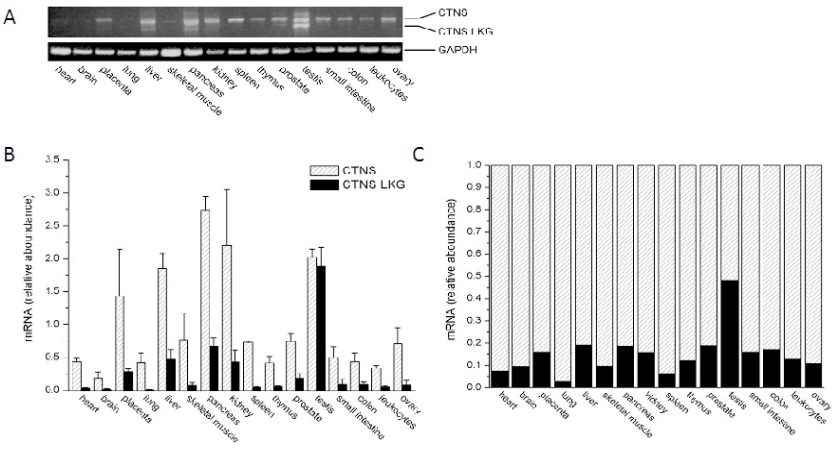
Final Report: Functional Characterization of Cystinosin-LKG

Principal investigator: Francesco Emma, MD

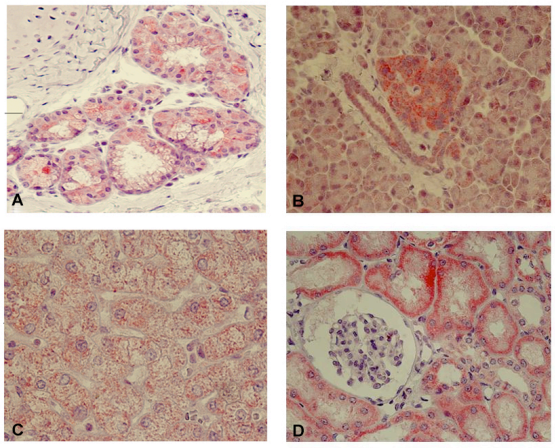
Co-investigator: Anna Taranta, Ph.D.

Cystinosin-LKG is a functional isoform produced by an alternative splicing of exon 12 in *CTNS* gene. In this study we investigated the abundance of this protein in different tissues, the subcellular distribution of the protein and its potential regulation by phosphorylation of serines in carboxi-terminal tail.

Tissue-specific expression of the two known cystinosin isoforms was carried out using a commercial cDNA library (Clontech Laboratories Inc.). PCR fragments were sequenced to confirm DNA sequence and relative expression was quantified by gel densitometry (A).

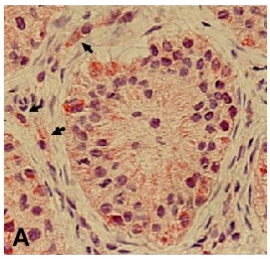


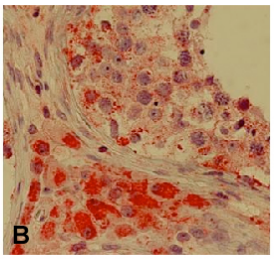
Cystinosin-LKG expression was weak in heart, brain, lung, skeletal muscle, spleen, thymus, leukocytes and ovary; stronger expression was observed in liver, pancreas, kidney and testis (B). In most tissues, cystinosin-LKG accounts for 10-20% of transcripts, while in the testis cystinosin-LKG represents about 50% of transcripts as shown by relative expression of the two isoforms in panel C.



In order to study the distribution of cystinosin-LKG, in different tissues, formaldehyde-fixed paraffin-embedded human tissue sections were deparaffinized and subjected to antigen retrieval. Tissue sections were incubated with an affinity-purified rabbit antiserum directed against a cystinosin-LKG specific sequence spanning amino acid (ARTGSGSRLRQDWAPSLQPKALPQ).

The APAAP (alkaline phosphatase - anti-alkaline phosphatase) method was used to reveal antigen expression; antibodies were detected with Fast Red chromogene substrate; tissue sections were counter-stained with hematoxylin. As shown in the panel, cystinosin-LKG expression was higher in the bronchial muco-serous glands (A), in the Langerhans islet cells (B), a diffuse granular staining was observed in hepatocytes (C) and a particular accumulation in the baso-lateral region of kidney tubular cells (D).





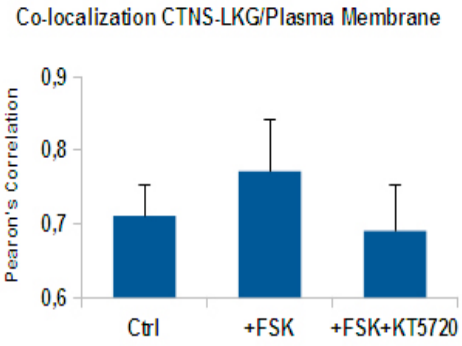
Analysis of male gonad tissues showed a weak expression of cystinosin-LKG in prepubertal testis (A). After puberty, strong immunostaining was observed in Leydigh cells and in the spermatogonium (B).

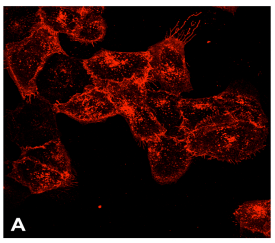
These data show a different distribution of the Cystinosin-LKG in different tissues, clearly linked to the functional state as reported in the last panel where the protein expression correlate with gonad features.

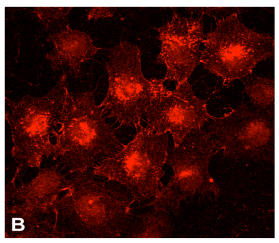
Based on our previous data (Taranta et al, 2008) showing different sub-cellular distributions of cystinosin-LKG, we hypothesized that the expression of this isoform can be functionally regulated by phosphorylation of its carboxy-terminal domain.

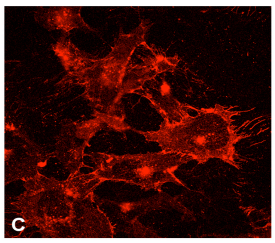
Candidate serine, threonine and tyrosine phosphorylation sites were identified with the NetPhos 2.0 software (http://www.cbs.dtu.dk/services/NetPhos/). High phosphorylation probability was detected in the cystinosin-LKG sequence. Numerous experiments of pulldown with anti-cystinosin and anti-RFP associated to tandem mass analysis were performed but we could not obtained, until now, a sufficient quantity of purified material to directly analyse the phosphorylation state in C-terminal tail.

Human kidney cells (HK2) stably transfected with cystinosin-LKG conjugated with RFP, after 24 hours of serum starvation, were treated with 100 μM Forskolin (FSK) that induces an increase of intracellular levels of cAMP by activating adenylyl cyclase. This stimulus induced a moderate accumulation of cystinosin-LKG at the plasma membrane level. Treatment with 0.5 μM KT5720, a PKA inhibitor, blocked the effect of FSK, showing that translocation of cystinosin-LKG was mediated by a phosphorylation PKA mediated.



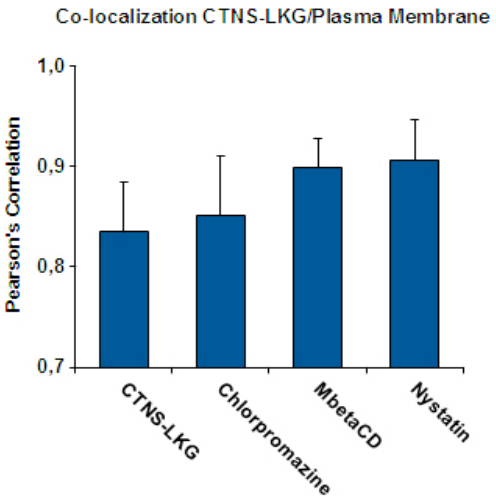


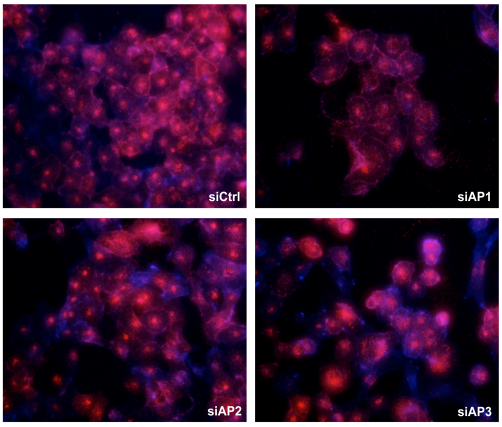




In order to identify specific sites responsible for protein translocation, we carried out a site-directed mutagenesis at Serine^{396} and/or Serine^{397} used to mimic constitutive phosphorylation (Ser > Asp) or dephosphorylation (Ser > Ala). Cells were acquired by confocal microscopy, images processing and analysis was performed with ImageJ software (http://rsbweb.nih.gov/ij/). Higher presence of cystinosin-LKG-RFP was observed on plasma membrane of stably transfected cells S397D mutant (C) respect to the wild-type (A) and S397A mutant (B), showing that phosphorylation of Serine^{397} could have a key role in the sorting of the carrier to plasma membrane.

Mammalian cells use two main pathways to internalize molecules from the plasma membrane and recycle them back to the surface, a clathrin and a caveolin dependant pathway. Treatments of HK2 stably transfected with cystinosin-LKG with specific inhibitors of endocytosis, chlorpromazine which inhibited clathrine, and nystatin or methyl-beta-cyclodextrin (MbetaCD) which inhibited caveolin mediated internalization, showed that cystinosin-LKG is internilezd from the plasma membrane primarily using a caveolin dependant pathway.





Furthermore, adaptor proteins (APs) are heterotetramers that mediate sorting of membrane proteins in the endocytotic and secretory pathways. We therefore performed silencing experiments targeting 3 APs to assess the impact on cystinosin-LKG expression in the plasma membrane. AP2 silencing, which is involved in the transport of proteins from plasma membrane to endosomal compartments, and silencing of AP3, which is involved in sorting proteins to lysosomes and lysosome-related organelles from the Golgi, induced an intracellular accumulation of cystinosin-LKG and a reduction at plasma membrane level.

Finally, we have performed Fluorescence Loss In Photobleaching (FLIP) and Fluorescence Recovery After Photobleaching (FRAP) experiments to analyze the inter-dependence of the lysosomal and non lysosomal compartments expressing cystinosin LKG. These experiments have provided evidence that the expression of cystinosin-LKG in these 2 compartments is relatively independent; loss of fluorescence in one of the 2 compartment is only very weekly compensate by translocation of the protein from the other compartment.

Conclusions

Taken together, these data strongly suggest that cystinosin-LKG expression in different sub-cellular compartments is highly regulated, which support the notion that this isoform has a specific role in cells that may be very relevant to cystinosis.

A first paper showing the expression of cystinosin-LKG has been published (Taranta et al, Histochem Cell Biol 2012) and a second manuscript summarizing the above results is in the process of being submitted.

We are deeply grateful to the CRN for its support and hope that these results will contribute to our understanding and treatment of cystinosis. In particular, based on these

results, Dr Cherqui is now studying the efficacy of the LKG isoform in rescuing the cystinotic phenotype in mice after bone morrow transplantation, and we are screening drug libraries to identify drugs that may revert some of the observed abnormalities.

Francesco Emma and Anna Taranta