Cystinosis Research Foundation, 18 month progress report

THE ROLE OF ALTERED CALCIUM AND mTOR SIGNALING IN THE PATHOGENESIS OF CYSTINOSIS

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Background

Several lines of evidence suggest that cystinosin, next to its cystine-transporting function, has a role in cellular signaling pathways. In this project we focus on two closely interfering pathways, namely on Ca^{2+} signaling and on mTOR (mammalian target of rapamycin) signaling.

The cytosolic [Ca^{2+}] is involved in the regulation of mTOR via Ca^{2+}/calmodulin-dependent protein kinase and AMP-activated protein kinase. Thus, abnormal Ca^{2+} signaling can be an upstream event in the pathogenesis of cystinosis, analogous to the other lysosomal storage disorders, and might alter mTOR activity.

On the other hand, in yeast, the cystinosin analog ERS1 interacts with the components of the EGO (exit from growth arrest) complex, located on the vacuole and controlling TOR signaling, a major signaling pathway that integrates metabolic processes with nutrient availability and growth-factor signaling. Inhibiting TOR signaling induces autophagy. Indeed, signs of increased autophagy have been reported in cystinosis. Our preliminary results indicate that mTOR is altered in cystinosis cells and that further inhibiting mTOR by rapamycin augments cystine accumulation.

In addition we have detected an abnormal distribution of endo-lysosomes in cells lacking or overexpressing cystinosin, which would suggest a role of cystinosin in controlling lysosome distribution and movement. These processes are under control of the GTPase Rab7, which in turn can regulate the mTOR pathway through its role in regulating the morphology and function of late endosomes/lysosomes.

Based on this background information and our preliminary data, we aim to investigate the role of Ca^{2+} and mTOR signaling in the pathogenesis of cystinosis.

Specific aims

- We will specifically study the mechanism of altered Ca^{2+} signaling in cystinosis cells. To this end we will study expression of Ca^{2+}-regulating proteins and Ca^{2+} fluxes in the endoplasmic reticulum (ER) and endo-lysosomal compartment.
- We will focus on the mechanism of altered mTOR signaling/autophagy in human cystinosis cells.
- Based on the obtained results we will test appropriate chemical compounds interfering with Ca^{2+} and mTOR signaling for improving cystinosis phenotype.

Results

1. Study of endocytosis in control and cystinosis ciPTEC with DQ-BSA

Previously we demonstrated altered receptor-mediated endocytosis in cystinosin deficient cells (ciPTEC derived from patients and CTNS knocked-down HK-2 cells) using recombinant GST-RAP ligand of megalin to monitor the uptake and processing of cargo by multiligand receptors. Cystinosis cells had decreased amount of GST-RAP bound to the cell surface after 30 min of incubation on ice to prevent internalization indicative for reduced surface expression of the receptors. When internalization was allowed by moving cells at 37°C, GST-RAP remained longer visible in cystinosis cells (at time points of 30 – 45 minutes), suggesting a delayed processing of internalized ligands.

We next asked whether lysosomal degradation of endocytic cargo was compromised in cystinosin-deficient cells. To monitor endosomal proteolysis, we incubated cells with DQ-BSA green substrate (Molecular Probes) according to Manufacturer's protocol. In its native form, DQ-BSA is quenched because of excessive labeling with fluorescent probe. However upon degradation by lysosomal proteases, fragments are released that results in bright green fluorescence that can be detected in living cells as soon as 30 minutes after presenting the substrate.

We detected no difference in intensity of DQ-BSA fluorescence between control and cystinosis ciPTEC at all tested time points (Figure 1). This result indicates that lysosomal degradation of substrate is unaffected in cystinosis cells. Treatment of cells with 100 nm of bafilomycin A1 (started 30 min before addition of DQ-BSA) inhibiting the acidification of the endolysosomes resulted in complete abolishment of fluorescence that confirms the specificity of the assay.

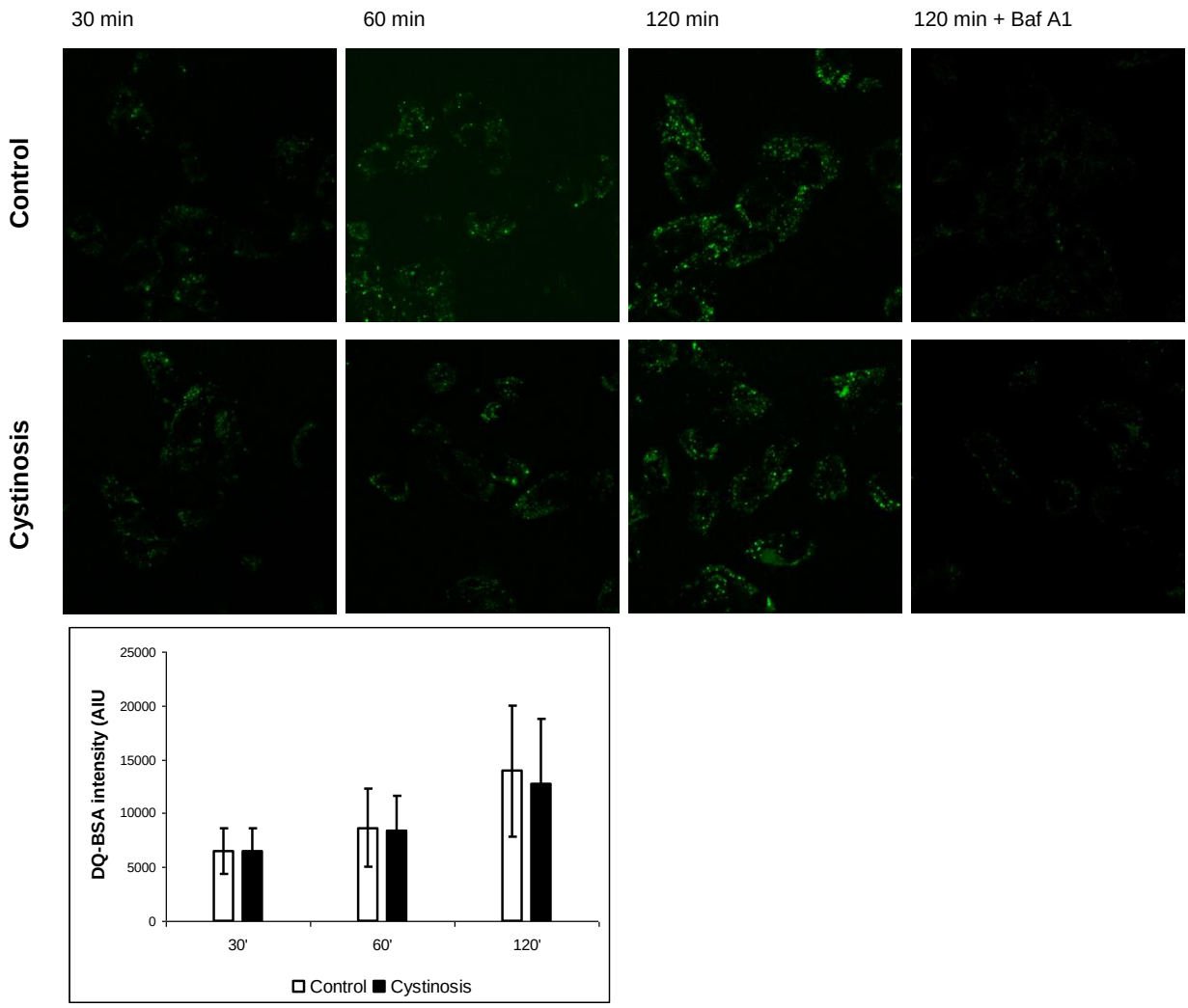

Figure 1. Study of lysosomal proteolysis in control and cystinosis ciPTEC with DQ-BSA. Above: examples of DQ-BSA staining of cellular sites of proteolysis (lysosomes) at different time points. Treatment with 100 nm of Bafilomycin A1 was used as negative control. Below: quantification of DQ-BSA fluorescence intensity (~ 180 cells in 2 independent experiments).

Study of internalization of fluorescently-labelled BSA (555-BSA), that was performed using the same protocol as for DQ-BSA, showed that accumulation of the fluorescent protein was more prominent in cystinosis cells in comparison with the control (Figure 2). This result is in agreement with previously observed delayed processing of RAP ligand.

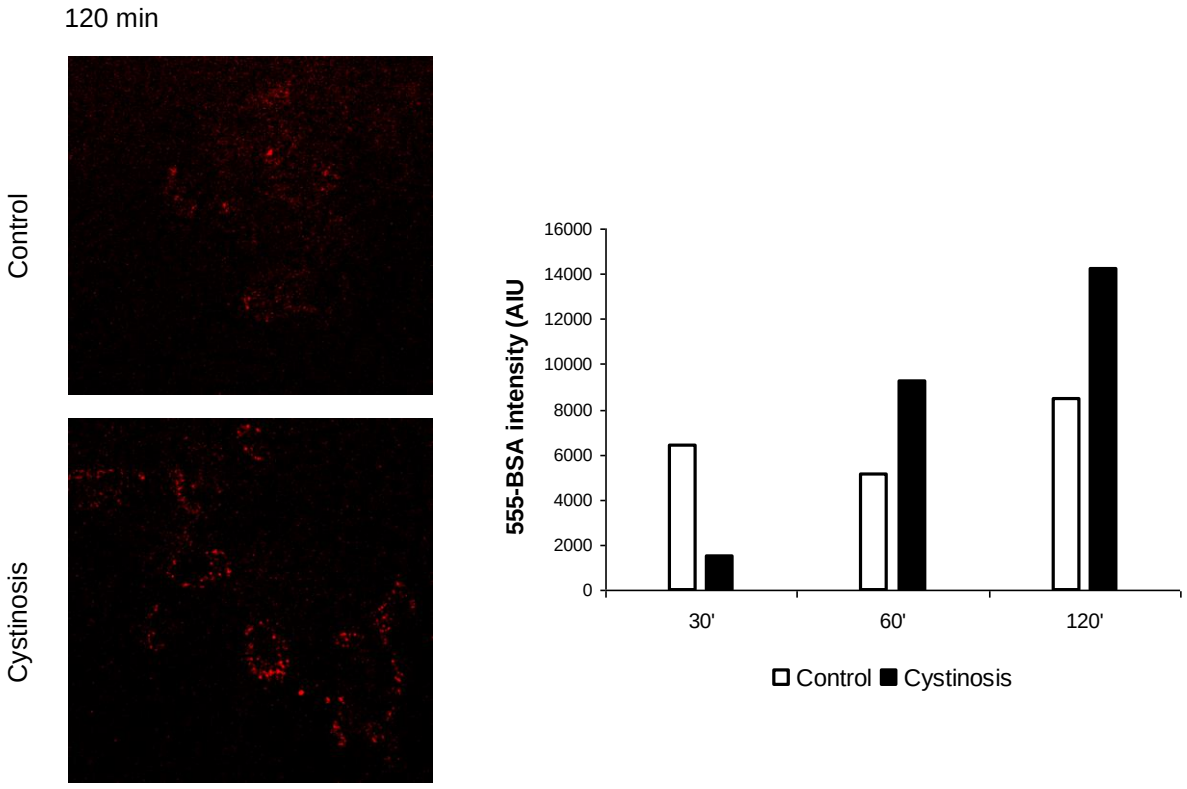

Figure 2. Endocytosis of fluorescent BSA by control and cystinosis ciPTEC. Left: example of 555-BSA staining after 120 min of incubation. Left: quantification of 555-BSA fluorescence intensity (~ 180 cells).

Together these observations suggest that lysosomal degradation is not affected in cystinosis. Delayed processing of endocytosis cargo might be explained by defective delivery of the internalized cargo to the degradation sites due to abnormal intracellular trafficking of the endosomes. Altered trafficking of intracellular vesicles might also explain the observed deficiency of surface expression of receptors that in normal situation constantly recycle between plasma membrane and late endosomes.

2. Study of autophagosomal-lysosomal fusion

We next asked whether lysosomal degradation of endogenous substrates is affected in cystinosis cells. Autophagosomal protein LC3 can be used as a marker substrate, as it is degraded upon fusion of autophagosomes with lysosomes. Control and cystinosis ciPTEC were transiently transfected with GFP-LC3, which was clearly visible on structures, corresponding to autophagosomes within 24 h after transfection. No visible difference in localization and level of expression was registered between control and cystinosis cells; in both cases, some GFP-LC3-positive structures co-localized with Lamp1-positive vesicles, indicative for autophagosomal-lysosomal fusion (Figure 2A). Incubation of cells with 100 nm bafilomycin A1 for 2 h resulted in significant increase of number of GFP-LC3-positive autophagosomes both in control and cystinosis cells. Treatment with lysosomal inhibitor results in accumulation of autophagosomes that otherwise would be degraded after fusion with lysosomes. This observation was confirmed by measurement of LC3-II level in control

and cystinosis cells, treated with vehicle alone or with 100 nm of bafilomycin A1 for 2 h. Incubation with the lysosomal inhibitor caused accumulation of LC3-II both in control and cystinosis cells (Figure 2B). Together these results suggest that fusion of autophagosomes with lysosomes is not affected in cystinosis.

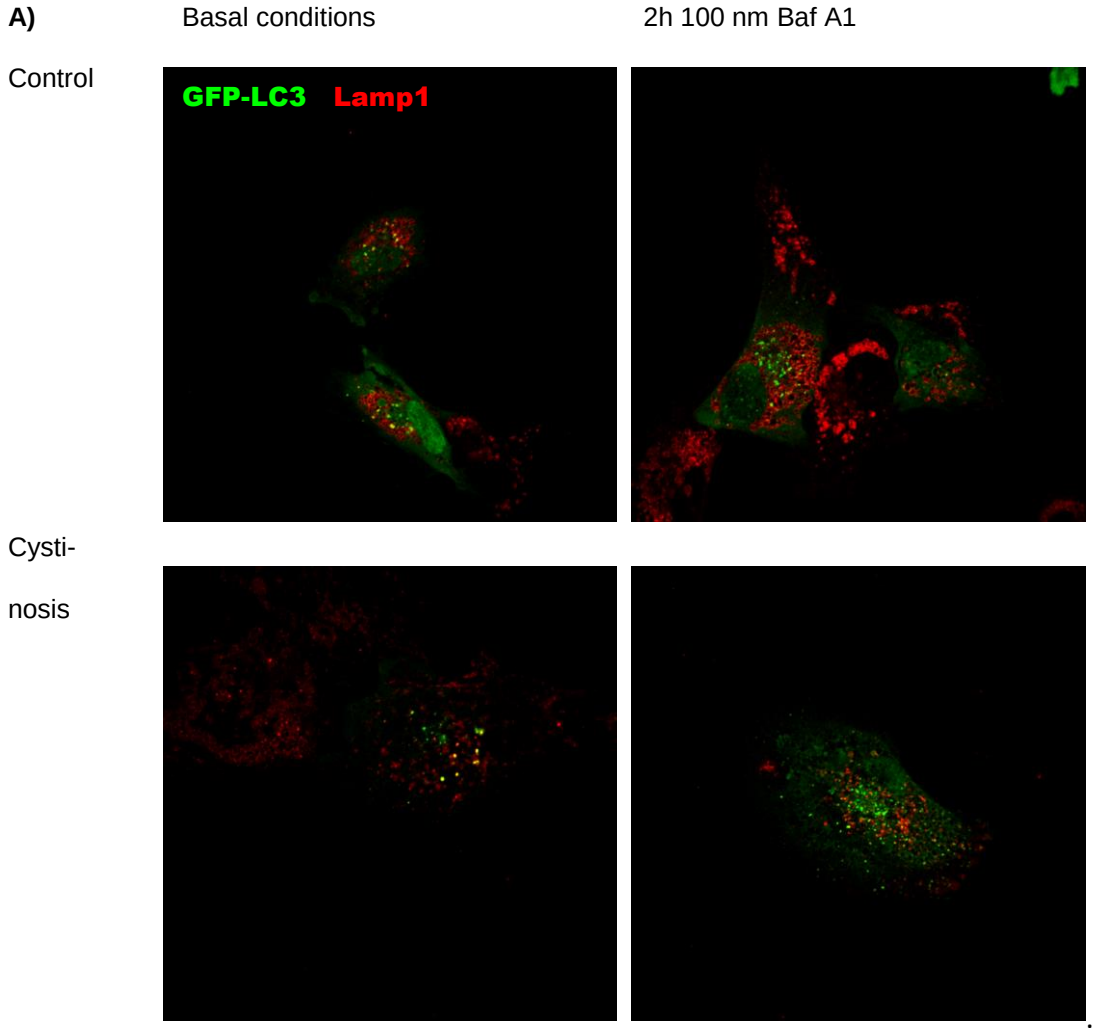

Figure 2. A) Study of autophagosomes in control and cystinosis ciPTEC by transient transfection with GFP-LC3. B) Western blot analysis of LC3-II concentration in ciPTEC cells incubated with vehicle or 100 nm bafilomycin A1 for 2 h. Accumulation of LC3-II is expressed as % increase of initial value.

3. Study of endosomal calcium content

As luminal concentration of Ca^{2+} can influence lysosomal function and fusion, we asked whether intralysosomal $Ca2^{+}$ is changed in cystinosis. To study lysosomal Ca^{2+} content, control and cystinosis ciPTEC were loaded with Fura2-AM Ca^{2+}–sensitive dye, pre-incubated with 2 µM of Thapsigargin to exclude the input of Ca^{2+} current from the ER stores and then treated with 200 µM of GPN. Cleavage of GPN in the lysosomes leads to osmotic disruption of these organelles and to release of Ca^{2+} into the cytoplasm, where it can be detected by Fura2-AM signal. We did not observe any difference in lysosomal Ca^{2+} content between control and cystinosis cells using the described method (Figure 3).

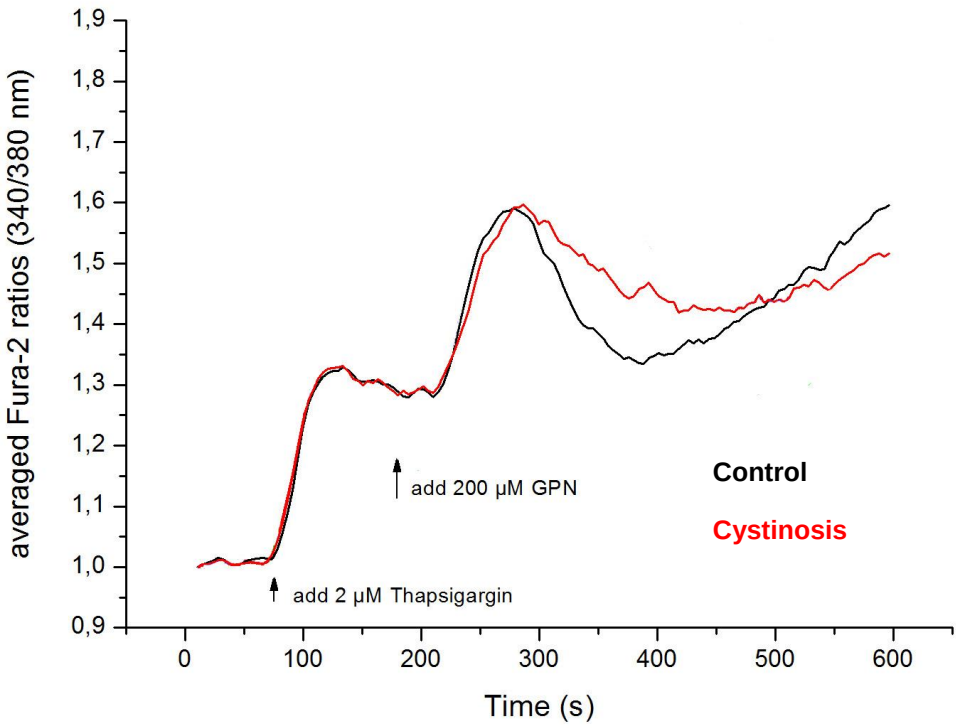

Figure 3. Study of lysosomal Ca^{2+} content in control and cystinosis ciPTEC (averaged response, n = 4). Increase of Fura-2 340/380 ratio after addition of GPN corresponds to the release of lysosomal Ca^{2+}.

We obtained the same results by using GPN alone and by substituting thapsigargin with ionomycin, an ionophore that disrupts the plasma and ER membranes, equalizing intracellular Ca^{2+} concentration, but leaves intact the lysosomal membranes that are protected with glycocalix (data not shown). Our results indicate that lysososmal Ca^{2+} content remains unchanged in cystinosis cells in comparison to control. However, it has to be taken into account that lysosomes appear enlarged and more numerous in cystinosis ciPTEC. Therefore, a more accurate measurement of Ca^{2+}concentration in individual lysosomes using

a Ca^{2+}-sensitive fluorescent probe and microscopy approach is needed to draw the final conclusion.

4. Study of motor proteins

Our previous results indicate that endosomal trafficking is affected in cystinosis. To further study the mechanisms of altered trafficking of intracellular vesicles, we performed immunostaining of the motor protein kinesin that is responsible for trafficking of vesicles towards the periphery of the cell. Our data indicate that in cystinosin deficient HK-2 cells kinesin shows an altered distribution and abnormally co-localizes with enlarged Lamp1-positive vesicles (Figure 4).

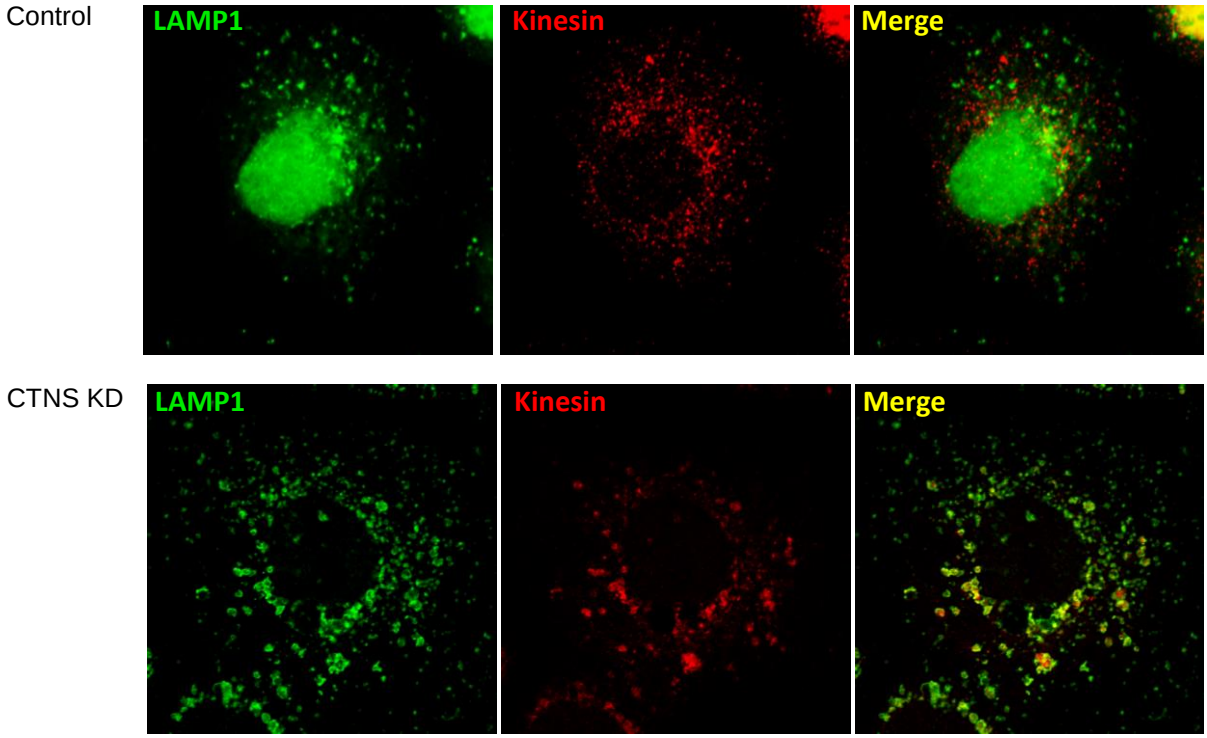

Figure 4. HK-2 cells were treated with siRNA specific for CTNS gene (see previous report). Control and cystinosin-deficient cells were stained with antibodies to the late endosomal/lysosomal marker Lamp1 and kinesin. In cystinosin-deficient cells kinesin was differently distributed and present on the enlarged Lamp1-positive vesicles.

This finding indicates that cystinosin might play an important role in endosomal/lysosomal trafficking and functioning of motor proteins. In the further experiments we will investigate the input of lysosomal storage of cystine by incubating the control and cystinosin-deficient cells with cysteamine and performing immunostaining of kinesin.

Output related to the project:

The work was presented at:

- Poster presentation, Gordon's Conference on Lysosomal Storage Disorders, April 2013, Lucca, Italy
- Accepted for poster presentation at ASN Conference, November 2013, Atlanta, USA.