Cystinosin is a Component of the Vacuolar H⁺-ATPase-Ragulator-Rag Complex Controlling Mammalian Target of Rapamycin Complex 1 Signaling

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ABSTRACT

Cystinosis is a rare autosomal recessive storage disorder characterized by defective lysosomal efflux of cystine due to mutations in the CTNS gene encoding the lysosomal cystine transporter, cystinosin. Lysosomal cystine accumulation leads to crystal formation and functional impairment of multiple organs. Moreover, cystinosis is the most common inherited cause of renal Fanconi syndrome in children. Oral cysteamine therapy delays disease progression by reducing intracellular cystine levels. However, because cysteamine does not correct all complications of cystinosis, including Fanconi syndrome, we hypothesized that cystinosin could have novel roles in addition to transporting cystine out of the lysosome. By coimmunoprecipitation experiments and mass spectrometry, we found cystinosin interacts with almost all components of vacuolar H⁺-ATPase and the Ragulator complex and with the small GTPases Ras-related GTP-binding protein A (RagA) and RagC. Furthermore, the mammalian target of rapamycin complex 1 (mTORC1) pathway was downregulated in proximal tubular cell lines derived from Ctns⁻/⁻ mice. Decrease of lysosomal cystine levels by cysteamine did not rescue mTORC1 activation in these cells, suggesting that the downregulation of mTORC1 is due to the absence of cystinosin rather than to the accumulation of cystine. Our results show a dual role for cystinosin as a cystine transporter and as a component of the mTORC1 pathway, and provide an explanation for the appearance of Fanconi syndrome in cystinosis. Furthermore, this study highlights the need to develop new treatments not dependent on lysosomal cystine depletion alone for this devastating disease.


The large serine/threonine kinase targeted by rapamycin, mammalian target of rapamycin (mTOR), exists in two independent complexes involved in the response to multiple growth and proliferation promoting factors, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is a multi-component protein kinase that integrates inputs from growth factors as well as nutrients to control many biosynthetic and catabolic processes. Upon amino acid stimulation, the small GTPases Ras-related GTP-binding proteins A–D (RagA–D) and the pentameric Ragulator complex recruit mTORC1 to the lysosomal membrane, close to its activator, Rheb GTPase.¹² mTORC1 activation stimulates the growth-promoting program by increasing protein synthesis while inhibiting autophagy. Conversely, starvation inactivates...
mTORC1, thereby inhibiting anabolic processes and releasing nutrient reserves by activating autophagy. In addition, it has recently been shown that the proton-pumping vacuolar ATPase (v-ATPase) is necessary for amino acids to activate mTORC1 through extensive amino acid–sensitive interactions with Rags and the Ragulator complex at the lysosomal membrane.

Cystinosis is a rare autosomal recessive lysosomal storage disorder characterized by cystine accumulation in lysosomes, which leads to functional impairment of multiple organs, including kidneys, endocrine glands, muscles, and the central nervous system. Cystinosis is the most frequent cause of the renal Fanconi syndrome, characterized by urinary loss of water and salts, bicarbonates, calcium, glucose, phosphate, carnitine, amino acids, and ultrafiltrated proteins due to defective renal proximal tubular reabsorption. Based on the age of onset and the severity of the symptoms, three clinical forms of cystinosis have been described. The most severe form, infantile cystinosis (OMIM 219800), appears between 6 and 12 months of age along with Fanconi syndrome, which, if untreated, leads to ESRD by the age of 10 years. Moreover, the accumulation of cystine crystals in the cornea leads to a severe and painful photophobia within the first 2 years of age. Juvenile cystinosis (OMIM 219900), a less severe form, is characterized by later onset of photophobia and glomerular impairment, not necessarily associated with Fanconi syndrome. Ocular non-nephropathic cystinosis (OMIM 219750) described primarily in adults is characterized by mild photophobia without renal anomalies.

The causative gene, CTNS, encodes a 367 amino acid–long lysosomal cystine–proton symporter, cystinosin. Cystinosin is the founding member of the "PQ-loop" protein family characterized by a seven-transmembrane (TM) domain structure and the presence of a duplicated "PQ-loop" motif. Reminiscent of its lysosomal localization, cystinosin contains 7-N-glycosylation sites in the amino-terminal region and a classic tyrosine-based lysosomal targeting signal (GYXX0) in the C-terminal tail. Interestingly, the fifth inter-TM loop was found also to play an essential role in the lysosomal localization of cystinosin. Deletion of each of the two motifs alone results in the partial mislocalization of cystinosin to the plasma membrane, whereas in the absence of both, cystinosin is no longer present at lysosomes.

The functional study of mutated forms of cystinosin revealed the correlation between cystine transport activity and severity of the disease. Indeed, most of the mutations leading to infantile cystinosis analyzed in this study abolished cystine transport, whereas mutations associated with juvenile or ocular forms strongly reduced transport, although some puzzling discrepancies have been observed between transport activity and clinical phenotype.

Cystinosis is the first lysosomal storage disorder for which a treatment based on substrate depletion efficiently delays disease progression. Oral cysteamine therapy, if used early in the disease and in high regular doses, reduces intracellular cystine concentration and delays the progression toward renal failure and the appearance of other clinical anomalies. However, as cysteamine does not correct all clinical manifestations of cystinosis, and in particular does not have any effect on Fanconi syndrome, we hypothesized that cystinosin could have other functions than its well characterized role in cystine transport out of the lysosome. Using a proteomic approach, we show here that cystinosin consistently interacts with the various members of the mTORC1 pathway and that these interactions are mainly lost with specific mutations of cystinosin. Our data show that cystinosin plays a role in the regulation of the mTORC1 pathway in renal tubular cell lines under nutrient supplementation, demonstrating a new function for this amino acid transporter.

RESULTS

Cystinosin Interacts with Multiple Proteins of the mTORC1 Signaling Pathway

Because of the lack of a reliable antibody to detect endogenous cystinosin, we transduced MDCK and 3T3 cells with lentiviral constructs to stably express the cystinosin-enhanced green fluorescence protein (EGFP) fusion protein and its mutants. Mass spectrometry analysis of anti-green fluorescence protein (GFP) immunoprecipitates consistently identified various components of the mTORC1 signaling pathway; (1) almost all subunits of the V1 domain; (2) the a, c, d subunits of the V0 domain of the v-ATPase and its accessory protein Ac45 (S1); (3) proteins p18, p14, Mp1, and HBXIP – but not C7orf59 – of the Ragulator complex (also known as LAMTOR1, 2, 3, 5, and 4, respectively); and (4) Rag GTPases RagA and RagC (Table 1). These interactions were further confirmed by coimmunoprecipitation (co-IP) experiments with anti-GFP and -RagC antibodies followed by Western blotting. By contrast, no interactions were found with the control late endosomal/lysosomal membrane proteins, EGFP-CDD3 and Lamp1-EGFP (Figure 1, A and B, Supplemental Figures 1, A and B and 2, Table 1).

As seen by mass spectrometry and Western blots, the deletion of both lysosomal targeting motifs (2m) of cystinosin, leading to its relocation to the plasma membrane, almost completely abolished interactions with its partners (Supplemental Figure 2, Table 1). However, while interactions were preserved when cystinosin lacked its C-terminal tail containing the classic tyrosine-based GYDQL lysosomal sorting motif, they were considerably decreased when cystinosin was deleted of the YFPQA or the YMNF sequences in the fifth inter-TM loop (ΔYFPQA, ΔYMNF) (Figure 2, A and B, Supplemental Figure 1, Table 1). In line with the previously described cellular localization of these mutants, although both ΔYFPQA and ΔGYDQL partially colocalized with the plasma membrane marker wheat germ agglutinin in 3T3 cells, a large fraction of these mutants remained lysosomal (Supplemental Figure 3A). Moreover, the identified interactions were abolished with the cystinosin mutant N288K localized in the fifth loop, but were maintained with the K280R mutant in the juxtamembrane portion of the same loop and
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Proteins identified by mass spectrometry from co-IP experiments with cystinosin-EGFP and its mutants, as well as EGFP-CD63 and Lamp1-EGFP controls, using GFP antibodies are reported for 3T3 and MDCK cells. The numbers represent the total number of peptides used for the identification of the respective protein. The accession numbers according to the database used are described. All biologic replicates (1–4) were performed at different times always in parallel with nontransduced cells as internal controls (not shown, as all values were null).

*3T3 cells.

†MDCK cells.
the N323K mutant in the sixth luminal loop, all mutations previously identified in patients with cystinosis and shown not to impair the lysosomal localization of the protein. Of note, although all these mutants were shown to completely abolish cystine transport, only the first one led to the most severe, infantile form of cystinosis, whereas the last two led to juvenile cystinosis. Moreover, N to A substitution at position 288 had no effect on partners co-IP with cystinosin. We showed that both N288K and N288A proteins were correctly localized to the late endosomal-lysosomal compartment in 3T3 cells, where they colocalized with Lamp1 and components of the mTORC1 signaling pathway (Supplemental Figure 3, B and C). Starting from MDCK data, in silico protein networks were built for the wild-type (WT) and different mutants of cystinosin (Figure 2C). Altogether, these data show the specificity of identified interactions and also point to a key role of the fifth inter-TM loop of cystinosin for interaction with the v-ATPase-Ragulator–Rag complex, and call for special attention to crucial amino acids within this loop, in particular N288.

The Lack of Cystinosin Alters mTOR Localization

All proteins identified by mass spectrometry as partners of cystinosin–EGFP are required for the proper activation of the mTORC1 signaling pathway. Two steps are important for the activation of the mTOR kinase in response to nutrient signals, the Ragulator–Rag–GTPase–dependent relocalization of mTORC1 to the lysosomal membrane and its subsequent activation by Rheb GTPase. Upon nutrient deprivation, mTORC1 is released from lysosomes where it relocalizes after restoration of the amino acid level. To evaluate the impact of cystinosin depletion on mTORC1 signaling, we analyzed mTOR subcellular localization and activity after nutrient starvation and reintroduction in Ctns+/+ and Ctns−/− mouse proximal tubular (MPT) cell lines generated in our laboratory by a thermosensitive SV40 immortalization strategy as already described. We first confirmed that Ctns+/+ and Ctns−/− mice-derived cell lines, grown in MPT cell–specific medium, similarly mimic a proximal tubule phenotype and acquire epithelial polarity with brush border differentiation, as indicated by γ-glutamyl transferase activity and phosphatase alkaline assays (Supplemental Figure 4, A and B). Second, Ctns−/− cells accumulated cystine (approximately eightfold increase in cystine level as compared with the Ctns+/+ cells [Supplemental Figure 4C]). Third, analysis of junctional complexes revealed a selective loss of tight junctions (Zonula Occludens-1 [ZO-1]) in Ctns−/− cells while adherent junctions (β-catenin) were better preserved (Supplemental Figure 4D). The loss of tight junction integrity has recently been described in primary cultures of Ctns−/− MPT cells. Thus, MPT cells appeared appropriate for nutrient deprivation/refeeding experiments.

Under the normal culture conditions in rich MPT cell–specific medium, mTOR is recruited to the lysosomal membrane in both Ctns+/+ and Ctns−/− cell lines (Supplemental Figure 5). Upon 30 minutes in RPMI starvation medium, mTOR (red, Figure 3A) exhibited a diffused pattern in all cell lines tested. At 30 minutes after reintroduction of amino acids alone (AA) or together with serum (AA/FBS), mTOR was relocalized to the lysosomal membrane in two Ctns+/+ MPT lines. In striking contrast, mTOR still assumed a diffuse (cytosolic) pattern in two Ctns−/− MPT lines (Figure 3A). Of note, the level of expression of mTOR remained stable in all cell lines upon starvation and reintroduction of nutrients (Supplemental Figure 6). Moreover, defective mTOR relocalization in Ctns−/− cells correlated with impaired downstream signaling. While S6K1, the main target of mTOR, became phosphorylated upon AA or AA/FBS reintroduction in control cells, phosphorylation was not detected in Ctns−/− cells (Figure 3, B and C). This difference persisted 60 minutes after AA or AA/FBS reintroduction. Moreover, a significant decrease in the activity of the mTORC1 pathway was also observed in Ctns−/− compared with Ctns+/+ when cells were grown at differentiating conditions (37°C) for 9 days (Supplemental Figures 7, A and B and 8). The possible explanation for some variations observed in the activity of the mTORC1 pathway between 33°C and 37°C could be the difference in the kinetics of the response to nutrient reintroduction in differentiating MPT cells. In addition, whereas no difference in the basal phosphorylation level of S6K1 could be

Figure 1. Cystinosin interacts and colocalizes with components of the mTORC1 pathway. Lysates of 3T3 cells stably expressing cystinosin-EGFP or EGFP-CD63 were immunoprecipitated with (A) anti-GFP or (B) anti-RagC antibodies, and coimmunoprecipitated proteins were analyzed by Western blotting (n=1). IP, immunoprecipitates; NT, nontransfected cells.
observed between Ctns+/+ and Ctns−/− cell lines grown at 33°C, it was significantly lower in Ctns−/− cell lines compared with controls in differentiating conditions (Supplemental Figure 7, A and B). Thus, cystinosin appeared essential for mTOR regulation by nutrients in MPT cell lines.

Cystinosin Acts Upstream of Rags

To test if cystinosin could act upstream of Rag GTPases, we generated cells overexpressing EGFP-RagA or its constitutively active form, EGFP-RagA Q66L (Supplemental Figure 9). By mimicking the GTP-bound state, the dominant active mutants of RagA/B GTPases constitutively activate the mTORC1 pathway, rendering it insensitive to amino acid starvation.2 As predicted, Ctns+/+ cells expressing EGFP-RagA Q66L were resistant to the starvation protocol (Figure 4, A–C). Moreover, while in Ctns−/− cells expressing EGFP-RagA the mTOR protein showed a diffuse cytosolic pattern even after nutrient reintroduction, expression of EGFP-RagA Q66L rescued mTOR recruitment to the lysosomal membrane bypassing cystinosin absence (Figure 4A). In agreement with mTOR lysosomal localization, its downstream signaling remained constitutively activated as shown by S6K1 phosphorylation in control and Ctns−/− cells regardless of nutrient level (Figure 4, B and C). We thus concluded that cystinosin acts upstream of Rags in the mTORC1 signaling pathway.

Cysteamine has no Effect on mTOR Signaling in Ctns−/− Cells

Finally, to rule out the possibility that lysosomal accumulation of cystine, not cystinosin absence per se, was responsible for dysregulation of the mTORC1 signaling pathway in Ctns−/− cells, we analyzed the effect of cysteamine treatment on the levels of S6K1 phosphorylation. Cysteamine converts the lysosomal cystine into cysteine and cysteamine-cystine mixed disulfide, which is analogous to lysine and thus able to exit lysosomes through the cationic amino acid PQLC2/LAAT-1 transporter.10 A 2-hour treatment with 1 mM cysteamine efficiently decreased the levels of cystine in Ctns−/− cells, yet had no impact on mTORC1 signaling, as S6K1 phosphorylation could not be restored in treated cells (Figure 5). This substrate depletion experiment excluded an indirect effect of impaired cystine transport and confirmed a direct role of cystinosin for structural and functional interactions with the mTOR pathway.

Figure 2. Mutations in the fifth inter-TM loop of cystinosin suppress interactions with the mTORC1 complex. (A) 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 and coimmunoprecipitated proteins were analyzed by Western blotting. Representative blots from two independent experiments are shown. (B) Schematic representation of cystinosin indicating point mutations and deletions used in the study. (C) Representation of differential protein networks of WT or mutated cystinosin and its partners. The connections irradiating from cystinosin can represent both direct and indirect physical interactions between cystinosin and the proteins partners identified by co-IP. The connections between the proteins partners represent direct and indirect interactions established according to literature archived in Ingenuity Knowledge Database. The red color code reflects the abundance of the protein in the complex, estimated according to the number of identified peptides (brighter red = higher abundance). IP, immunoprecipitates; NT, non transfected cells.
Cystinosis, a devastating hereditary lysosomal storage disorder, is the most frequent cause of renal Fanconi syndrome in children. Even though the currently used cysteamine-based therapy efficiently decreases cellular cystine levels in affected organs, it only delays progression to renal failure. We thus hypothesized that cystinosin could have additional role(s) independent of cystine transport, which could explain why some deleterious effects of cystinosis are not prevented despite compliant cysteamine treatment. Here, mass spectrometry enabled us to identify cystinosin as a component of a complex, recently characterized lysosomal nutrient-sensing machinery, as we found cystinosin to bind to almost all components of the v-ATPase, to four subunits of the pentameric Ragulator, and to both RagA and RagC small GTPases. In line with our hypothesis of an important role of cystinosin in the mTORC1 pathway, the yeast ortholog of cystinosin, ErS1, was found to complement for the absence of the ortholog of p18, MeH1/Ego1.18,19 Interestingly, yeast lacking ERS1 or MEH1 are sensitive to hygromycin B that, in both cases, can be completely complemented by expression of either ErS1 or MeH1, strongly suggesting that both proteins are functioning in the same cellular mechanism/pathway.

We found that deletions leading to disruptions of the fifth inter-TM loop of cystinosin weakened the identified interactions and this effect was even more drastic for the N288K mutation, but not for the K280R or N323K mutations. Previous studies from our laboratory revealed that although all three mutations disrupted cystinosin transport activity in vitro, only N288K led to the most severe infantile form of cystinosis, whereas the two others resulted in a juvenile form without Fanconi syndrome.11 Likewise, we found that only N288K abolished interactions with components of the mTORC1 pathway. These results clearly suggest that the development of the Fanconi syndrome characteristic for the most severe infantile form of cystinosis does not simply result from lack of cystine transport activity, but also from dysregulation of the mTORC1 pathway in the absence of interactions between cystinosin and the v-ATPase-Ragulator-Rags complex. Moreover, these results perfectly explain discrepancies between cystine transport activity of some mutants (i.e., K280R and N323K) and the clinical phenotype.11 Surprisingly, when alanine was substituted for a glutamine residue in the 288 position (N288A), interactions were maintained at a level comparable with WT cystinosin. These results suggest that the substitution of a positively charged amino acid for an uncharged, bulky one at this particular position may introduce structural changes in cystinosin, hampering interaction with its binding partners. Although our results demonstrate a crucial role of the fifth loop of cystinosin in the interaction with the mTORC1 complex, this interaction remains to be precisely mapped. So far we have been unsuccessful using the yeast two-hybrid system to evidence any

**DISCUSSION**

Cystinosis, a devastating hereditary lysosomal storage disorder, is the most frequent cause of renal Fanconi syndrome in children. Even though the currently used cysteamine-based therapy efficiently decreases cellular cystine levels in affected organs, it only delays progression to renal failure. We thus hypothesized that cystinosin could have additional role(s) independent of cystine transport, which could explain why some deleterious effects of cystinosis are not prevented despite compliant cysteamine treatment. Here, mass spectrometry enabled us to identify cystinosin as a component of a complex, recently characterized lysosomal nutrient-sensing machinery, as we found cystinosin to bind to almost all components of the v-ATPase, to four subunits of the pentameric Ragulator, and to both RagA and RagC small GTPases. In line with our hypothesis of an important role of cystinosin in the mTORC1 pathway, the yeast ortholog of cystinosin, ErS1, was found to complement for the absence of the ortholog of p18, MeH1/Ego1.18,19 Interestingly, yeast lacking ERS1 or MEH1 are sensitive to hygromycin B that, in both cases, can be completely complemented by expression of either ErS1 or MeH1, strongly suggesting that both proteins are functioning in the same cellular mechanism/pathway.

We found that deletions leading to disruptions of the fifth inter-TM loop of cystinosin weakened the identified interactions and this effect was even more drastic for the N288K mutation, but not for the K280R or N323K mutations. Previous studies from our laboratory revealed that although all three mutations disrupted cystinosin transport activity in vitro, only N288K led to the most severe infantile form of cystinosis, whereas the two others resulted in a juvenile form without Fanconi syndrome.11 Likewise, we found that only N288K abolished interactions with components of the mTORC1 pathway. These results clearly suggest that the development of the Fanconi syndrome characteristic for the most severe infantile form of cystinosis does not simply result from lack of cystine transport activity, but also from dysregulation of the mTORC1 pathway in the absence of interactions between cystinosin and the v-ATPase-Ragulator-Rags complex. Moreover, these results perfectly explain discrepancies between cystine transport activity of some mutants (i.e., K280R and N323K) and the clinical phenotype.11 Surprisingly, when alanine was substituted for a glutamine residue in the 288 position (N288A), interactions were maintained at a level comparable with WT cystinosin. These results suggest that the substitution of a positively charged amino acid for an uncharged, bulky one at this particular position may introduce structural changes in cystinosin, hampering interaction with its binding partners. Although our results demonstrate a crucial role of the fifth loop of cystinosin in the interaction with the mTORC1 complex, this interaction remains to be precisely mapped. So far we have been unsuccessful using the yeast two-hybrid system to evidence any

**Figure 3.** The mTORC1 pathway is downregulated in Ctns<sup>−/−</sup> MPT cells. Ctns<sup>+/−</sup> or Ctns<sup>−/−</sup> MPT cells were either AA-/FBS-starved for 30 minutes, or starved and then allowed to recover in AA- or AA/FBS-containing medium for 30 minutes. (A) Cells were coinmunolabeled with mTOR and Lamp1 antibodies (confocal microscopy; scale bars = 10 µm); similar results were obtained for two Ctns<sup>−/−</sup> and two Ctns<sup>+/−</sup> lines in three independent experiments. Zoomed areas and merge images are presented on the right of the panels to better appreciate the lysosomal or diffuse localization of mTOR in the various experimental conditions. (B) Total cell lysates were analyzed by Western blotting with anti-S6K1 and anti-S6K1P antibodies to evaluate the phosphorylation levels of the S6K1 protein. Ctns<sup>−/−</sup> 1 and 2 are two cell lines derived from different Ctns<sup>−/−</sup> mice. Representative blots from at least three independent experiments are shown. (C) Quantification of phosphorylation levels of S6K1 protein (each bar represents the mean±SEM from four independent experiments; *P<0.05; **P<0.01).
Figure 4. Cystinosin acts upstream of RagA in the mTORC1 signaling pathway. Ctns+/+ or Ctns−/− MPT cells stably expressing EGFP-RagA or its constitutively active form (EGFP-RagA Q66L) were either AA/FBS-starved for 30 minutes, or starved and then allowed to recover in medium containing AA or AA/FBS for 30 minutes. (A) Cells were coimmunolabeled for mTOR and Lamp1 (confocal microscopy; scale bars = 10 μm); similar results were obtained for two Ctns−/− and two Ctns+/+ lines in two independent experiments.
direct interaction of the loop motif, with either p18 and Rag proteins or the cytosolic part of a1 subunit of v-ATPase (data not shown). Two explanations remain possible: (1) these proteins do not interact directly with the loop motif; and/or (2) in the yeast two-hybrid system, the loop motif fails to acquire a native conformation essential for the identified interactions. Further studies using other v-ATPase subunits and/or different systems would be necessary to characterize direct partner(s) and the interacting domain of cystinosin.

Functionally, we further demonstrated that relocation of mTOR to lysosomes after starvation/refeeding is dramatically altered in Ctns<sup>−/−</sup> cells as compared with control cells. Moreover, defective lysosomal recruitment of mTOR upon amino acid re-introduction impacted on the downstream signaling pathway, as shown by the delay of phosphorylation of S6K1, the main mTORC1 target. We also showed that it is the very absence of cystinosin – not accumulation of cystine – that accounted for the deregulation of mTORC1 signaling, because efficient cystine depletion by cysteamine treatment failed to restore the response of Ctns<sup>−/−</sup> cells to refeeding. This supports our hypothesis on a role of cystinosin in the mTORC1 pathway beyond cystine transport.

Recent studies brought further light to a complex relationship between mTOR-v-ATPase-dependent signaling and apical endocytosis. Lysosomal mTOR-v-ATPase signaling was shown to control the expression of megalin and thereby regulating apical protein uptake in Drosophila epithelial cells and proximal tubular cells in mice. Moreover, detailed analysis of the progression toward a Fanconi syndrome in Ctns<sup>−/−</sup> mice revealed a gradual loss of megalin and cubulin in kidney proximal tubules, resulting in extending the defect of endocytosis and tubular proteinuria. Together with these observations, our data indicate that defective mTORC1 signaling upon absence of functional cystinosin could result in impaired megalin expression in proximal tubular cells, leading to proteinuria. Moreover, the lack of effect of cysteamine on mTORC1 signaling in Ctns<sup>−/−</sup> cells offers a plausible explanation as to why some symptoms of cystinosis, like Fanconi syndrome, are not improved by this drug. An alternative, nonmutually exclusive explanation for the loss of megalin and cubulin has been
proposed, based on nuclear retention of the ZO-1 partner, ZONAB, a corepressor of expression of these receptors. It is tempting to imagine the existence of a cellular mechanism regulating expression of endocytic receptors linking mTOR signaling and ZONAB activity, which could be impaired in cystinosis. However, it is very likely that the deregulation of the mTORC1 pathway is not limited to proximal tubular cells. Additional studies should be performed to shed light on the impact of mTORC1 signaling in other renal and extrarenal cell types from cystinotic patients and/or mice.

It is further conceivable that other lysosomal amino acid transporters have a role on amino acid–dependent signaling similar to that of cystinosis. For example, two members of the SCL38 family, proton-assisted amino acid transporter 1 (PAT1) and SLC38A9 have recently been found to directly interact with components of the Rag-Ragulator complex and to be involved in the regulation of mTORC1 activity. Importantly, SLC38A9 was shown to interact with Rag-Ragulator in an amino acid–dependent manner, and to act as a positive regulator of mTORC1 activity, most likely independently of v-ATPase. The interaction between SLC38A9 and v-ATPase seems much less important than that of cystinosin and v-ATPase, suggesting that the mechanism of regulation of the mTORC1 pathway differs between these two amino acid transporters. Moreover, the recent discovery of a novel PQLC family member lysine/cationic amino acid transporter PQLC2/LAAT-1 at the lysosomal membrane raises the possibility that other proteins bearing the loop motif could also be implicated in the mTORC1 signaling pathway.

In conclusion, our data clearly show that cystinosin, via its interaction with v-ATPase, Ragulator, and Rag GTPases, plays an important role in nutrient sensing, at least in kidney proximal tubular cells. Thus, cystinosin cellular function is more complex than merely cystine transport out of the lysosome. This sobering conclusion highlights the need for developing new treatments besides lysosomal cystine depletion.

**CONCISE METHODS**

**Materials**

For all antibodies and reagents used in the study see Supplemental Material.

**Cell Culture**

MDCK and 3T3 cells (obtained from the American Type Culture Collection) were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM l-glutamine. MPT cell lines were generated in our laboratory from renal cortex of ctsA58 transgenic Immortomouse and Ctns+/+ mice bearing at least one copy of the SV40 T antigen gene. MPT cell lines were grown in DMEM/F12 (1:1) supplemented with 2% certified FBS, 1 x ITS (0.5 mg/L insulin, 5.10⁵ U/L transferrin, 5 mg/L sodium selenite), 4 pg/L tri-iodothyronine, 10⁻⁸ M dexamethasone, 10 μg/ml EGF, 20 U/ml IFN-γ, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM l-glutamine (MPT cell–specific medium) on culture dishes coated with rat tail type-I collagen. Cells were grown either in growth-promoting conditions (at 33°C with 20 U/ml IFN-γ) that enable the expression of the IFN-γ inducible SV40 T antigen gene or in differentiating conditions (37°C–39°C without IFN-γ). For characterization of the MPT cell lines, refer to Supplemental Material.

**Generation of Stable Cell Lines**

For the expression of the tagged cystinosin protein, cystinosin-EGFP was subcloned into the lentiviral pRRL.SIN.cPPT.PGK/WPRE vector. Cystinosin-EGFP mutants DYPFPQA, DYMNF, AGYDQL, 2m (DYPFPQA + AGYDQL), K280R, N288K, N323K, and N288A were obtained by the modification of the pRRL.SIN.cPPT.PGK/WPRE-CTNS-EGFP construct with specific mutagenic primers using the Stratagene Quick Change Site-Directed Mutagenesis Kit, according to the manufacturer’s recommendations. Lentiviruses were produced in HEK293T cells by cotransfecting pRRL.SIN.cPPT.PGK/WPRE-CTNS-EGFP–derived constructs with pMDL, pREV, and pVSVG helper vectors as described. MDCK and 3T3 cells were transduced by lentiviral particles containing CTNS-EGFP or its mutant forms at an moi of 3.5 or 7, respectively, in the presence of 8 μg/ml polybrene. Full-length cDNA encoding WT human CD63 was kindly provided by Michael J. Caplan, and subsequently subcloned into the EGFP-C3 vector (Clontech). Full-length cDNA encoding WT human Lamp1 was subcloned into the EGFP-N vector (Clontech). 3T3 and/or MDCK cells were then transduced with EGFP-CD63 or Lamp1-EGFP constructs using 6 μl of Lipofectamine 2000 reagent (Life Technologies), according to the manufacturer’s protocol, in antibiotic-free medium. Cells stably expressing EGFP-CD63 or Lamp1-EGFP were selected by 2 mg/ml genetin (Life Technologies).

**co-IP for Mass Spectrometry Analysis and Western Blotting**

MDCK or 3T3 cells stably expressing the various fusion proteins (see above) were rinsed twice with PBS and lysed in ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, and one tablet of Complete Protease Cocktail (Roche Diagnostics) per 50 ml). Lysates were cleared by 10 minutes of centrifugation at 1000g, 4°C. Protein concentration was determined by bicinchoninic acid assay (BCA Protein Assay Kit; Thermo Fisher Scientific, Rockford, IL), according to the manufacturer’s instructions.

co-IP was performed using μMACS GFP or Protein A Microbeads Isolation Kit (Miltenyi Biotec) for the isolation of GFP-tagged proteins or RagC, respectively. Briefly, 50 μl of MicroBeads conjugated to anti-GFP monoclonal antibody were incubated with lysate containing 3 mg total proteins at 4°C for 1 hour, then applied onto μMACS separation columns. Protein isolation was further performed according to the manufacturer’s recommendations. For RagC immunoprecipitation, RagC antibodies were coupled to Protein A microbeads.
with disuccinimidyl suberate as crosslinking reagent, according to the manufacturer’s protocol. Lysates containing 3 mg total protein were incubated overnight at 4°C with beads coupled to RagC antibodies (1:150). Protein isolation was performed as above. Precipitated proteins were resolved by SDS-PAGE on 10% gel for mass spectrometry and immunoblotting.

**Mass Spectrometry by LTQ Orbitrap**
Coimmunoprecipitated protein samples prepared as above were concentrated on the top of a 10% SDS-PAGE gel, excised and subjected to trypic digestion with sequencing grade modified trypsin (Promega), as described previously. Nano-LCMS/MS data-dependent analysis of in-gel digested samples was performed on an Ultimate 3000 Rapid Separation Liquid Chromatography system coupled to LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). The in-house Mascot search engine (version 2.2.07; Matrix Science) and Proteome Discoverer software (version 1.2) were used for protein identification. Only proteins identified with at least one unique peptide and with the ion score higher than 20 were retained. Protein networks were generated using Ingenuity software (www.ingenuity.com). For each protein, the expression value corresponds to the average number of peptides obtained by mass spectrometry analysis across biologic replicates. For peptide extraction and analysis by mass spectrometry, see Supplemental Material.

**Amino Acid Starvation/Stimulation**
MPT cells were plated in 12-well culture dishes coated with rat tail type-I collagen in MPT cell–specific medium (see above) and treated or not for 2 hours with 1 mM cysteamine hydrochloride. According to Wilmer et al., cysteine effectively decreases cystine levels for up to 8 hours after addition. Cells were then rinsed twice, starved with AA/FBS-free RPMI for 30 minutes, then stimulated either: (1) by addition of 1 × AA (MEM amino acids 50× liquid) or AA/FBS (2% certified FBS) for 30 minutes, or (2) by RPMI medium with extra 1× AA (MEM amino acids 50× liquid) or AA/FBS (2% certified FBS) for an indicated time (30–60 minutes), and used for lysate preparation or immunofluorescence analysis. For cystine level assay, MPT cells were plated in parallel in rat tail type-I collagen-coated 10-cm culture dishes and treated or not for 2 hours with 1 mM cysteamine hydrochloride, rinsed twice with normal complete medium, incubated for 1 hour with MPT cell–specific medium, then processed for cystine assay as described in the Supplemental Material.

The level of S6K1 phosphorylation was determined by quantification of the S6K1 to S6K1 ratios obtained from quantification of the Western blot signals of total cell lysates. Statistical analysis of log-transformed data using a paired t test was performed with Prism Version 5 (GraphPad software). The threshold for statistical significance was set to P<0.05.

**Immunofluorescence**
MPT and 3T3 cells were plated at 300,000 cells/dish in 12-well tissue culture dishes containing collagen-coated glass coverslips and noncoated glass coverslips, respectively. After 24–38 hours, slides were rinsed once with PBS++, (supplemented by 0.49 mM MgCl₂ and 0.9 mM CaCl₂) and fixed with 4% formaldehyde in PBS++ at room temperature for 15 minutes, then quenched with PBS++ supplemented with 50 mM NH₄Cl for 10 minutes. After rinsing twice with PBS++, slides were incubated with primary antibodies diluted 1:200 for MPT cells and 1:100 for NIH/3T3 cells, in PBS++ containing 0.075% saponin and 0.1% BSA, overnight at 4°C. After three rinses with PBS++, samples were incubated with secondary antibodies diluted 1:200 in PBS++ with 0.075% saponin and 0.1% BSA for 1 hour at room temperature, and washed thrice with PBS++. Glass coverslips were mounted on microscope slides using FLUOPREP (bioMérieux) and imaged on a ZEISS LSM 700 scanning laser confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

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**DISCLOSURES**

None.

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