Impaired autophagy bridges lysosomal storage disease and epithelial dysfunction in the kidney

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The endolysosomal system sustains the reabsorptive activity of specialized epithelial cells. Lysosomal storage diseases such as nephropathic cystinosis cause a major dysfunction of epithelial cells lining the kidney tubule, resulting in massive losses of vital solutes in the urine. The mechanisms linking lysosomal defects and epithelial dysfunction remain unknown, preventing the development of disease-modifying therapies. Here we demonstrate, by combining genetic and pharmacologic approaches, that lysosomal dysfunction in cystinosis results in defective autophagy-mediated clearance of damaged mitochondria. This promotes the generation of oxidative stress that stimulates Gα12/Src-mediated phosphorylation of tight junction ZO-1 and triggers a signaling cascade involving ZO-1-associated Y-box factor ZONAB, which leads to cell proliferation and transport defects. Correction of the primary lysosomal defect, neutralization of mitochondrial oxidative stress, and blockage of tight junction-associated ZONAB signaling rescue the epithelial function. We suggest a link between defective lysosome-autophagy degradation pathways and epithelial dysfunction, providing new therapeutic perspectives for lysosomal storage disorders.

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The epithelial cells lining the proximal tubules (PT) of the kidney constitute a paradigm of effective communication between the environment and endomembrane compartments, allowing the reabsorption of essential nutrients. By processing incoming substances and recycling receptors and transporters at the apical plasma membrane, the endolysosomal system dictates cell differentiation, hence the maintenance of homeostasis. The PT uptake accounts for ~80% of the clearance of small proteins and peptides, which are continuously filtered and completely reabsorbed by apical endocytosis involving the multi-ligand receptors, megalin, and cubilin. Alterations in these transport processes lead to generalized PT dysfunction (an entity named renal Fanconi syndrome, RFS), causing urinary loss of solutes and low-molecular-weight (LMW) proteins, often complicated by dehydration, electrolyte imbalance, rickets, growth retardation, and development of chronic kidney disease (CKD). Such PT dysfunctions are typically encountered in congenital disorders due to defective endolysosomal transporters, particularly in nephropathic cystinosis.

Cystinosis is a lysosomal storage disease (LSD) caused by recessive, inactivating mutations in the CTNS gene coding for the proton-driven transporter cystinosin that exports cystine out of lysosomes. The loss of cystinosin causes an accumulation of cystine in tissues, leading to renal failure, diabetes, hypothyroidism, myopathy, and central nervous system deterioration. Infantile (MIM #219800) and juvenile (MIM #219900) forms of cystinosis represent a frequent cause of congenital PT dysfunction and RFS, most often complicated by CKD.

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**Fig. 1** Abnormal lysosome dynamics and autophagy in CTNS-deficient PT cells. a Left: confocal microscopy and three-dimensional (3D) reconstruction of Ctns mPTCs labeled with anti-LAMP1 (red) antibody. Right: quantification of changes in vesicle size (top, each point representing the average size of LAMP1+ vesicles in a cell) and lysosome positioning (bottom, percent of perinuclear or peripheral distribution) (n = 30 cells pooled from 3 Ctns kidneys per group; two-tailed unpaired t-test, \( P < 0.0001 \) relative to Ctns+/+ mPTCs). b-f Ctns mPTCs were cultured in normal growth (Fed; 8 h) or nutrient-deprived cell medium (Starved; 8 h). b Representative confocal micrographs (left) and quantification (right) of numbers of LC3+ structures (green) in Ctns mPTCs (n = 100 cells pooled from three Ctns kidneys per group; each point representing the number of LC3+ vesicles in a cell; one-way analysis of variance (ANOVA) followed by Bonferroni's posthoc test, \( **P < 0.001 \) relative to Ctns+/+ mPTCs under fed conditions; NS, not significant). c Western blotting and densitometric analyses of LC3 protein levels. \( \beta \)-Actin was used as a loading control. Two-tailed unpaired Student's t-test, \( *P < 0.05 \) relative to Ctns+/+ mPTCs under fed conditions, \( n = 3 \) independent experiments. d Representative electron micrographs (left) and quantification (right) of numbers of autophagic vacuoles per cell (n = 10 micrographs per each condition; one-way ANOVA followed by Bonferroni's posthoc test, \( **P < 0.01 \) and \( \#P < 0.0001 \) relative to Ctns+/+ mPTCs under fed conditions; NS, not significant). e Representative confocal micrographs and quantification of SQSTM1+ structures (red) in Ctns mPTCs (n = 100 cells pooled from three Ctns kidneys per group; two-tailed unpaired Student's t-test, \( \#P < 0.001 \) relative to Ctns+/+ mPTCs). f Representative western blotting of SQSTM1 in Ctns mPTCs. GAPDH was used as a loading control, \( n = 3 \) independent experiments. Plotted data represent mean ± SEM. Nuclei are counterstained with DAPI (blue). Scale bars are 10 μm in a, b, and e, and 2 μm in d. Unprocessed scans of original blots are shown in Supplementary Fig. 13.
strategy to counteract cystine storage is oral administration of cysteamine, which allows cystine to exit lysosomes. However, cysteamine treatment is hampered by side effects and poor tolerance, and it does not treat nor prevent PT dysfunction. Thus, there is an urgent need to identify novel therapeutic strategies for this devastating disorder. Recent studies based on a Ctns mouse model that recapitulates multiple features of cystinosis have demonstrated that the loss of cystinosin is associated with aberrations of the endolysosomal compartment, and abnormal proliferation and dysfunction of PT cells. Despite the identification of cellular defects associated with cystinosin in different models and cell systems, a unifying mechanism linking loss of cystinosin, lysosomal dysfunction, and defective epithelial transport has not been deciphered.

In most mammalian cells, the endolysosomal system captures and degrades intracellular worn-out constituents through autophagy. This homeostatic process is particularly active in PT cells, whose intense reabsorptive and transport properties require the maintenance of mitochondrial network. The PT cells, whose intense reabsorptive and transport properties are required for protecting PT from acute tubular injury, whereas deletion of essential autophagy genes damages PT cells through defective mitochondrial clearance and increased reactive oxygen species (ROS). Of note, accumulation of distorted mitochondria and of autophagy receptor SQSTM1/p62 has been described in kidney biopsies and urinary cells from cystinotic patients, suggesting a possible involvement of autophagy. In addition, recent evidences show that cystinosin is a component of the lysosomal mammalian target of rapamycin complex, a hub that regulates autophagy-lysosome functions and nutrient transport in renal epithelial cells. Altogether, these data suggest potential interactions between cystinosin function, the autophagy-lysosome degradation pathways, and the transport properties in PT epithelial cells.

In the present study, we decipher a pathway linking loss-of-function of cystinosin, lysosome–autophagy dysfunctions, mitochondrial oxidative stress, disruption of tight junction integrity, and activation of a signaling cascade causing epithelial cell

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**Fig. 2** Cystinosin deficiency alters autophagy in proximal tubules of mouse and zebrafish kidneys. (a) Representative confocal micrographs and quantification of numbers of LC3+ (left panel; red) or SQSTM1+ structures (right panel; red) in LTL+ (green) proximal tubules in Ctns mouse kidneys (n = 30 proximal tubules pooled from three kidneys per group; each point representing the number of LC3+ or SQSTM1+ vesicles in a proximal tubule; two-tailed unpaired Student’s t-test, ***P < 0.001 relative to Ctns+/+ kidneys). Nuclei counterstained with DAPI (blue). (b) Western blotting and densitometric analyses of LC3 protein levels in pronephric tubules from 3-month-old Ctns+/+ and Ctnsdel8/del8 zebrafish embryos at 5 dpf (two-tailed unpaired Student’s t-test, *P = 0.029 relative to Ctns+/+ kidneys, n = 3 mice per group). Representative images of ctns+/+ and ctnsdel8/del8 zebrafish embryos at 5 dpf and quantification of cystine levels by HPLC in ctns+/+ and ctnsdel8/del8 zebrafish embryos at 5 dpf (two-tailed unpaired Student’s t-test, ***P < 0.001 relative to ctns+/+ embryos, n = 8 ctns+/+ zebrafish and n = 7 ctnsdel8/del8 zebrafish). (e) Western blotting and densitometric analyses of LC3 protein levels in pronephric tubules from 3-month-old ctns+/+ and ctnsdel8/del8 zebrafish (n = 4 per group; two-tailed unpaired Student’s t-test, *P = 0.03 relative to ctns+/+ kidneys). (f) Representative electron micrographs and quantification of numbers and size of autophagic vacuoles in pronephric tubules of ctns+/+ and ctnsdel8/del8 zebrafish embryos at 5 dpf. White squares contain images at higher magnification. Yellow arrowhead indicates autophagic vacuoles. Number of autophagic vesicles: n = 43 (ctns+/+) and n = 42 (ctnsdel8/del8) randomly selected micrographs were pooled from 11 ctns+/+ and 12 ctnsdel8/del8 zebrafish. Average vesicle size: n = 32 (ctns+/+) and n = 33 (ctnsdel8/del8) randomly selected micrographs were pooled from 11 ctns+/+ and 12 ctnsdel8/del8 zebrafish. Two-tailed unpaired Student’s t-test, ***P < 0.001 and *P < 0.0001 relative to ctns+/+ zebrafish. β-Actin was used as a loading control in b and e. Plotted data represent mean ± SEM. Scale bars are 50 μm in a and 5 μm in f.
dysfunction and loss of transport capacity. These insights offer new therapeutic strategies for treating epithelial dysfunction in nephropathic cystinosis and endolysosomal disorders.

**Results**

**Loss of cystinosin alters lysosomal dynamics and autophagy.** We first investigated the consequences of Ctns deletion on the lysosomal–autophagy pathways in epithelial cells. The loss of cystinosin, which was reflected by the accumulation of cystine in mouse kidneys and derived PT cells (mPTCs), induced a phenotype switch associating abnormal proliferation and apical dedifferentiation, leading to defective receptor-mediated endocytosis and urinary loss of LMW proteins in vivo (Supplementary Fig. 1a–g). These changes, which confirmed the validity of the Ctns mouse model and derived mPTCs, were associated with a dramatic modification in lysosomal dynamics as evidenced by enlarged lysosomes, clustered into the perinuclear region (Fig. 1a and Supplementary Movies 1–2).

As the intracellular positioning of lysosomes coordinates autophagy in response to nutrient availability, we tested whether the perinuclear clustering of lysosomes reflects changes in autophagy. We used mPTCs, because this primary culture system provides a particularly suited model to decipher the molecular mechanisms underpinning the endolysosome disorders within epithelial cells. Autophagy was assessed by quantifying the conversion of the non-lipidated form of LC3-I to the lipidated, autophagosome-associated form LC3-II, and the numbers of LC3 vesicles in mPTCs cultured in nutrient-rich media (hereafter referred to as “fed”) or in nutrient-deprived conditions (hereafter referred to as “starved”). Compared with wild-type cells, Ctns−/− cells showed higher numbers of punctate LC3 structures and steady-state levels of LC3-II, which did not further increase in starved conditions (Fig. 1b, c), as well as more electron microscopy (EM) structures compatible with autophagic vacuoles (Fig. 1d). Likewise, Ctns−/− cells showed larger numbers of aggregates positive for the autophagy receptor SQSTM1 (Fig. 1e) and higher SQSTM1 protein levels, which did not further increase in starved conditions (Fig. 1f).

Fig. 3 Cystinosin deficiency delays the clearance of autophagosomes by impairing lysosome function. a Western blotting and densitometric analyses for LC3 and SQSTM1 protein levels in Ctns mPTCs cultured in fed or starved medium in the presence or in the absence of 250 nM BfnA1 for 4 h (n = 3 independent experiments). Two-tailed unpaired Student’s t-test, **P < 0.01 (SQSTM1) and *P < 0.05 (LC3) relative to fed Ctns+/+ mPTCs, and *P = 0.05 (SQSTM1) and **P = 0.009 (LC3) relative to starved Ctns+/+ mPTCs. b Correlative light-electron microscopy (CLEM) in Ctns mPTCs co-transduced with GFP-tagged Lamp1 (LAMP1/RFP, red) GFP-tagged Map1lc3b (Map1lc3b/GFP, green) bearing adenoviral particles for 2 days. The colocalization of GFP-LC3 and RFP-LAMP1 was monitored by confocal microscopy. Selected cells were further processed and serial sections analysed by electron microscopy. Dotted black squares contain images at higher magnification. Scale bars are 2 μm (top panel) and 500 nm (bottom panel). c Western blotting and densitometric analyses of CtsD protein levels in Ctns mPTCs; two-tailed unpaired Student’s t-test, **P < 0.01 relative to Ctns+/+ mPTCs, n = 3 independent experiments. d Ctns mPTCs were loaded with Bodipy-FL-PepA (1 μM, for 1 h at 37 °C, green), immunostained with anti-LAMP1 antibody (red) and analysed by confocal microscopy. Quantification of numbers of PepA/LAMP1+ structures (in percentage of total lysosomes; n = 5 randomly selected fields per condition, with each containing ~20–25 cells; two-tailed unpaired Student’s t-test, *P < 0.0001 relative to Ctns+/+ mPTCs). e ctns zebrafish embryos expressing cdh17::GFP (green, pronephric tubule marker) were injected at 5 dpf with Cy5-tagged-β-lactoglobulin. At 120 min after the injection of the tracer, zebrafish embryos were imaged using light sheet fluorescent microscopy. A similar rate of internalization of Cy5-tagged-β-lactoglobulin was observed at 20 min in both ctns+/+ and ctnsdels/delz zebrafish embryos, validating the uptake of the tracer by pronephric tubules. Representative micrographs (left) and quantification (right) of numbers of β-lactoglobulin+ structures (red) in ctns zebrafish pronephric tubule (n = 6 zebrafish per group; two-tailed unpaired Student’s t-test, **P = 0.01 relative to ctns+/+ zebrafish). GAPDH was used as loading control in a and c. Plotted data represent mean ± SEM. Nuclei are counterstained with DAPI (blue). Yellow indicates colocalization. Scale bars are 10 μm in d and 50 μm in e.
The accumulation of LC3-marking autophagosomes and SQSTM1-positive aggregates was consistently detected in the PT of Ctns−/− kidneys (Fig. 2a, b). To further explore the consequences of cystinosin deletion in vivo, we established a novel ctns knockout zebrafish model using the TALENs (transcription activator-like effector nucleases) technique (Supplementary Fig. 2a–c). One mutant zebrafish line showed an 8 bp TALEN-driven deletion (ctnsdel8/del8), resulting in a premature stop codon within exon 3 of ctns, which would result in a truncated protein deprived of the transmembrane domains of cystinosin. Microinjection of wild-type (but not mutant) human CTNS messenger RNA decreased the cystine storage in ctns-deficient embryos to the same extent than cysteamine treatment (Supplementary Fig. 2d). The protein levels of LC3-II and the numbers of autophagic vesicles (AVs) were remarkably increased in cystine-accumulating pronephric tubules in ctns-deficient zebrafish (Fig. 2c–f), demonstrating the evolutionary conservation of this connection. These results demonstrate that the deletion of cystinosin alters lysosomal dynamics and autophagy in PT cells, both in vitro and in vivo.

**Cystinosin deficiency perturbs autophagic clearance in PT cells.** An increased number of AVs may arise from stimulation of autophagosome biogenesis or from alteration of their degradation by lysosomes. To discriminate between these two possibilities, we treated Ctns mPTCs with Bafilomycin A1 (BfnA1), a proton pump inhibitor that prevents lysosome degradation and thus increases autophagic cargoes and substrates exclusively when autophagy is active. Treatment with BfnA1 heightened the amounts of LC3-II and SQSTM1 in nutrient-deprived Ctns−/+ mPTCs, whereas it did not change the already elevated levels of these proteins in nutrient-deprived Ctns−/− cells (Fig. 3a).

We next performed a pulse-chase assay to monitor the degradation of resting autophagosomes in Ctns mPTCs. Cells
were starved to form autophagosomes and then treated with the class III phosphoinositide 3-kinase (PI3K) vacuolar protein sorting 34 (Vps34) kinase inhibitor SAR-405 to prevent the formation of new autophagosomes. We validated the selectivity of SAR-405 by determining the intracellular phosphatidylinositol-3-phosphate pools (PtdIns-3P, the end-product generated by Vps34 kinase activity) in mPTCs (Supplementary Fig. 3a). The degradation of the formed autophagosomes following SAR-405 treatment was tracked by time-lapse confocal microscopy: in contrast to the autophagic-mediated degradation observed in starved Ctns−/− cells, the Ctns−/− cells retained almost all the formed autophagosomes (Supplementary Fig. 3b, c). Upstream events regulating autophagosome biogenesis, such as conjugation (ATG7) and phagosome formation (ATG16L + vesicles), including Beclin1, remained unchanged in control and Ctns−/− PT cells (Supplementary Fig. 4a–c). Taken together, these results indicate that the accumulation of autophagosomes in Ctns−/− cells results predominantly from a slower autophagosome clearance rather than augmented biogenesis.

Defective lysosomal function impends autophagy in Ctns−/− cells. A blockade in LC3-II degradation can occur at any step after autophagosome formation and can be induced by delayed trafficking of autophagosomes to lysosomes and/or reduced fusion between both compartments. We thus explored whether the delayed autophagosome clearance in Ctns−/− cells might be caused by an impaired autophagosome delivery to lysosomes. To test this hypothesis, we examined the subcellular distribution of LC3-marking autophagic structures and LAMP1-labeled lysosomes in response to short incubations with non-saturating...
concentrations of BfnA1 (50 nM for 1 h)26. Confocal microscopy analysis showed that cystinosin deletion did not decrease the colocalization of LC3 and LAMP1-labeled lysosomes in BfnA1-treated PT cells but rather increased the number of large LAMP1 + vesicles filled by LC3 in Ctns–/– PT cells (Supplementary Fig. 5a). Electron tomography microscopy and three-dimensional (3D) reconstructed tomograms confirmed the existence of enlarged, single-membrane structures engulled with partially digested cellular debris in Ctns–/– cells (Supplementary Fig. 5b and Supplementary Movie 3). Accumulation of autophagic material in autolysosomes of Ctns–/– cells was also confirmed by correlative light-EM, where GFP-LC3-positive autophagosomes containing cellular constituents coalesced within enlarged, single-membrane RFP-LAMP1-positive organelles (Fig. 3b), indicating that the transport and/or autophagosome-lysosome fusion are not compromised.

One mechanism by which the autophagic cargo clearance might be impeded is defective lysosomal degradation capacity11. To substantiate the delayed autophagosome degradation in Ctns–/– cells, we analyzed trafficking, processing, and maturation of lysosomal cathepsins. Western blot analyses of cathepsin-D (CtsD) showed a decreased proteolytic generation of the 32 kDa mature CtsD in Ctns–/– compared with Ctns+/+ cells (Fig. 3c). We next tested the lysosomal CtsD activity by incubating mPTCs with Bodipy-FL-PepstatinA (PepA), a fluorescence-tagged PepA that binds to the active site of CtsD in acidic lysosomes22. Although the majority of lysosomes were co-stained with PepA-labeled CtsD in Ctns+/+ cells, the number of PepA-labeled vesicles and the colocalization of active CtsD with LAMP1 were substantially lower in Ctns–/– cells (Fig. 3d). These changes in lysosome function were observed despite normal trafficking of CtsD between Golgi and LAMP1 compartments (Supplementary
Fig. 7 Augmented phosphorylation rate of ZO-1 promotes epithelial dysfunction in Ctns<sup>−/−</sup> cells. a Tight junction ZO-1 protein was immunoprecipitated from Ctns<sup>+/+</sup> and Ctns<sup>−/−</sup> mPTCs, and its phosphorylation rate was examined by western blotting, n = 2 independent experiments. b Confocal analysis of endogenous phosphorylation of ZO-1 by proximity ligation assay (PLA), and c of ZO-1/LAMP<sup>1</sup> structures (in percentage of the total lysosomes) and d of ZONAB<sup>+</sup> nuclei (in percentage of the total nuclei) in Ctns mPTCs. Quantifications in b, c, and d were obtained from five randomly selected fields per condition, with each containing ~25–25 cells; two-tailed unpaired Student’s t-test, <i>p</i><0.0001 relative to Ctns<sup>+/+</sup> mPTCs. e The mRNA levels of Lrp2, Cdcl1, and Pcna in Ctns microdissected proximal tubules were analysed by real-time PCR, n = 3 mice per group. Two-tailed unpaired Student’s t-test, *<i>p</i><0.05, **<i>p</i><0.01, and ***<i>p</i><0.001 relative to Ctns<sup>+/+</sup> microdissected proximal tubules. f Growth curves of Ctns mPTCs. Two-tailed unpaired Student’s t-test, *<i>p</i><0.05 and ***<i>p</i><0.001 relative to Ctns<sup>+/+</sup> mPTCs, n = 3 independent experiments. g-i Ctns mPTCs were transduced with Scmb or Csda adenoviral shRNAs for 5 days. g Confluent analysis and quantification of ZONAB<sup>+</sup> nuclei and h of PCNA<sup>+</sup> nuclei in Ctns mPTCs. The quantifications (expressed in percentage of the total nuclei) were obtained in g and h from four randomly selected fields per condition, with each containing ~25–25 cells. i The cells were loaded with Al647-BSA (50 μg ml<sup>−1</sup> for 15 min at 37 °C) and imaged by confocal microscopy. Quantification of numbers of Al647-BSA<sup>+</sup> structures (n = 45–50 cells pooled from three mouse kidneys per condition; each point representing the number of BSA<sup>+</sup> structures in a cell). One-way ANOVA followed by Bonferroni’s post hoc test, */<i>p</i><0.01, ***/<i>p</i><0.001, and ###<i>p</i><0.0001 relative to Ctns<sup>+/+</sup> mPTCs transduced with Ad-Scmb shRNA or to Ctns<sup>−/−</sup> mPTCs transduced with Ad-Scmb shRNA. Plotted data are mean ± SEM. Nuclei counterstained with DAPI (blue). Scale bars are 10 μm.

Fig. 5c, d). Similarly, the lysosome-based processing of the LMW β-lactoglobulin, which is normally internalized and degraded by PT endolysosomes<sup>23</sup>, was dramatically reduced in proxenephic tubules of ctns<sup>del1/del1</sup> compared with ctns<sup>+/+</sup> zebrafish (Fig. 3e).

The key role of cystinosin depletion in lysosomal–autophagy dysfunctions was assessed by transducing Ctns<sup>−/−</sup> cells with an adenovirus that expresses mouse hemagglutinin (HA)-tagged cystinosin (Ad-Ctns-HA; Fig. 4). The functional re-expression of HA-CTNS protein at late endosomal/lysosomal compartments in Ctns<sup>−/−</sup> cells (Fig. 4a; Supplementary Fig. 6) lowered the intracellular cystine content (Fig. 4b), rescued the lysosomal dynamics (Fig. 4c, d), activated cathepsin-B within lysosomes (Fig. 4e), and augmented the lysosome-mediated degradation of autophagy substrates SQSTM1 and LC3-II (Fig. 4f) when compared with Ctns<sup>−/−</sup> cells transduced with an empty vector. These features were abolished by treating the HA-CTNS expressing Ctns<sup>−/−</sup> cells with BfnA1 (Fig. 4f), indicating that cystinosin deficiency blocks autophagosome clearance by compromising lysosome function.

Disruption of autophagy causes epithelial cell dysfunction. As autophagy mediates cellular homeostasis, we tested whether and how its disruption may cause epithelial dysfunction. Inactivation of basal autophagy was obtained by transducing mPTCs with an adenovirus expressing a short hairpin RNA (Ad-shRNA) against Atg7 or by inhibiting the autophagy Beclin1/Vps-34 complex with SAR-405 or Spautin-1.

We confirmed that either shRNA-mediated knockdown of Atg7 (Fig. 5a) or autophagy inhibitors (Supplementary Fig. 7a–b) prevented the conversion of LC3-I to LC3-II and increased the levels of the SQSTM1 protein, and of aggregate-forming SQSTM1 and ubiquitin-positive inclusions (Fig. 5a–c and Supplementary Fig. 7c) in mPTCs. Disruption of autophagy led to the accumulation of dysfunctional mitochondria, as shown by increased levels of prohibitin (an inner mitochondrial membrane protein; Fig. 5d) and decreased resting mitochondrial membrane potential (ΔΨm, assessed by quantitative confocal imaging of tetramethylrhodamine methyl ester (TMRM) dye; Fig. 5e and Supplementary Fig. 7d), and the induction of a major mitochondrial oxidative stress, as scored by elevated mitochondrial ROS levels (MitoSOX; Fig. 5f and Supplementary Fig. 7e).

Importantly, either the genetic or the pharmacological impairment of autophagy induced the nuclear translocation of tight-junction ZO-1-associated Y-box factor ZONAB (Fig. 5g) and its activity (as measured by the regulation of the Ccdn1, Pcna, and Lrp2 targets<sup>27,28</sup> (Fig. 5h and Supplementary Fig. 7f). These changes were reflected by a phenotype switch that included abnormal cell proliferation, as evidenced by bromodeoxyuridine (BrDU) incorporation (Fig. 5i and Supplementary Fig. 7g), and dedifferentiation, as testified by reduced expression of the apical endocytic receptor megalin (Fig. 5j and Supplementary Fig. 7j).
and by decreased endocytic uptake (Fig. 5) and Supplementary Fig. 7h). Collectively, these data demonstrate that the maintenance of mitochondrial function and homeostasis by autophagy is required for the terminal differentiation, hence regulating the reabsorptive capacity of PT epithelial cells.

**Dysfunctional mitochondria drive oxidative stress in Ctns−/− cells.** Having established that disruption of autophagy recapitulates the phenotype switch of proliferation dedifferentiation, we investigated how defective lysosomal degradation of autophagy substrates might lead to epithelial dysfunction in cystinosis.

As autophagy delivers protein aggregates and organelles to endolysosome for cellular degradation, we analyzed the ability of Ctns−/− mPTCs to clear polyubiquitinated aggregates and compromised mitochondria. Correlating with the stalled autophagy flux, we found an accumulation of ubiquitin-forming aggregates and abnormal mitochondria with disorganized cristae in Ctns−/− compared with Ctns+/+ cells (Fig. 6a–c). The defective mitochondria in Ctns−/− cells were retained within enlarged, dysfunctional lysosomes (Fig. 6d) and showed a major decrease in Δψm (Fig. 6e). Seahorse metabolic flux analyses measuring the overall consumption rates (OCR) confirmed markedly decreased mitochondrial bioenergetics (baseline respiration, ATP turnover, and total respiratory capacity) in Ctns−/− compared with Ctns+/+ mPTCs (Fig. 6f). These changes were paralleled by a major mitochondrial oxidative stress, as scored by the elevated mROS levels (MitosOX; Fig. 6g) and increased anti-oxidant response (heme oxygenase-1 (HO1); Fig. 6h). Overall, these findings indicate that impaired lysosome-mediated autophagic degradation of dysfunctional mitochondria causes a major oxidative stress in cystinosin-deficient cells.

**Abnormal tight junction signaling leads to epithelial dysfunction.** Oxidative stress has been shown to induce the
phosphorylation of the zona occludens-1 (ZO-1) adaptor protein, disrupting the integrity of the tight junction complex.\textsuperscript{29} As ZO-1 traps the Y-box transcription factor ZONAB that is known to regulate the differentiation of PT cells\textsuperscript{27,28}, an overproduction of ROS by dysfunctional mitochondria might alter tight junction integrity and trigger ZONAB-mediated signaling.

To test this hypothesis, we immunoprecipitated ZO-1 from mPTCs and noted that it was more heavily phosphorylated in Ctns\textsuperscript{-/-} mice (Fig. 7a) or with the mitochondrial complex I inhibitor Rotenone (ROT)-stimulating mitochondrial ROS (Supplementary Fig. 8d,e). The cells were immunostained for PCNA and imaged by confocal microscopy. Quantification of numbers of Al488-BSA\(^+\) structures (n = 100 cells pooled from three mouse kidneys per condition; each point representing the number of BSA\(^+\) structures in a cell). Nuclei counterstained with DAPI (blue) in d and f. Plotted data show mean \(\pm\) SEM. One-way ANOVA followed by Bonferroni’s post hoc test, *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\), and \(\#P<0.0001\) relative to mPTCs from vehicle-treated Ctns\(^{+/+}\) or Ctns\(^{−/−}\) mice. Scale bars are 10 \(\mu\)m.

The link between defective autophagy, compromised mitochondria, and oxidative stress was substantiated by the increased phosphorylation rate of ZO-1 induced by the shRNA-mediated knockdown of Atg7 in mPTCs (Supplementary Fig. 8a) or by the treatment of the cells with autophagy inhibitors (SAR-405 and Spautin-I; Supplementary Fig. 8b), with H\(_2\)O\(_2\) (Supplementary Fig. 8c) or with the mitochondrial complex I inhibitor Rotenone (ROT)-stimulating mitochondrial ROS (Supplementary Fig. 8d,e). Treatment with ROT induced ZONAB translocation and signaling (Supplementary Fig. 8f, g), reflected by increased proliferation (Supplementary Fig. 8h) and decreased endocytic uptake (Supplementary Fig. 8i).

The relevance of the abnormal tight junction signaling in cystinosis was confirmed by gain- and loss-of-function experiments in mPTCs. shRNA-mediated knockdown of Csda encoding ZONAB (Fig. 7g-i and Supplementary Fig. 9a-c) or overexpression of exogenous Tjp1 encoding ZO-1 (Supplementary Fig. 9d, e), which functionally inhibits ZONAB\textsuperscript{27,28}, significantly decreased the proliferation markers and rescued the endocytic uptake in Ctns\(^{−/−}\) cells. Conversely, depletion of ZO-1 (shRNA-mediated knockdown of Tjp1; Supplementary Fig. 10a-c) or overexpression of Csda (Supplementary Fig. 10d) triggered the activation of ZONAB signaling (Supplementary Fig. 10e) with
increased cell proliferation and decreased endocytic uptake of albumin (Supplementary Fig. 10f) in wild-type cells.

Mitochondrial ROS disrupt tight junctions through Gα12/Src activation. Finally, we investigated how aberrant mitochondria-producing ROS disrupt tight junctions in cystinosis epithelial cells. Given the accumulation of abnormal mitochondria-producing ROS (Fig. 6) and higher phosphorylation rate of ZO-1 (Fig. 7) in Ctns−/− PT cells, we hypothesized that high levels of mitochondrial ROS may stimulate the Gα12 protein that triggers the disruption of tight junctions through Src-mediated phosphorylation of ZO-1 (20-21). This would in turn activate ZONAB signaling and drives the epithelial dysfunction.

Consistent with this hypothesis, mPTCs from Ctns−/− kidneys (Fig. 8a, b) displayed an increased protein abundance of Gα12 and activated Src tyrosine kinase (monitored by the increased phosphorylation of pTyr416-Src; Fig. 8a, b). Likewise, wild-type mPTCs treated with ROT (Fig. 8c) or accumulating aberrant mitochondria-producing ROS (shRNA-mediated knockdown of Atg7; Fig. 8d) also exhibited an abnormal activation of Gα12/Src. This activation paralleled the increased phosphorylation of ZO-1, tight junction disruption, and epithelial dysfunction described above (Fig. 5 and Supplementary Fig. 8).

Having verified that the high level of mitochondrial ROS drives epithelial dysfunction by activating Gα12/Src kinase pathway, we tested whether the neutralization of mitochondrial ROS/Gα12/Src signaling loop may avert the loss of tight junction integrity and rescue the function of cystinosis cells. We incubated Ctns−/− mPTCs with mitochondria-localized-oxygen scavenger Mito-TEMPO (MT, 10 μM for 24 h; Supplementary Fig. 11a, b32) or with a selective c-Src family kinase inhibitor SU6656 (5 μM for 24 h33), or transduced the cells with Ad-shRNA against Gna12.

The blockage of the mitochondrial ROS/Gα12/Src signaling cascade by these pharmacologic or genetic interventions reversed the abnormal activation of Gα12/Src (Fig. 8a, b), preventing the phosphorylation of ZO-1 (Fig. 8e and Supplementary Fig. 11c) and its lysosomal accumulation, which increased ZO-1 abundance at cell boundaries (Supplementary Fig. 11d). In turn, these changes inhibited the nuclear translocation of ZONAB (Fig. 8f) and restored the differentiation and endocytic uptake of albumin in cystinosis cells (Fig. 8g, h). Of note, the rescue induced by MT treatment was prevented by overexpressing Csda-HA in Ctns−/− mPTCs (Supplementary Fig. 12a, b), supporting the role of ZONAB.

Taken together, these data suggest that the excess of mitochondrial ROS, resulting from impaired lysosome-mediated autophagic degradation of damaged mitochondria, triggers Gα12/Src-mediated phosphorylation of ZO-1, which disrupts tight junction integrity and activates ZONAB signaling, causing epithelial dysfunction in cystinosis cells.

Neutralizing mitochondrial ROS improves epithelial function in cystinosis. To enhance the translational potential of these findings, we administered MT (1 mg kg−1 body weight) to 16-week-old Ctns mice by daily intraperitoneal injections (Fig. 9a). After 30 days of treatment, mPTCs were isolated from the Ctns−/− kidneys to score the epithelial function by measuring the endocytic uptake of albumin. Treatment with MT effectively reduced ROS production and oxidative stress (Fig. 9b, c), lowered levels of the kidney injury marker lipocalin-2 (LCN234) (Fig. 9c), abrogated abnormal cell proliferation (Fig. 9d) and restored epithelial viability, resulting in improved albumin uptake (Supplementary Fig. 13a, b).

Fig. 10 A working model depicting the link between defective lysosome–autophagy pathways and epithelial dysfunction in cystinosis. The black arrows identify the sequence of events occurring within cystinosis PT cells. The loss of the cystinosin (CTNS) transport system leads to lysosomal dysfunction. This impairs the cellular clearance of autophagosomes containing SQSTM1 aggregates and/or damaged mitochondria. Similarly, pharmacologic and genetically mediated failure of autophagy (with SAR-405 and Spautin-1 inhibitors or with shRNA-mediated knockdown of Atg7) increases the SQSTM1 aggregates and/or damaged mitochondria-producing ROS. The mitochondrial oxidative stress enables the Gα12/Src-mediated phosphorylation of the tight junction adapter protein ZO-1, resulting in its misrouting to endolysosomal compartment. The disruption of tight junction integrity releases the ZO-1-associated Y-box factor ZONAB, which promotes cell proliferation and represses apical endocytic receptors such as LRPs2, causing epithelial dysfunction in PT cells. Conversely, neutralization of excessive mitochondrial oxidative stress (with mitochondria-targeted oxygen scavenger Mito-TEMPO) or blockade of the Gα12/Src regulatory loop (with a selective Src-kinase inhibitor Su6656) restores the differentiation and transport properties in cystinosis PT cells.
abundance of endocytic receptor megalin (LRP2; Fig. 9e), reflected by a significant recovery of the ligand uptake (Fig. 9f) in Ctns−/− cells. Thus, modulation of mitochondrial oxidative stress ameliorates epithelial function in nephropathic cystinosis.

Discussion

The endolysosomal system regulates the transport activity of specialized epithelial cells, sustaining their role in homeostasis. Congenital defects in lysosomal transporters, as exemplified by cystinosis, cause PT dysfunction and RFS. By combining genetic and pharmacologic approaches in vitro and in vivo, we decipher the link between lysosomal disease and epithelial dysfunction in cystinosis. We demonstrate that lysosomal dysfunction results in defective autophagy-mediated clearance of damaged mitochondria-producing ROS, disruption of tight junction integrity, and activation of a signaling pathway causing epithelial cell proliferation and dedifferentiation, with loss of reabsorptive capacity (Fig. 10). These data reveal the fundamental importance of the autophagosome-lysosome fusion in maintaining epithelial differentiation and offer novel therapeutic perspectives to restore epithelial transport capacity downstream of the primary lysosomal defect.

Cystinosin deficiency induces a major alteration in lysosomal dynamics, paralleled by increased numbers of autophagosomes in the PT cells of Ctns−/− mice. Similarly, abnormal lysosomes and LC3-marking autophagosomes heighten in cystine-accumulating pronephric tubules in ctns-deficient zebrafish, demonstrating the evolutionary conservation of this connection across vertebrates. Contrasting with cystine storage and defective lysosome–autophagy pathways, the ctns zebrafish larvae appear morphologically normal and display neither obvious development defects nor signs of PT dysfunction. As cystinosis is typically a storage disease, longitudinal studies are needed to provide a more comprehensive characterization of the phenotype after 5 days post fertilization (dpf) in this model.

The accumulation of the autophagy substrate SQSTM1, which is normally degraded within autolysosomes, suggests an abnormal autophagy flux in cystinosin-deficient PT cells, in line with recent observations stating an impairment of autophagy flux in many lysosome storage diseases and evidence in human cells and kidney biopsies from cystinosis patients. Evidence supporting the failure to degrade autophagy cargoes in primary PT Ctns−/− cells include the following: abnormally elevated numbers of mature autophagosomes under normal growth conditions; failure to clear AVs formed after starvation-induced autophagy, mimicking BfnA1 action; inability of BfnA1 to further induce the LC3-II protein levels; and impaired degradation of the resting AVs by a selective P3K3/Vps34 inhibitor. In contrast, upstream events regulating autophagosome biogenesis including Atg7–Atg12 conjugation and phagophore formation were not affected. Altogether, these data indicate that the functional loss of cystinosin, causing lysosomal cystine storage, delays the autophagy flux, causing an accumulation of autophagosomes in vitro and in vivo. Of note, the autophagy flux was found to be fully functional in fibroblasts derived from Ctns mice, in contrast with the predictions based on the defective mTORC1 signaling in cystinosis cells. The reason for these discrepancies would require further studies to understand the tissue and cell-specific effects of cystinosin depletion.

The question remained how the lysosomal defect could impair autophagy flux and lead to epithelial dysfunction. A blockage of the degradation of autophagic material may occur at any step after autophagosome formation, due to factors regulating the trafficking of autophagosomes to lysosomes or the fusion between both compartments. For instance, accumulation of LC3-positive organelles unable to fuse with lysosomes has been observed in many LSDs. Accordingly, a potential cause for the impaired autophagy flux observed in cystinosis could be a delayed fusion between lysosomes and autophagosomes. However, when treating Ctns−/− cells with short incubations of BfnA1, which accurately measure the autophagy flux, we observed a substantial co-localization of LC3-positive vesicles with the lysosome marker LAMP1, suggesting that the delivery and autophagosome–lysosome fusion is not compromised in cystinotic cells. Another indication is the perinuclear lysosomal clustering in Ctns−/− cells; such centripetal movement of lysosomes has been shown to mediate the autophagic degradation of macromolecules.

As the autophagy flux also relies on the degradative capacity of lysosomes, an impaired lysosomal function may explain the accumulation of autophagosomes in cystinosis cells. We postulated that cystinosin deficiency may affect lysosome function either by controlling the delivery of newly synthesized lysosomal cathepsins from Golgi to the lysosome or by inhibiting the processing/maturatoin of cathepsins within endolysosome compartments. Our results support the latter hypothesis, indicating an impairment of lysosome proteolysis (observed both in vitro and in vivo) as a result of defective cathepsin activation despite an efficient delivery of newly synthesized cathepsins from Golgi to lysosomes. Furthermore, the rescue of lysosomal cystine homeostasis through the transient expression of cystinosin in Ctns−/− cells resulted in multi-level reactivation of the autophagy–lysosome degradative pathways. Taken together, these findings support a role of cystinosin—beyond its function in cystine transport—in maintaining the lysosomal response to the arrival of the autophagy cargoes, hence in cellular homeostasis.

The conjugation of impaired lysosomal dynamics and altered lysosomal degradative capacity is strikingly similar to the cellular changes resulting from the accumulation of mononclonal light chains (κLCs) within endolysosomes of PT cells, causing a similar epithelial dysfunction. Furthermore, the uncontrolled increase in lysosomal PtdIns(4,5)P2 that results from the loss-of-function of the PtdIns(4,5)P2 5-phosphatase oculo cerebro renal Lowe syndrome (OCRL), leads to lysosomal dysfunction and autophagosome accumulation in PT cells from patients with Lowe syndrome, another congenital disorder causing PT dysfunction and RFS. These data suggest that lysosomal accumulation of cystine or specific LCs or PtdIns(4,5)P2 may have similar functional consequences on the epithelial phenotype, emphasizing the role of endolysosomes as crucial signaling hub to ensure cellular homeostasis. As lysosomal acidification is necessary for the activation of cathepsins, one could speculate that the vacuolar-type H+-ATPase (V-ATPase) complex might be altered in absence of cystinosin. Potential factors affecting V-ATPase efficiency in LSD may include the following: storage of cholesterol in the endolysosome membranes; luminal oxidation of lysosomal thiols by free cystine, and accumulation of cystine sustaining mTORC1 activity and negatively regulating lysosome biogenesis by suppressing TFE/MITF signaling. Recent studies showing that cystinosin is a member of the lysosomal machinery that controls mTORC1 activity in vitro, and that overexpression of transcription factor EB (TFEB) stimulates lysosomal cargo processing support the concept. Regardless of the mechanism, impaired cellular clearance leads to accumulation of autophagy substrates, such as dysfunctional mitochondria and ubiquitinated proteins. Although autophagy deficits have been reported in LSDs, the impact of lysosome–autophagy defects on epithelial cell function remains unknown. Basal autophagy operates to preserve the integrity of subcellular compartments, including damaged mitochondria. Lack of autophagy completion, due to impaired lysosomal degradation
capacity or through inactivation of essential genes, leads to the persistence of ubiquitinated proteins (including p62/SQSTM1) and dysfunctional, ROS-producing mitochondria. Our results demonstrate the importance of this cellular quality-control mechanism for the function of epithelial cells. Genetic or pharmacologic blockade of basal autophagy resulted in accumulation of ubiquitinated proteins and damaged/dysfunctional mitochondria, leading to abnormal cell proliferation and apical differentiation reflected by decreased uptake capacities. Similar to the changes observed in autophagy-deficient cells, there was a remarkable accumulation of SQSTM1- and ubiquitin-forming Y-box transcription factor that interacts with ZO-1, modulating the function of epithelial junctions. An abnormal activity of ZONAB has also been evidenced when Tjp1−/− mice and their derived mPTCs with mitochondrial-targeted mCherry (mPTCs) accumulated within dysfunctional endolysosomes22. Therefore, the blockade of autophagy-mediated quality control of mitochondria, which are crucial for the high transport activities performed by specialized epithelial cells. They also demonstrate the cross-talk between mitochondria and tight-junction associated signal transduction pathways regulating the epithelial phenotype. The effect of an antioxidant compound specifically targeting mitochondria on the function of Ctns−/− cells provides a promising approach to alleviate the loss of vital metabolites in nephropathic cystinosis.

Methods

Antibodies and reagents. The following antibodies were used in this study: rat anti-LAMPI (sc-19992, Santa Cruz Biotechnology; 1:500); rabbit anti-LC3 (PM006, MBL; 1:100); rabbit anti-LC3 (NB100-2331, Novus Biologicals; 1:200), rabbit anti-p62/SQSTM1 (PM045, MBL; 1:200); rabbit anti-GAPDH (2118, Cell Signaling Technology; 1:1,000); mouse anti-β-actin (A2228, Sigma-Aldrich; 1:10,000); goat anti-GstC (sc-6486, Santa Cruz Biotechnology; 1:500); rabbit anti-GM130 (ab52649, Abcam; 1:500); mouse anti-Ubiquitin (sc-8017, Santa Cruz Biotechnology; 1:1,000); mouse anti-PCNA (M0879, DAKO; 1:500); rabbit anti-Histone-3 (H3; ab4729, Abcam; 1:1,000); mouse anti-α-tubulin (T5168, Sigma-Aldrich; 1:1,000); rabbit anti-Prohibitin (ab28172, Abcam; 1:1,000); rabbit anti-ATG16L (D6D5) (8089, Cell Signaling; 1:400); rabbit anti-ATG7 (A2856, Sigma-Aldrich; 1:200); mouse anti-βECNI (sc-48341, Santa Cruz Biotechnology; 1:500); rat anti-HA (AB1167423001, Roche; 1:500); rabbit anti-Calnexin (C4731, Sigma-Aldrich; 1:500); mouse anti-Phospho-Tyr99 (PV99; sc-7020, Santa Cruz Biotechnology; 1:500); rabbit anti-ZO-1 (sc-10804, Santa Cruz Biotechnology; 1:100); rabbit anti-ZONAB (A303-070A, Bethyl Laboratories; 1:100); mouse anti-Gt12 (sc-515610, Santa Cruz Biotechnology; 1:200); rabbit anti-Phospho-Src Family (pTyr416; 6943, Cell Signaling; 1:100); rabbit anti-Src (2109, Cell Signaling; 1:500); mouse anti-Pdlns-3P (Z-P003, Echelon Biosciences; 1:200); rabbit anti-HO1 (ab13243, Abcam; 1:500); rabbit anti-LC2 (ab63929, Abcam; 1:500); and goat anti-green fluorescent protein (GFP) (AB0020-500, SIGCEN; 1:500). Sheep anti-megalin antibody (LRP2; 1:1,000) was kindly provided by P. Verroust and R. Kozyraki (INSERM, Paris, France). Compounds included BfnA1 (ALX-380-030, Enzo Life Sciences), PIK3C3/Vps34 inhibitor SAR-405 (A8883, APExBIO), autophagy inhibitor Spautin-1 (S7888, Selleckchem.com), hydrogen peroxide (H2O2; HI1009 Sigma-Aldrich), ROT (R8875, Sigma-Aldrich), SU6656 (S6962, Sigma-Aldrich), and Mito-TEMPO (MT; ALX-430-150-M005, Enzo Life Sciences).

Generation and maintenance of ctns zebrafish. ctns-specific left and right TALENs (ctns-TALENs) were constructed in accordance with Golden Gate TALEN assembly protocol and using the Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene, Kit 38142). CrisprGoldTALyzer was a gift from Daniel Carlson & Stephen Ekker (Addgene, plasmid 38142). TALENs were designed with the TAL assembly protocol and using the Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene, Kit 3810-030, Enzo Life Sciences), PIK3C3/Vps34 inhibitor SAR-405 (A8883, APExBIO), autophagy inhibitor Spautin-1 (S7888, Selleckchem.com), hydrogen peroxide (H2O2; HI1009 Sigma-Aldrich), ROT (R8875, Sigma-Aldrich), SU6656 (S6962, Sigma-Aldrich), and Mito-TEMPO (MT; ALX-430-150-M005, Enzo Life Sciences).
fish (Hubrecht Institute) were approved by the Animal Care and Use Committee of the Royal Netherlands Academy of Arts and Sciences.

**Rescue experiments in ctns zebrafish.** The plasmids containing wild type or mutant (ΔADVVF346-349) human CTSN complementary DNA were kindly provided by C. Anticaglia (INSERM, Paris, France). The T3 promoter sequence was cloned into the Xhol restriction site of the enhanced GFP vector by using forward primer: 5'-TGCGAGATCTCAATCCATGATTTGAGGAC-3' and reverse primer: 5'- TGGATTCCCCCTGAGGTGTTAATGGATCC-3'. Either wild type or mutant CTSN mRNA was synthesized by using the mMESSAGE mMACHINE T3 transcription Kit (Invitrogen). The microinjection of either wild type or mutant CTSN mRNA was performed in ctns Wt and ctns embryos at one-cell stage and subsequently collected at 5 dpf for cystine measurements. Where indicated, a working solution of cysteamine at 1 mM was prepared by diluting the stock solution in E3 medium. After dechorionation, cts zebrafish larvae at 2 dpf were incubated in the E3 medium in the presence or in the absence of cysteamine until to the sampled day (5 dpf), and the zebrafish medium was renewed daily. Twenty zebrafish embryos per group were pooled and homogenized by sonication, and prepared for cystine measurements.

Mouse model. Experiments were conducted on age- and gender-matched C57BL/6 and C57Bl/6 litterates (C57BL/6 background). Mice were maintained under temperature- and humidity-controlled conditions with 12 h light/12 h dark cycles with free access to appropriate standard diet in accordance with the institutional guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Mice aged 16 weeks were treated by daily intraperitoneal injection of MitoTracker Green FM (M2529, Thermo Fisher Scientific) or with 1 μM Magic Red-(RR)2 substrate (MR-CtsB; 938, Immunochemistry Technologies) according to the manufacturer’s specifications. After 15 min, the cells were pulsed with 1 μM Magic Red-(RR)2 in Live Cell Imaging (A14291DJ, Thermo Fisher Scientific) medium for 1 h at 37 °C followed by fixation and immunostaining with anti-LAMPI or anti-HA antibody, and subsequently analyzed by confocal microscopy.

Endocytosis assay. The endocytic uptake was monitored in mPTCs cells following incubation for 60 min at 4 °C with 50 μg/ml bovine serum albumin (BSA)–Alexa-Fluor-488 (A37703, Thermo Fisher Scientific) or with 50 μg/ml BSA–Alexa-Fluor-488 (A28425, Thermo Fisher Scientific) in complete HEPES-buffered Dulbecco’s modified Eagle’s medium. The cells were given an acid wash and warmed to 37 °C in growth cell medium for 15 min before being fixed and processed for immunofluorescence analyses.

Lysosomal activity. The detection of lysosomal activity was performed in live mPTCs cells using BODIPY-FL-PepA (P22712, Thermo Fisher Scientific) or Magic Red-(RR)-substrate (MR-CtsB; 938, Immunocytochemistry Technologies) according to the manufacturer’s instructions. Where indicated, the cells were incubated with 50 μg/ml BSA–Alexa-Fluor-488 (A28425, Thermo Fisher Scientific) in complete HEPES-buffered Dulbecco’s modified Eagle’s medium. The cells were given an acid wash and warmed to 37 °C in growth cell medium for 15 min before being fixed and processed for immunofluorescence analyses.

Extracellular flux analysis and metabolic measurement. OCR was measured in XpF Extracellular Flux Analyzers (Agilent Seahorse Biosciences) in mPTCs incubated with XF-Base Medium (non-buffered RPMI 1640 containing either 2 mM l-glutamine, 1 mM sodium pyruvate and 10 mM glucose, pH 7.4). Three measurements were assessed under basal conditions and upon addition of 2 μM FCCP, 1 μM Rot/AA (25 μg/ml) and 1 μM Oxymycin (Oligo), 0.5 μM FCP, and 1 μM ROT/AA (ANT). All the reagents were provided by XpF Cell Mito Stress Test Kit (Agilent Seahorse Biosciences). OCR measurements were normalized to the numbers of the cells (TC10 automated cell counter, Bio-Rad).

Mitochondrial membrane potential measurement. The mitochondrial membrane potential (ΔΨ) was measured in accordance with the manufacturer’s specifications. The cells were pulsed with 50 μM TMRE perchlorate (1666 Thermo Fisher Scientific) for 30 min in live-cell imaging at 37 °C. After washing, the cells were subsequently analysed by confocal microscopy. The fluorescence intensity

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was quantified by using the open-source cell image analysis software CellProfiler as described below.

ROS detection. The cells were pulsed with 5 μM CellROX Green Reagent (C10444, Thermo Fisher Scientific) or with 2.5 μM MitoSOX Red (C10447, Thermo Fisher Scientific) for 10 min in live-cell imaging conditions (2.5 °C). After washing, the cells were subsequently analysed by confocal microscopy. The fluorescence intensity was quantified by using the open-source cell image analysis software CellProfiler as described below.

Immunofluorescence and confocal microscopy. Fresh mouse kidneys were fixed by perfusion with 50–60 ml of 4% PFA in PBS (1×18271, Sigma-Aldrich), dehydrated and embedded in paraffin at 56 °C. Paraffin blocks were sectioned into consecutive 5-μm-thick slices with a Leica RM2255 rotary microtome (Thermo Fisher Scientific) on Superfrost Plus glass slides (Thermo Fisher Scientific). Before staining, slides were deparaffinized in changes of CitriSolv (22-143-975, Thermo Fisher Scientific) and 70% isopropanol. Antigen retrieval was accomplished by incubating in sodium citrate buffer (1.8% 0.1 M citric acid, 8.2% 0.1 M sodium citrate (P36930, Thermo Fisher Scientific) for 10 min in live-cell imaging conditions (2.5 °C). After washing, the cells were subsequently analysed by confocal microscopy. The fluorescence intensity was quantified by using the open-source cell image analysis software CellProfiler as described below.

Immunoelectron microscopy. The mPTCs were incubated overnight with the appropriate primary antibodies. Following incubation with primary antibodies, the samples were thawed on ice, normalized for protein (20 μg per lane), dissolved in Laemmli sample buffer, and separated by SDS-PAGE under reducing conditions. After blotting onto polyvinylidene difluoride and blocking with 5% non-fat milk (1706494, Bio-Rad Laboratories) diluted in PBS, the membranes were incubated overnight at 4 °C with primary antibodies, washed, incubated with peroxidase-labeled secondary antibody, and visualized with enhanced chemiluminescence (WBKLS0500, Millipore, Life Technologies). For re-probing, the membranes were rinsed, incubated for 30 min at 55 °C in a stripping buffer (62.5 mM tris- HCl pH 7.5, 1% SDS, 100 mM mercaptoethanol, adjusted to pH 7.4), before incubation with primary antibodies. Quantitative analyses were performed by scanning the blots and measuring the relative density of each band normalized to actin, and detection were performed using the Duolink In Situ Detection Reagents Red (Olink Bioscience). The PLA assay was performed using the Duolink in situ Detection Reagents (Olink Biosciences) in according with the manufacturer instructions. The PLA assay was performed using the Duolink in situ Detection Reagents (Olink Biosciences) in according with the manufacturer instructions. The PLA assay was performed using the Duolink in situ Detection Reagents (Olink Biosciences) in according with the manufacturer instructions.
PtdIns-3P detection. The cells were fixed with 2% formaldehyde in PBS for 15 min at room temperature. After washing with PBS containing 50 mM NH₄Cl, the cells were permeabilized for 5 min by the addition of 20 µM digitonin in buffer A (20 mM Pipes pH 6.8, 137 mM NaCl, 2.7 mM KCl). Digitonin was removed by three rinses in buffer A and cells were blocked for 45 min with buffer A supplemented with 5% (v/v) FBS and 50 mM NH₄Cl. Monoclonal mouse anti-PtdIns-3P antibody (α-PtdIns-3P; Invitrogen, Carlsbad, CA) was diluted in buffer with 5% FBS for 1 h (1:300). After washing with buffer A for three times, antibody was blocked second (1:400). Cells were incubated in buffer with 5% FBS (1:400) for 30 min. Cells were subjected to postfixation for 5 min in 2% PFA, washed with PBS containing 50 mM NH₄Cl, and then mounted with the Prolong Gold Anti-fade reagent. PtdIns-3P-3 levels were analyzed by using a Leica SP8 confocal laser scanning microscope and quantified by using CellProfiler.

Proliferation assay. To measure cell proliferation, the cells were seeded in 24-well plates at a density of 2.0 × 10⁴ cells per well. The cells were cultured for 3 days and cell medium was renewed daily. Afterwards, the cells were trypsinized every 24 h and quantified using the Countess automated cell counter TC10 automated cell counter (Bio-RAD). The time-course experiments were repeated three times using cells derived from three individual mice per each group. Where indicated, the cultures were incubated with BrdU (1.5 µg·mL⁻¹; Sigma-Aldrich) in accordance with the manufacturer’s protocol. BrdU-labeled cells were detected by immunostaining using rat anti-BrdU antibody (Oxford Biotechnology; 1:500), followed by the specific secondary biotinylated goat anti-rat antibody (1:300; Jackson Immunoresearch) and mounted with the Prolong Gold Anti-fade reagent. The slides were analyzed by using a Leica SP8 confocal laser scanning microscope and quantified by using CellProfiler.

Cystine measurement. Kidney tissues from mice kidney tissue or from zebrafish embryos, or primary cultured cells were homogenized and lysed with N-ethylmaleimide (NEM) solution containing 10 mM NEM in 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, and 0.1% NaN₃. NEM-ethylated cystine was measured by using a lithium high performance physiological column (Biochrom) followed by post-column derivatization with ninhydrin. The amino acids were identified according to the retention time and the ratio of the area between the two wavelengths (570 nm and 440 nm) and quantified by using EZChrom Elite software (Agilent Technologies Inc., Pleasanton, California, USA). Cystine concentration was normalized to the protein concentration and reported in nmol per mg protein. N-ethylmaleimide

Data analysis and statistics. The quantitative data were expressed as means ± SEM. Differences between experimental groups were evaluated using one-way analysis of variance followed by Bonferroni or Dunn’s post hoc test, when appropriate. When only two groups were compared, unpaired or paired two-tailed Student’s t-tests were used as appropriate. No statistical methods were used to predetermine the sample size. We estimated the sample size considering the variation and mean of the samples. Assumptions for statistical analyses were met (that is, normal distribution and equal variance). The sample size (number of cells or number of biological replicates derived from distinct mice or zebrafish) of each experimental group is described in figure legends. The results are representative of at least three independent experiments, unless specified in the figure legends. None of the samples/animals was excluded from the experiment, and the animals were not randomized. The investigators were not blinded to allocation during the experiments and outcome assessment. GraphPad Prism software was used for all statistical analyses. Statistical significance was set at a P < 0.05.

Data availability statement. The data that support the findings of this study are available on reasonable request from the corresponding authors (O.D. and A.L.) available on reasonable request from the corresponding authors (O.D. and A.L.)
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**Author contributions**

O.D. and A.L. supervised the entire project. R.P.F., O.D., and A.L. designed the research concept, planned the overall experimental design, performed cellular and mouse studies, analysed and interpreted the data, and wrote the paper with inputs and final approval from all the authors. Z.C., G.V.D.H., and R.H.G. generated and analyzed the ctns knockout zebrafish model and contributed to the interpretation and analysis of the data. M.B. and J.A.P. performed primary cultures of PT cells and immunoblot experiments. N. T. performed the seahorse metabolic flux analyses, H.D. performed RNA and cellular studies, and contributed to the interpretation and analysis of the data. A.C. performed the measurements of the cystine levels on primary cells and kidney tissues. A.R. performed transmission and tomography microscopy studies, and contributed to the interpretation and analysis of the data. N.N. provided and analyzed Ctns mice, and contributed to the interpretation of the data.

**Additional information**

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**Competing interests:** The authors declare no competing financial interests

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