Progress Report:

Molecular mechanisms to repair the vesicular transport system in cystinosis

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Aim 1 | *To analyze vesicular transport and exocytosis of conventional lysosomes in cystinosis*

Aim 2 | *To study exocytosis as a mechanism of membrane repair in cystinotic cells*

Aim 3 | *Improvement of vesicular transport and lysosomal fusion in cystinotic cells by upregulation of vesicular trafficking and exocytic pathways*

Cystinotic cells have decreased Rab27a expression

One of the hypotheses proposed to explain the cellular defects observed in lysosomal storage disorders is that vesicular trafficking is affected by lysosomal overload (17). Here, to investigate whether cystinosis is underlined by abnormalities in vesicular trafficking regulators, we analyzed the expression of Rab GTPases that are involved in the endocytic or exocytic pathway of lysosomes. Given the pathophysiological importance of kidneys in cystinosis, we first evaluated Rab GTPases expression using lysates from kidneys of wild type or *Ctns*$^{-/-}$ mice. Western blot analysis demonstrated a significant decrease in the level of expression of Rab27a but not other Rab GTPases regulators of the exocytic (Rab3a) or endocytic (Rab7) pathways (Figs. 1A and B). Because defects in renal proximal tubular cells (PTCs) are central to the pathophysiology of late cystinosis, we next determined the expression of Rab27a in PTCs from cystinotic patients, which also showed a significant reduction in Rab27a expression when compared to PTCs from healthy controls (Fig. 1C). To analyze the distribution of Rab27a in kidneys in further detail, we performed immunofluorescence analysis of endogenous Rab27a in murine kidneys and identified Rab27a expression in polarized, megalin-positive PTCs (Fig. 1D). Rab27a was distributed at both basal and apical punctate structures resembling vesicular organelles. A more detailed analysis detected Rab27a in colocalization with the lysosomal marker LAMP-1 in kidney PTCs from wild type and *Ctns*$^{-/-}$ mice (Fig. 1E).

Upregulation of lysosomal transport decreases the UPR in cystinosis

To analyze whether correction of intracellular vesicular transport is an effective mechanism to improve cellular function in *Ctns*$^{-/-}$ cells, we next tested the hypothesis that upregulation of the Rab27a-dependent trafficking pathway protects cystinotic cells and decreases ER stress. To this end, we analyzed the UPR in wild type and cystinotic cells expressing constitutively active Rab27a. Using Western blot analysis, we showed that constitutively active Rab27a expression significantly reduces the expression of both Grp78 and Grp94 UPR chaperones (Figs. 5A-C). Using immunofluorescence analysis and confocal microscopy, we also found that *Ctns*$^{-/-}$ cells in which Rab27a-dependent lysosomal trafficking was upregulated have a marked reduction in the expression of UPR-induced chaperones (Fig. 5D and E). Altogether, our data suggest that the upregulation of lysosomal trafficking mediated by Rab27a reduces endoplasmic reticulum-stress and decreases the need for the UPR in cystinotic cells, and supports the idea that the correction of vesicular transport mechanisms in LSDs has the potential to improve cellular function.

Lysosomal trafficking mediated by Rab7 contributes to the improvement of the cellular phenotype in cystinosis

We have already shown that upregulation of the Rab27a trafficking mechanism rescues cellular defects in cystinosis. Next, we determined whether Rab7, a Rab known to mediate lysosomal bidirectional microtubule-associated movement through interaction with its effectors RILP and FYCO1 (9,47), could contribute to the rescue of the defective phenotype in cystinosis. To this end, we analyzed the effect of constitutively active Rab7 expression on lysosomal kinetics and ER stress in *Ctns*$^{-/-}$ cells. Here, we show that the expression of constitutively active Rab7 increases lysosomal trafficking in *Ctns*$^{-/-}$ cells (Figs. 6A and B, and Supplementary movie 4). This is in agreement with a previous study showing trafficking repair mechanisms induced by Rab7 in a different storage disease (12). Furthermore, active Rab7 expression significantly reduced the UPR (Fig. 6C) 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 secretion may have additional positive effects over Rab7 which regulates lysosomal trafficking but not exocytosis.

Calcium-induced exocytosis of readily releasable lysosomes is not affected in cystinotic cells

Exocytosis has been proposed to decrease lysosomal overload in lysosomal storage disorders (40). To determine whether the lysosomal exocytic pathway is impaired in cystinotic cells, we first evaluated calcium-induced exocytosis of readily releasable lysosomes in *Ctns*$^{-/-}$ fibroblasts. Here we show that exocytosis of these lysosomal pool was not impaired in cystinotic fibroblasts stimulated with calcium ionophores (Fig. 7A), which induces exocytosis of plasma membrane proximal lysosomal (25). Moreover, although Rab27a was proposed to regulate both trafficking and exocytosis of secretory organelles (64) including lysosomes (35), cells overexpressing constitutively active Rab27a showed no significant upregulation of readily releasable lysosomes exocytosis in response to calcium ionophores (Fig. 7A). In addition, the expression of synaptotagmin 7 (Syt7), a exocytosis regulatory protein of the readily releasable lysosomal pool (36), was not downregulated in cystinosis. Thus, all Syt7 isoforms (16,60) were expressed in cystinotic kidney lysates at similar or higher levels than those observed in wild type cells (Fig. 7B). Next, we showed that the subcellular localization of Syt7 in cystinotic fibroblasts has a similar distribution pattern to that observed in wild type cells (Fig. 7C). 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 (Fig. 7C). Altogether, our data rule out putative defects in Syt7 expression or localization, exclude exocytosis of membrane proximal lysosomes as an impaired mechanism in cystinosis and suggest that Rab27a does not play a major role in calcium-induced secretion of readily releasable proximal lysosomes in cystinotic cells.

Figures and References: Please see attached Manuscript

A Manuscript including these results has been submitted to the journal *Molecular and Cellular Biology*. The complete manuscript is included in a separate attachment. The manuscript is under final revision after minor modifications. Final acceptance letter will follow soon.