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Project title: Small molecule regulators of vesicular trafficking to enhance lysosomal exocytosis in cystinosis

The aim of the project "Small molecule regulators of vesicular trafficking to enhance lysosomal exocytosis in cystinosis" is to use strategies directed at upregulating the exocytic pathway in cystinotic cells, in order to improve vesicular transport, force cystine release and restore normal cell function. Our hypothesis, in fact, states that cells that have accumulated cystine share the intracellular traffic defects that characterize other lysosomal storage disorders and that correction of intracellular vesicular trafficking would lead to improvement of the pathological conditions.
In my previous fellowship report I showed that CTNS-deficient cells have enlarged and increased number of lysosomes, which correlated with impaired lysosomal trafficking in these cells. Importantly, overexpression of the constitutively active form of the small GTPase Rab27a (Rab27aQ78L) was able to rescue proper lysosomal trafficking in CTNS-deficient cells.

Preliminary results from our lab obtained prior to the beginning of the fellowship award showed that cystinotic cells have high levels of ER stress and a marked upregulation of the Unfolded Protein Response (UPR). Several lines of evidence suggest that defects in vesicular transport mechanisms lead to ER stress, probably induced by accumulation of misfolded proteins in the ER caused by inefficient protein trafficking. In my previous report, I showed that Rab27aQ78L expression was able to decrease the UPR in cystinotic cells. Since Rab27a was also able to induce a small but significant decrease of cystine in these cells, we propose that amelioration of vesicular transport and decrease of cystine induced by the activation of Rab27a-dependent pathways can contribute to decrease ER-stress and ameliorate the phenotype observed in cystinotic cells. Here, I report new significant findings that help elucidate further the mechanisms linking ER-stress and lysosomal trafficking/exocytosis in cystinosis:

CTNS-deficient mouse kidneys and proximal tubular cells derived from cystinotic patients show increased ER-stress
To establish the clinical relevance of putative defects in the trafficking mechanism and ER stress development in cystinosis, we assessed whether the increased UPR and ER-stress found in CTNS-deficient mouse fibroblasts was recapitulated *in vivo* in cystinosis-affected organs. To this end, kidney extracts from WT or CTNS-deficient mice were subjected to WB using antibodies recognizing the UPR target genes Grp78 and Grp94. As shown in Figure 1A-C, the levels of both chaperones Grp78 and Grp94 were highly increased in CTNS-deficient mouse kidneys, confirming our previously reported findings using mouse fibrobalsts. Next, using an independent approach, I analyzed the expression of calnexin, a chaperone previously associated with stress-induced apoptosis. Immunofluorescence analysis demonstrated significantly higher levels of calnexin expression in proximal tubular cells (PTCs) from CTNS-deficient mouse kidney sections

(Figure 1D-E), although less pronounced than those observed for the UPR chaperones Grp78/94.
Importantly, we also found significant upregulation of UPR-induced chaperones in human PTCs from cystinotic patients (Figure 1F), indicating that this phenotype is also observed in cystinotic patients and therefore it is relevant to the human pathology.
Altogether, our data indicate that the ER stress phenotype observed in cystinotic fibroblasts correlates with a similar phenotype in cystinosis target organs and cystinotic human PTCs, which adds further clinical relevance to our previous findings.

Exocytosis of the readily releasable, Synaptotagmin VII-dependent, pool of lysosomes is not impaired in CTNS-deficient cells

In my previous CRF project report we showed that activation of Rab27a-dependent pathways is beneficial to improve lysosomal transport and mediate cystine release in CTNS-deficient cells, which correlated with an amelioration of the phenotypes found in these cells, including ER-stress. To add further details to our understanding of the mechanism by which Rab27a can contribute to lysosomal exocytosis and ER-stress decrease, we followed different approaches. To understand whether Rab27a was involved in the release of readily releasable lysosomes, I stimulated lysosomal exocytosis in WT and CTNS-/- cells, or in CTNS-deficient cells infected with mock or Rab27aQ78L-containing lentiviruses, with the ionophore A23187, which only promotes exocytosis of lysosomes located in proximity to the plasma membrane. Figure 2A shows that Rab27aQ78L expression did not further increase lysosomal exocytosis induced by A23187, indicating that Rab27a is involved in constitutive lysosomal exocytosis rather than exocytosis of readily releasable lysosomes. In addition, the data in Figure 2A also indicate that CTNS-deficient cells do not show any defects in ionophore-induced lysosomal exocytosis. Furthermore, I also found that Synaptotagmin VII (SytVII), a major regulator of exocytosis of readily releasable lysosomes, was expressed in cystinotic kidneys at similar or higher levels than those observed in kidneys from WT mice (Figure 2B). In addition, the subcellular localization of Syt7 in cystinotic fibroblasts had a similar distribution pattern to that observed in WT cells (Figure 2C). Thus, Syt7 was observed in punctate internally distributed structures as well as in close proximity to or at the plasma membrane, most likely representing readily releasable lysosomes (Figure 2C). Altogether, our data rule out possible defects in Syt7 expression/localization and in exocytosis of proximal lysosomes in cystinosis and suggest that Rab27a does not play a major role in calcium-induced secretion of readily releasable proximal lysosomes. These data also suggest that the beneficial effect of Rab27aQ78L expression on ameliorating ER-stress is mainly due to improvement of lysosomal transport and/or the activation of a constitutive secretory pathway that ultimately leads to cystine release.

Amelioration of vesicular trafficking by expression of constitutively active Rab7 can partially rescue UPR in CTNS-deficient cells

To assess whether amelioration of the phenotypes described above was Rab27a-specific or it could be extended to other molecules known to be involved in vesicular transport, we focused on the small molecule Rab7, a Rab known to mediate lysosomal bidirectional microtubule-associated movement through interaction with its effectors RILP and

FYCO1. To this end, we analyzed the effect of constitutively active Rab7 expression on lysosomal kinetics and ER stress in *Ctns*$^{-/-}$ cells. As shown in Figure 3A, the expression of constitutively active Rab7 increases lysosomal trafficking in *Ctns*$^{-/-}$ cells. Furthermore, active Rab7 expression significantly reduced the UPR (Fig. 3B) albeit to a less extent than the reduction observed in cells overexpressing Rab27a. These data support the idea that upregulation of the lysosomal transport system has the potential to reduce cell defects induced by lysosomal overload. The data also suggest that Rab27a, a GTPase with dual role in trafficking and exocytosis may have additional positive effects over Rab7, which regulates lysosomal trafficking but not exocytosis.

Rab27a localizes at lysosomes and is downregulated in cystinotic cells and tissues

Next, because of its important regulatory mechanism in cystinotic cells, we examined the expression levels of Rab27a in cystinotic cells and tissues. To this end, we compared Rab27a expression in WT and CTNS-/- kidney lysates by WB. As shown in Figure 4A-B, the levels of Rab27a, but not that of other Rabs involved in the endocytic and exocytic pathways, were downregulated in CTNS-/- kidneys. Importantly, Rab27a expression levels were also found to be downregulated in PTCs from cystinotic patients, suggesting that Rab27a downregulation could be at least in part responsible for impaired vesicular trafficking in these cells. This hypothesis is further supported by the observation that Rab27a colocalizes with the lysosomal marker LAMP1 in PTCs from both WT and CTNS-/- mouse kidneys (Figure 4C), which also makes Rab27a a good target for pharmacological intervention aimed to improve vesicular transport and cystine release in cystinosis affected tissues.

Future objectives

Future experiments will be directed at elucidating the molecular mechanisms regulating Rab27a-dependent improvement of vesicular transport and cellular function in cystinosis. I will focus my attention on the role played by the Rab27a effectors JFC1 and Munc13-4 in lysosomal exocytosis and on the potential action of small molecules that modulate these interactions.

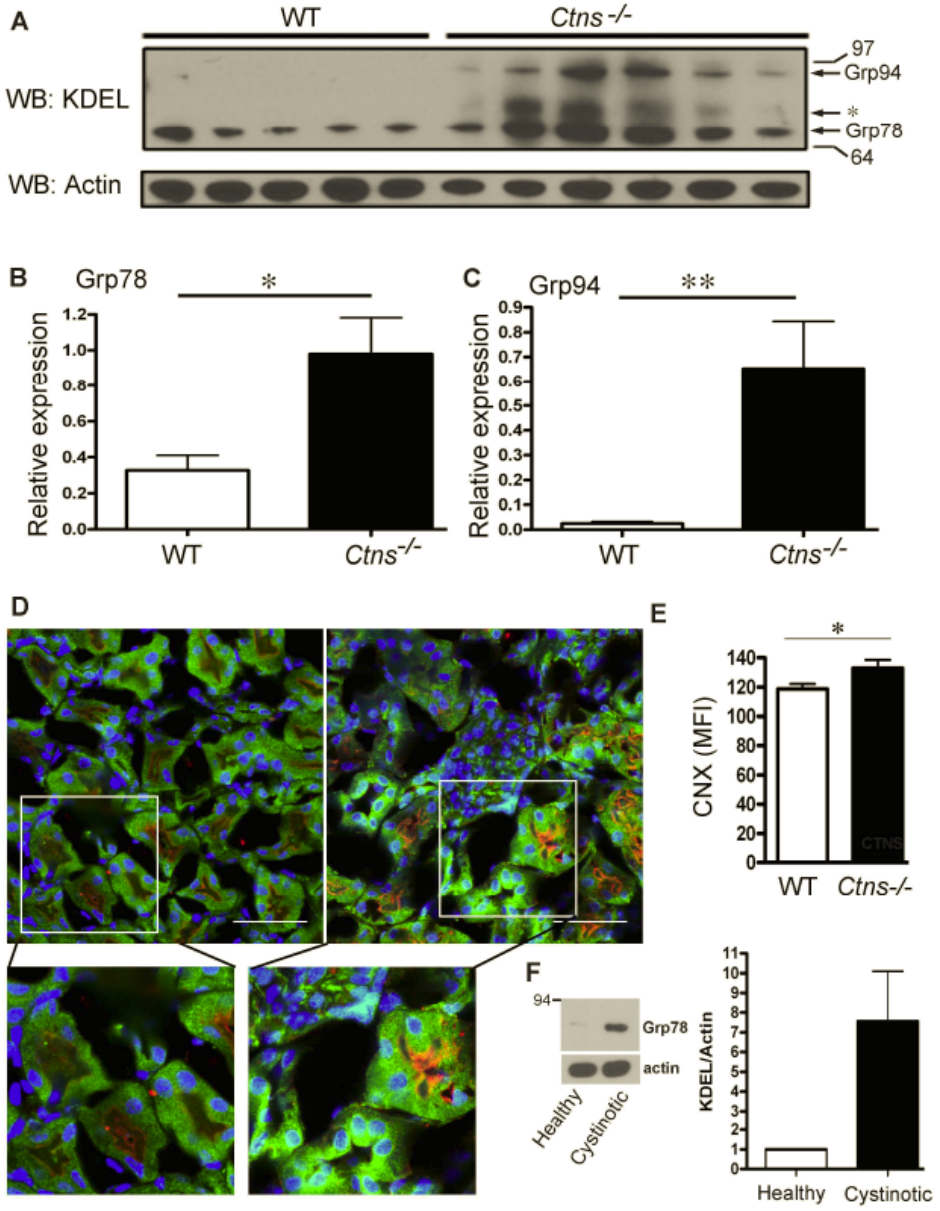

Figure 1. *In vivo upregulation of the UPR in cystinosis*
A, Western blot analyses of the expression of Grp94 and Grp78 in kidney lysates from wild type and *Ctns*$^{-/-}$ mice. Each lane corresponds to an individual sample from independent mice. B and C, Chaperone expression (relative to the actin signal in the same lane) was quantified by densitometry. *, $p<0.05$; **, $p<0.005$, Mann-Whitney test. D, Immunofluorescence analysis of the expression of endogenous calnexin (CNX) in PTCs from wild type and cystinotic mice. Scale bar = 50 μm. E, Quantification of the mean fluorescence intensity (MFI) of endogenous calnexin. Four to six proximal tubules from at least 5 different areas from 3 independent wild type (WT) or *Ctns*$^{-/-}$ mice were analyzed. A total of 69 and 58 proximal tubules from wild type and *Ctns*$^{-/-}$ kidneys, respectively, were included in the analysis. Mean ± SEM, ∗, $p<0.02$. F, Western blot analysis of the expression of Grp78 in human proximal tubule cells (PTCs). $n=2$, mean ± SD.

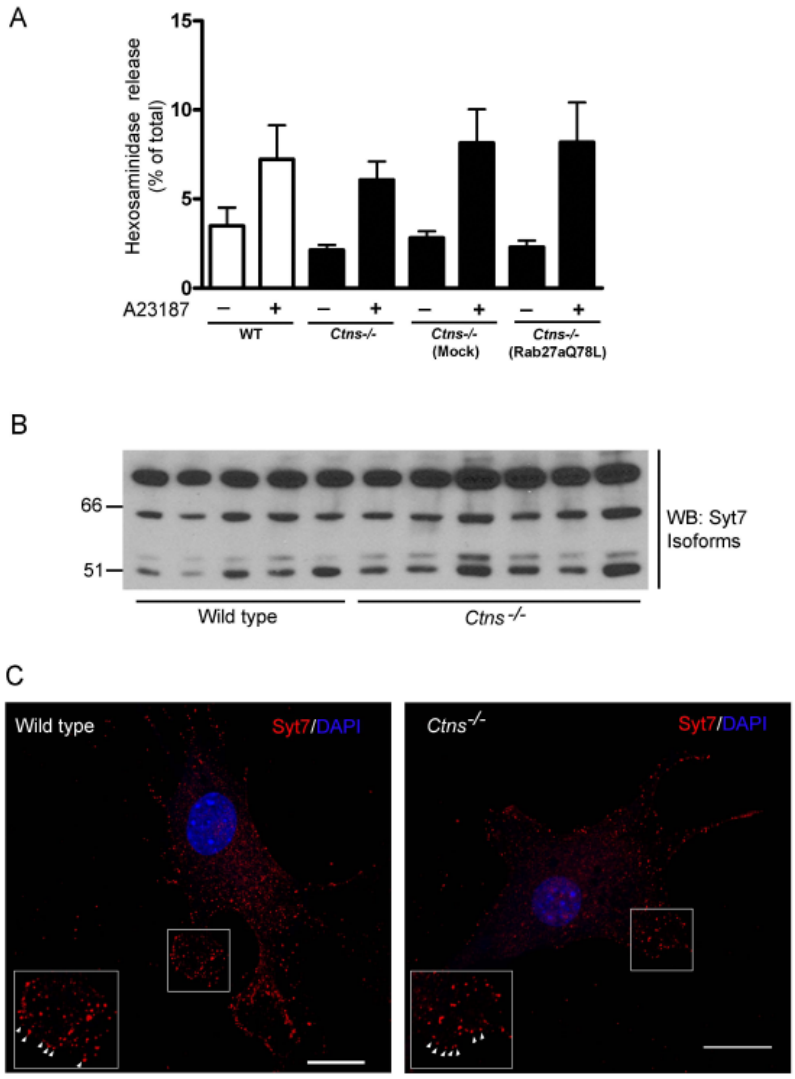

Figure 2. *Exocytosis of the readily releasable, Synaptotagmin VII-dependent, pool of lysosomes is not impaired in CTNS-deficient cells*

A, Lysosomal exocytosis was evaluated in fibroblasts stimulated with the ionophore A23187 in the presence of 1 mM Ca^{2+}. Hexosaminidase in the supernatants and cell lysates was analyzed. Mean ± SEM, $n = 3$. B, Analysis of the expression of synaptotagmin 7 (Syt7) in kidneys from wild type and *Ctns*$^{-/-}$ mice. Each lane corresponds to an individual sample from independent mice. Similar to previous studies several isoforms of Syt7 were identified. None of this isoforms were downregulated in *Ctns*$^{-/-}$ cells. C, The subcellular localization of Syt7 was further analyzed by immunofluorescence. Endogenous Syt7 was detected in punctate structures in proximity to the plasma membrane of fibroblast (insets, arrowheads), as well as internally, in both wild type and cystinotic cells. Scale bar = 20 μm.

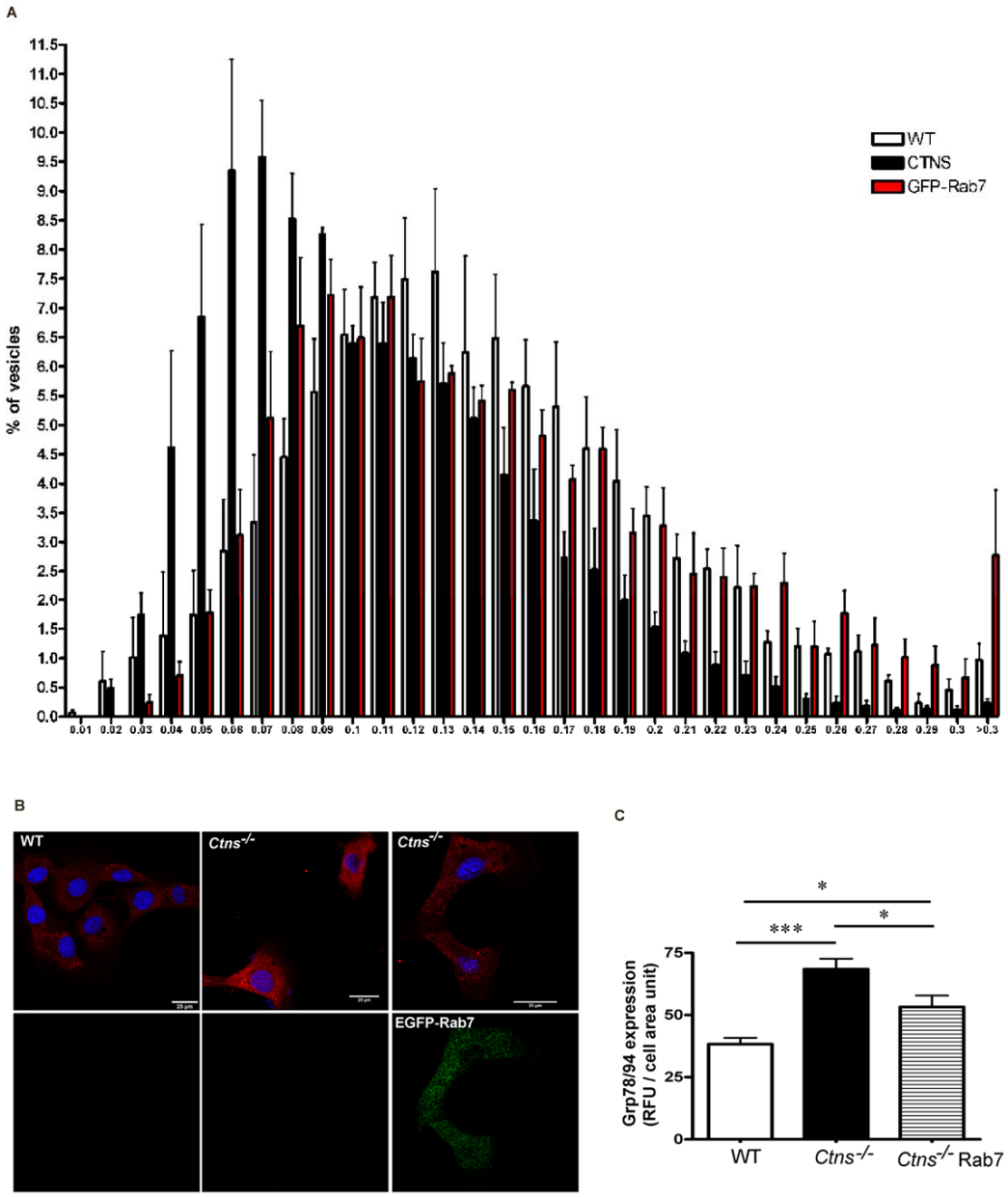

Figure 3. *Amelioration of vesicular trafficking by expression of constitutively active Rab7 can partially rescue UPR in CTNS-deficient cells*

A, Murine fibroblasts were labeled using LysoTracker and lysosomal dynamics were analyzed by TIRFM. The dynamics of the labeled lysosomes were followed for 2 min and the kinetics of lysosomal movement was analyzed using Imaris software. Experiments were repeated twice with similar results. Histograms of lysosomal speeds from wild type cells (black columns), *Ctns*$^{-/-}$ cells (red columns) and *Ctns*$^{-/-}$ cells expressing constitutively active Rab7 (green columns) are shown. The speeds of lysosomal movement were binned in 0.01-μm/s increments and plotted as a percentage of total granules for a given cell. Results are represented as mean ± SEM. ∗ $p<0.05$. B and C, Immunofluorescence analysis of the expression of Grp78/94 was performed as described in Nuclei were stained with DAPI. The level of expression of the UPR target genes Grp78 and Grp94, which are upregulated in *Ctns*$^{-/-}$ cells (arrowheads) was significantly decreased in cells expressing EGFP-Rab7 (arrows). Scale bar=40 μm.

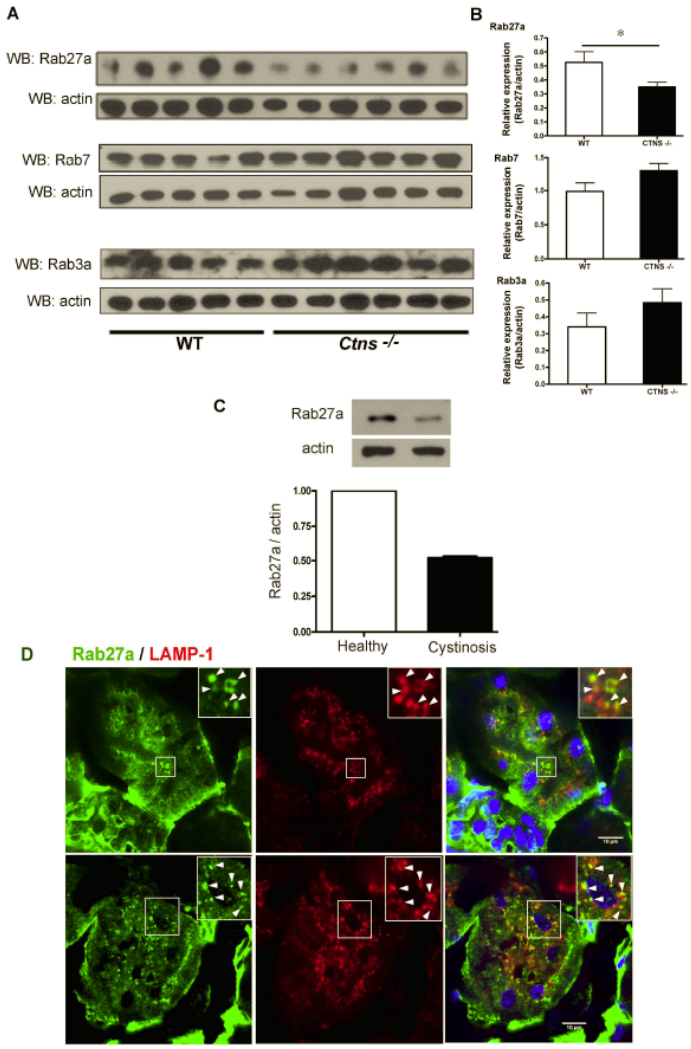

Figure 4. *Rab27a localizes at lysosomes and is downregulated in cystinotic cells and tissues*

A, Expression of endogenous Rab GTPases in kidney lysates from wild type or *Ctns*$^{-/-}$ mice. Each lane corresponds to kidney lysates from individual wild type (WT) or cystinotic mice (*Ctns*$^{-/-}$) B, Quantitative densitometry analysis of the immunoblots presented in A. Relative expression refers to the ratio between specific Rabs and actin signal in the same lane. Results are mean ± SEM (error bars). *, $p<0.05$ (Mann-Whitney test). C, Western blot analysis of the expression of Rab27a in human renal proximal tubular cells (PTCs) from a patient with cystinosis and a healthy control. $n=2$; mean ± SD. D, Immunofluorescence analysis of mouse kidneys showing colocalization (insets, arrowheads) of Rab27a with the lysosomal marker LAMP1 in PTCs. Scale bar = 10 μM.