response to nutrient signal in *Ctns*$^{-/-}$ cells both at 33°C and 37°C

(A) Total cell lysates were analyzed by western blotting with anti-S6K1 and anti-S6K1P antibodies to evaluate phosphorylation levels of S6K protein. *Ctns*$^{-/-}$ 1 and 2 are two cell lines derived from different *Ctns*$^{-/-}$ mice. Representative blots from at least 3 independent experiments are shown. **(B)** Quantification of phosphorylation levels of S6K protein (*,P < 0.05; **, P < 0.01; ***, P< 0.001; ns, non significant, each bar represents the mean ± S.E.M from at least 3 independent experiments).The analysis of the basal level of phosphorylation of S6K protein under the normal culture condition at 33°C in rich DMEM/F12 medium, specific for MPT cell lines, did not reveal significant difference between *Ctns*$^{+/+}$ and *Ctns*$^{-/-}$ cells. Of note, the protocol was here modified from the initial protocol (see fig.3-5), as the addition of AAs alone for more than 30min was not sufficient to allow the survival of MPT cell lines and did not permit to analyze the mTOR activity at later time points. Hence, to obtain stronger activation of the pathway, after starvation the medium was changed into RPMI medium comprising an additional dose of AAs with or without serum (2xAAs or 2xAA/FBS) for indicated time. Upon 60min incubation in 2xAA/FBS medium, the basal phosphorylation level of S6K1 was restored in *Ctns*$^{+/+}$ cell lines, still no phosphorylation was observed in *Ctns*$^{-/-}$ cell lines. Whereas no diffrence in the basal level of phosphorylation of S6K1 protein could be observed between *Ctns*$^{+/+}$ and *Ctns*$^{-/-}$ cell lines at 33°C, it was significantly lower at 37°C in *Ctns*$^{-/-}$ compared to *Ctns*$^{+/+}$ cell lines. The activity of the mTOR pathway in 2xAAs or 2xAA/FBS media was still significantly lower at 37°C in *Ctns*$^{-/-}$ compared to *Ctns*$^{+/+}$ cell lines.

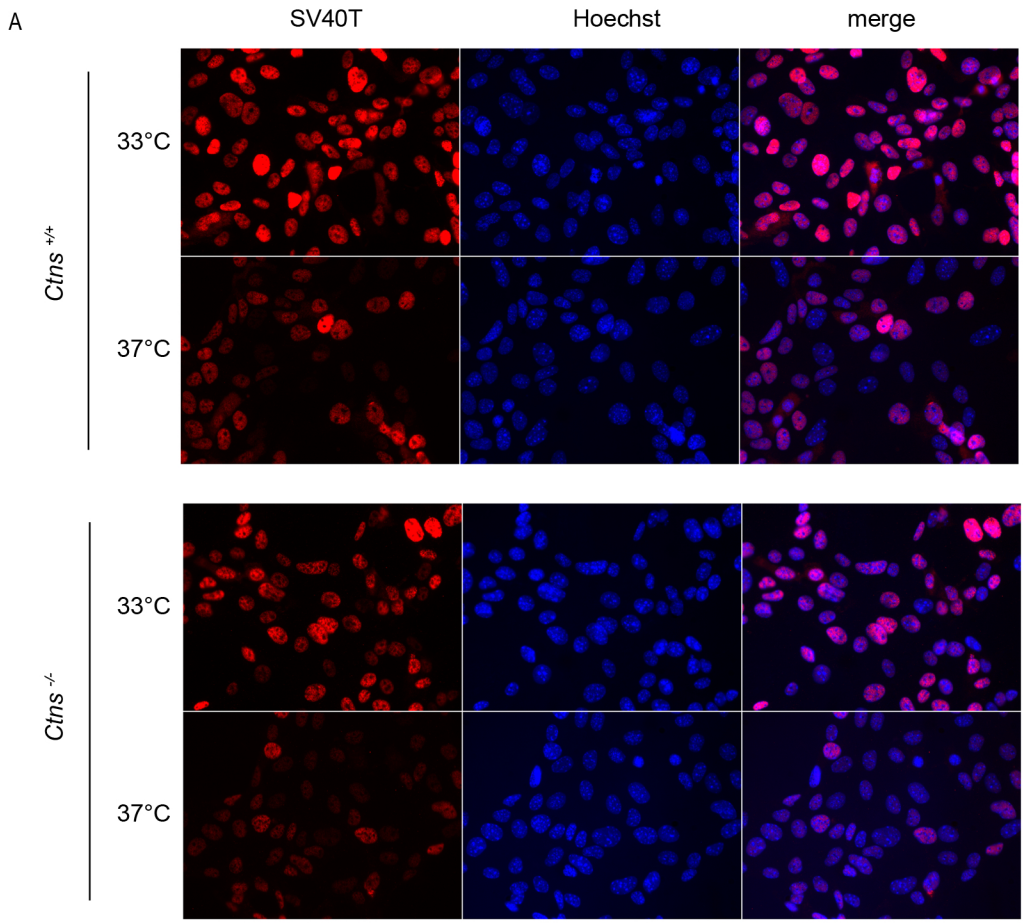


Figure S8. Decreased expression of SV40 T antigen in MPT cell lines at 37°C
The expression of thermosensitive SV40 T antigen was analysed by immunofluorescence in MPT cell lines cultivated at 33°C or 9 days at 37°C.

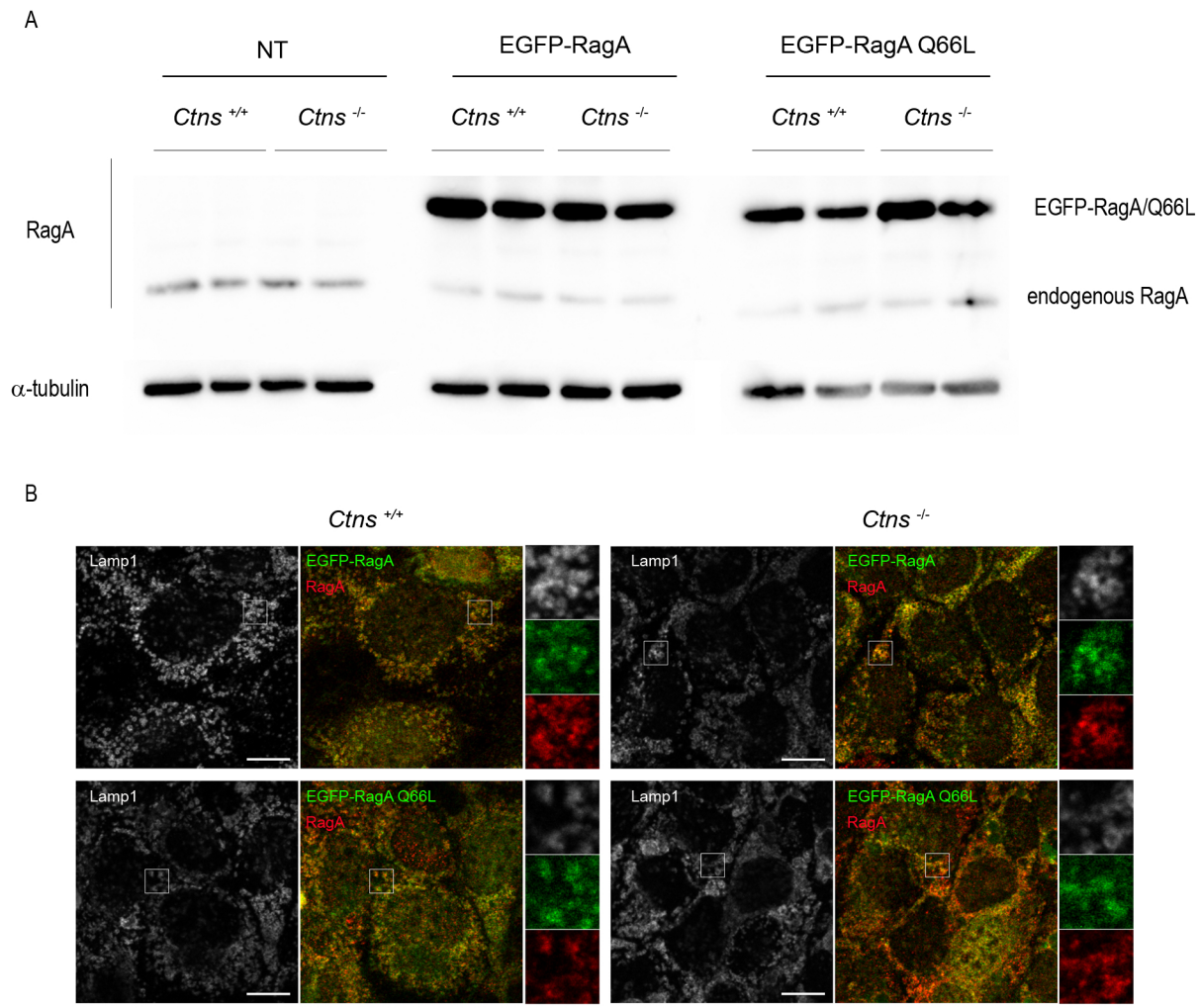


Figure S9. Characterization of EGFP-RagA and EGFP-RagA Q66L constructs
Both EGFP-RagA and EGFP-RagA Q66L constructs are recognized by RagA antibody. **(A)** Expression level of RagA was analyzed by western blotting in lysates of non-transduced (NT) and EGFP-RagA or EGFP-RagA Q66L stably expressing *Ctns*$^{+/+}$ and *Ctns*$^{-/-}$ cells. The lower band corresponds to the endogenous RagA and the higher band to EGFP fusion proteins. **(B)** EGFP-RagA and EGFP-RagA Q66L are correctly localized onto lysosomes. *Ctns*$^{+/+}$ and *Ctns*$^{-/-}$ cells stably expressing EGFP-RagA or EGFP-RagA Q66L were immunolabeled for RagA and Lamp1 to test for lysosomal localization (confocal microscopy; scale bars=10 μm).