Advances in molecular understanding of cystinosis: implications for therapy

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Diagnosis and management of inherited kidney diseases: What's new?
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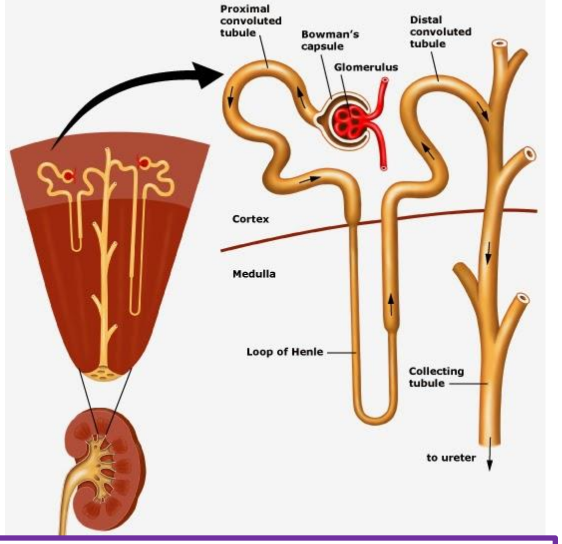
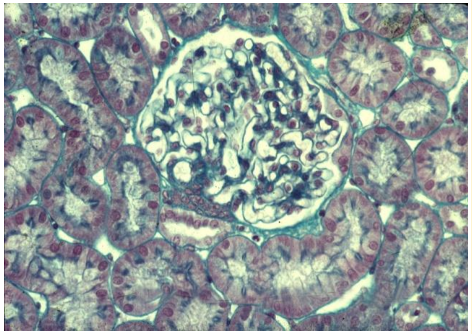
Cystinosis

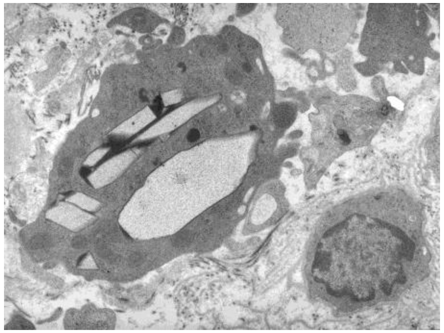
- Rare autosomal recessive lysosomal storage disorder
- Defective lysosomal efflux of cystine
- Three clinical forms:
    - infantile or nephropathic (Fanconi syndrome)
    - juvenile
    - ocular non-nephropathic
- Multisystem disease



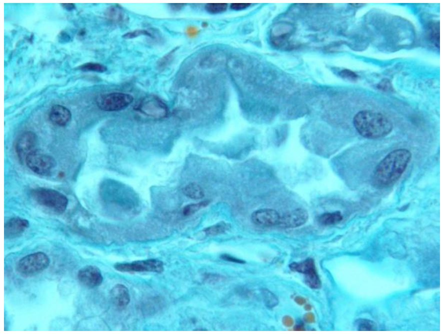
Normal kidney



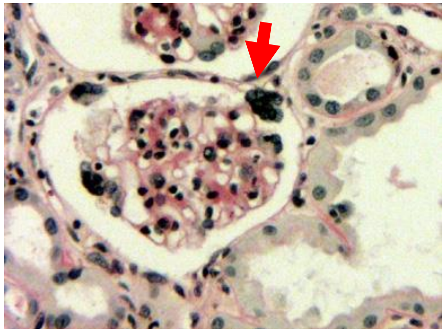
Cystinotic kidney



Cystine crystals in interstitial cells



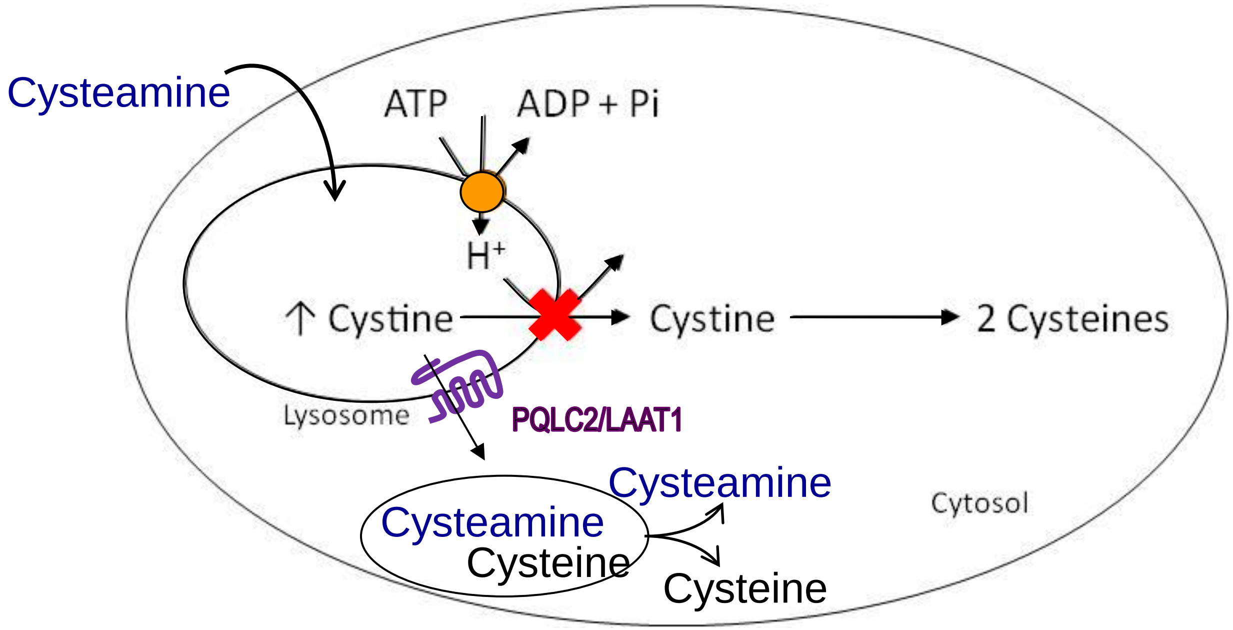
Irregular tubular epithelium



Multinucleated giant podocytes

- No genetic heterogeneity: complementation studies in somatic cell hybrids between fibroblasts from patients with different forms of cystinosis (Pellet,Smith *et al.* 1988)

Treatment - cysteamine

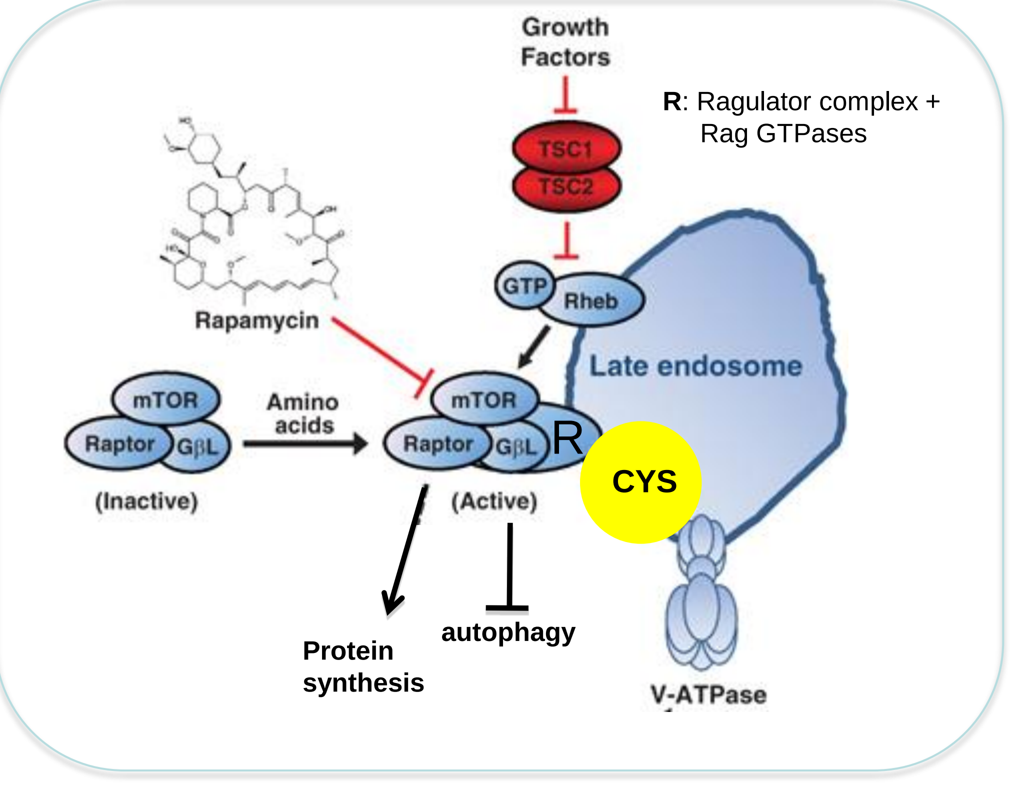


- Oral administration: improves growth & glomerular filtration rate; delays ESRD and the appearance of other clinical anomalies
- Eye drops dissolve corneal cystine crystals
- Side effects & need of regularly spaced doses (each 6h for oral cysteamine and each 1h for eye drops)
- **No effect on Fanconi syndrome**
- New delayed-release form administrated twice a day (Dohil, Gangoiti *et al.* 2010)

Cystinosin, the gene product of *CTNS* mutated in cystinosi

- Lysosomal membrane protein with two targeting motifs (Cherqui et al., *JBC,* 2001)
- Proton-cystine symporter active at low pH, allowing cystine export from lysosomes (Kalatzis *et al.,* EMBO J, 2001)

- Component of the vATPase-Ragulator-Rag complex controlling the mTORC1 complex (Andrzejwska *et al.,* JASN, 20017)

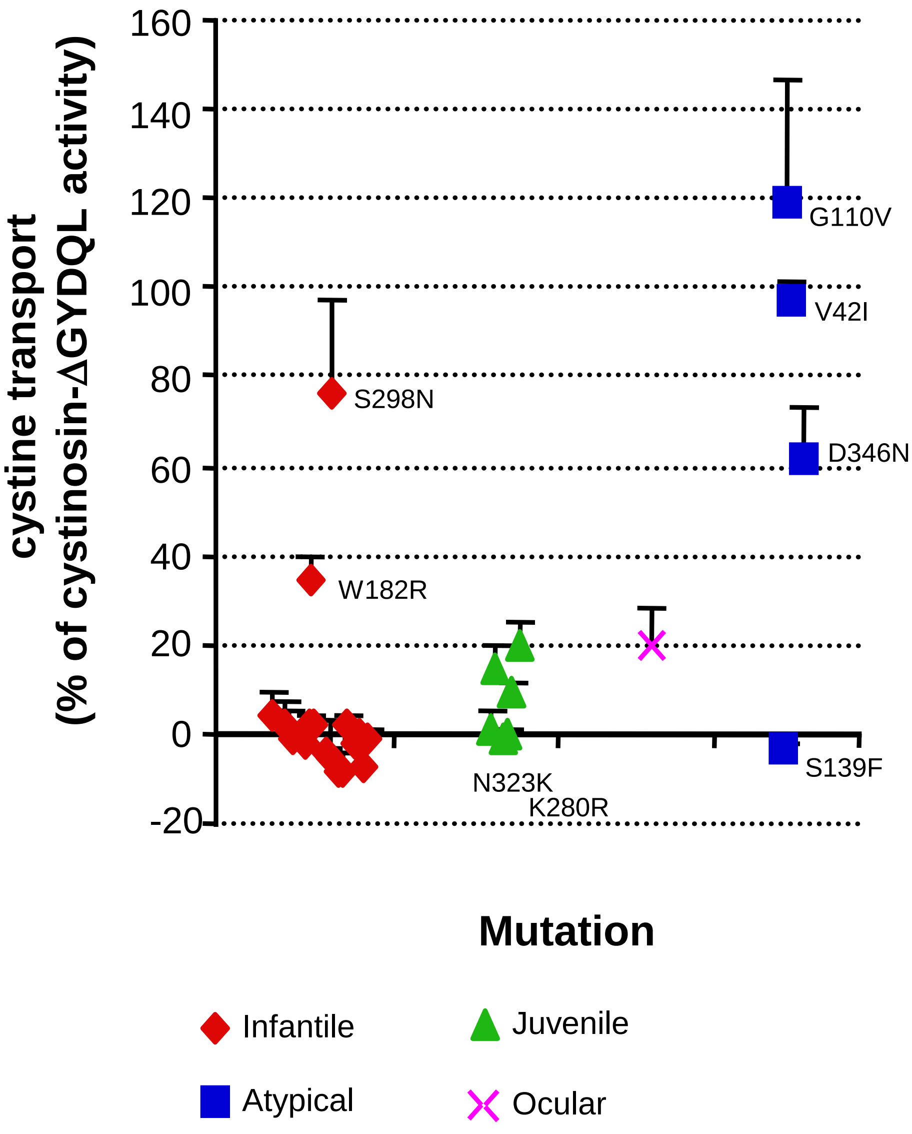


Cystinosis: Mutations in the *CTNS* gene encoding cystinosin

- ~120 different mutations in cystinosis patients all over the world
- 57kb “European” deletion (56 to 76% in Northern Europe)
- Several recurrent mutations in addition to the “European” deletion
- Maternal uniparental heterodisomy of chrom 17

- Clear phenotype-genotype correlations:
  - Two « severe » mutations in the infantile forms
  - Two « mild » mutations or one « severe » and one « mild » mutation in the other forms

Functional studies of missense mutations



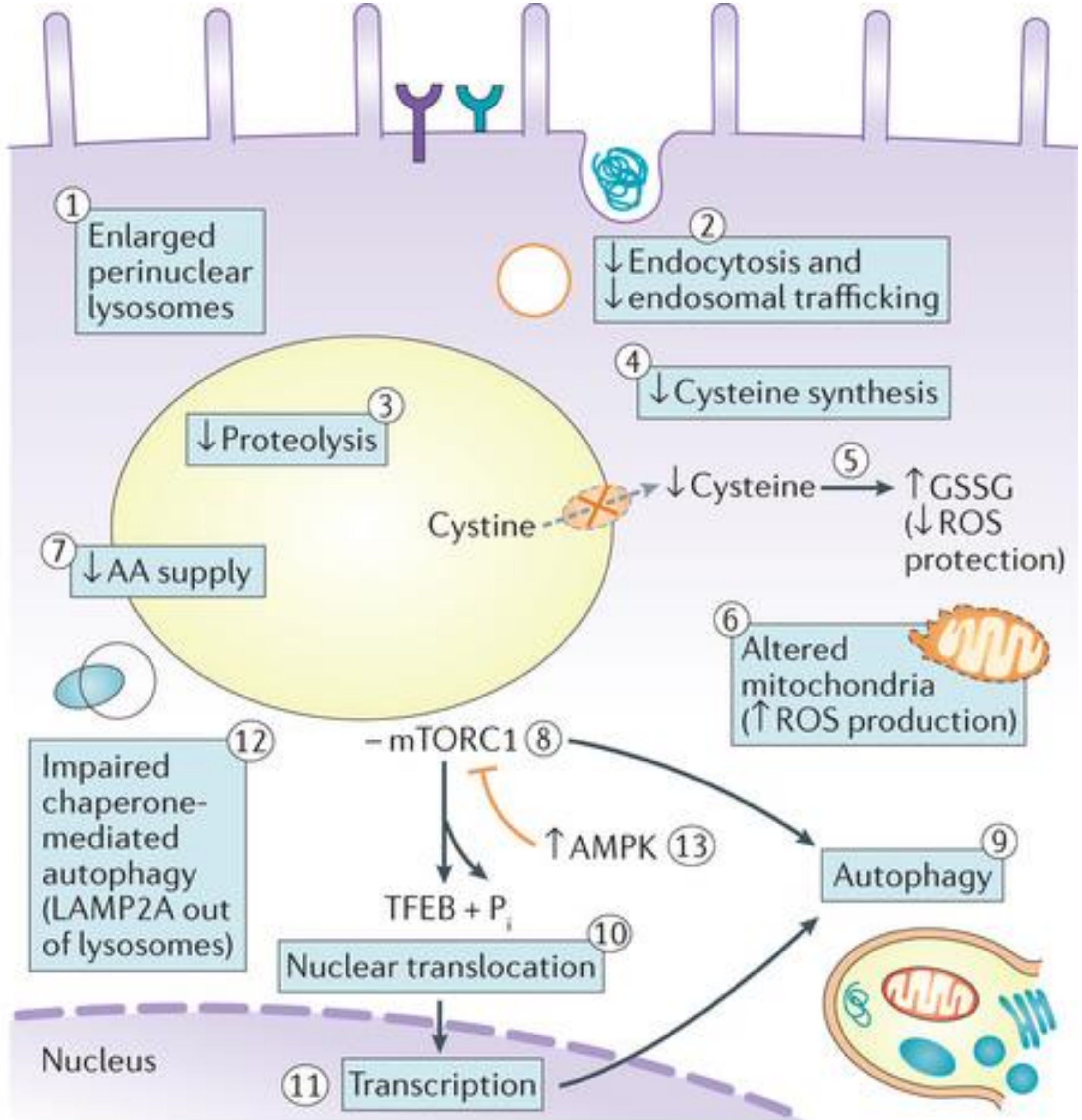
- Good genotype-phenotype correlation but some exceptions:
  - 2 mutants associated with infantile cystinosis are partially or fully active (additional, unidentified mutations in these patients? - less severe phenotype ?)
  - 3 mutants associated with juvenile or atypical cystinosis do not transport cystine (additional role of cystinosin beyond cystine transport ?)

Proposed cellular dysfunctions in cystinosis

- Impact of cystine accumulation on glutathion synthesis and oxidative stress (Chol *et al.*, 2004; Laube *et al.*, 2006; Mannuci Pastores *et al.,* 2006; Bellomo, Corallini *et al.*, 2010)
- Increased apoptosis (Thoene *et a*l., Mol Genet Metab 2007; Sansanwal *et al*., Pediatric Nephrology 2010; Taub and Cutuli, BBRC 2012)
- Increased ER stress (Wei *et al.,* HMG, 2007)
- Implication of autophagy including chaperone-mediated autophagy (CMA) (Sansanwal *et al.,* JASN, 2010; Sansanwal and Sarwal, Pediatric Nephrology, 2012; Napolitano *et al*., EMBO Mol Med, 2015, Zang *et al.,* J Biol Chem, 2017)
- Reduced TFEB (master regulator of the autophagy –lysosomal pathway) expression and induced nuclear translocation (Rega *et al.,* KI, 2016)
- Impaired lysosomal transport (Johnson *et al*., MCB, 2013)
- Involvement in the mTORC1 pathway (Andrzejewska *et al.* JASN, 2015; Ivanova *et al.,* JIMD 2016)

Direct impact of **cystine crystal** accumulation and/or the absence of **cystinosin**?

Proposed cellular dysfunctions in cystinosis



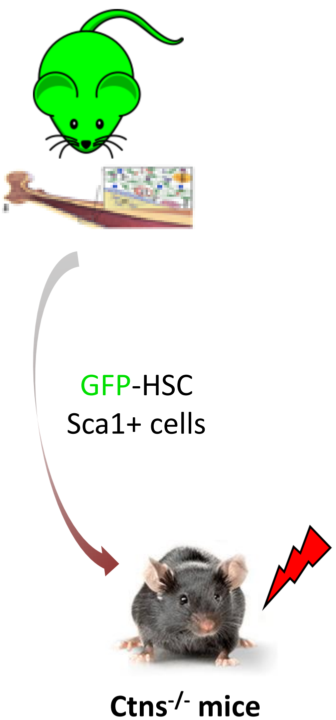
From Cherqui & Courtoy, **Nature Reviews** | Nephrology 2017

New potential therapeutic interventions

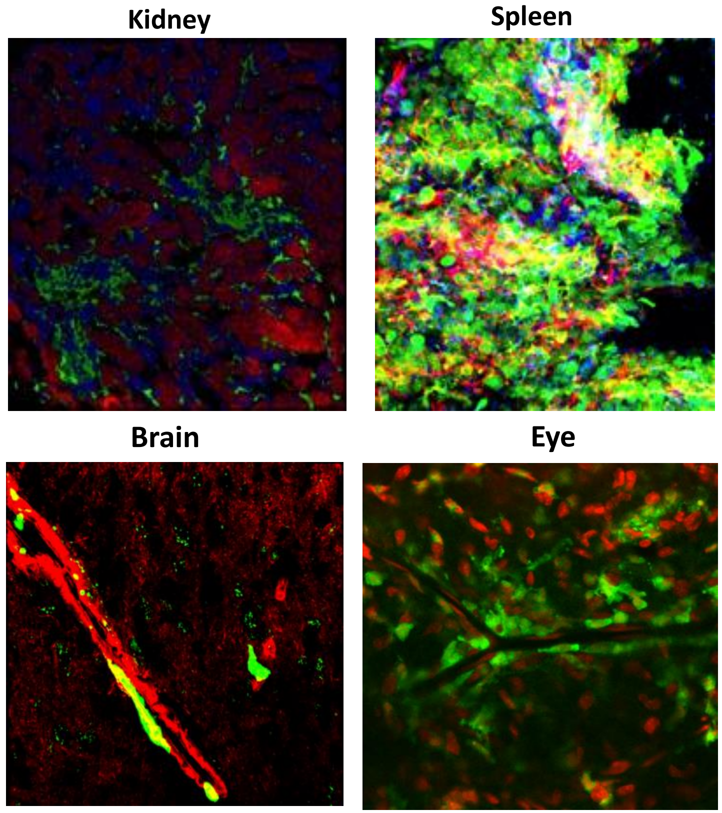
- Additional therapies to cysteamine
  - cysteine supplements
  - anti-oxydants
  - triggers of lysosome biogenesis,
  - CMA modulators
  - TFEB expression modulators (genistein

- **Stem cell therapy :** How delivering a lysosomal transmembrane gene product to every tissue?

Hematopoietic stem cell (HSC) transplantation in *Ctns*$^{-/-}$ mice

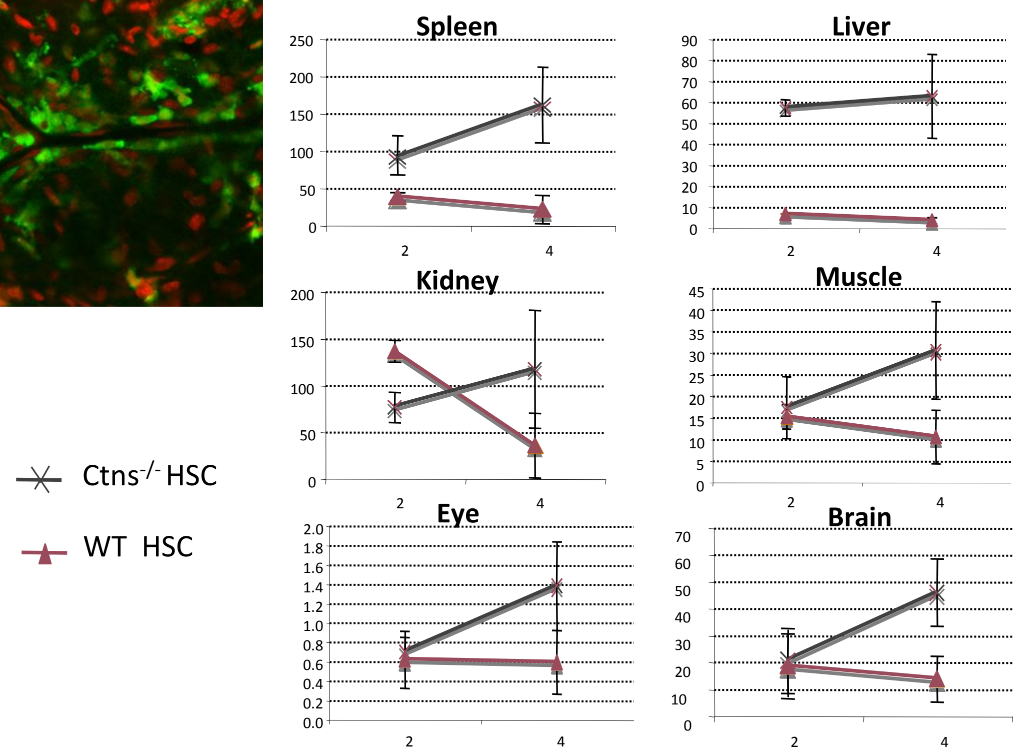


Confocal Microscopy 4 months post-transplantation



GFP+ cells
Actin
(Phalloidin)
Endothelial cells
(vWF)
Nuclei (Dapi)

Cystine content at 2 and 4 months post-transplant



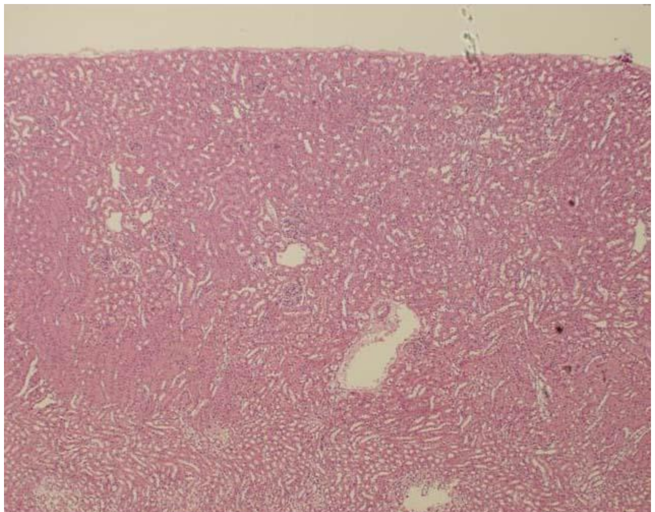
Long term significant reduction of cystine levels in all organs

Syres et al., *Blood*, 2009

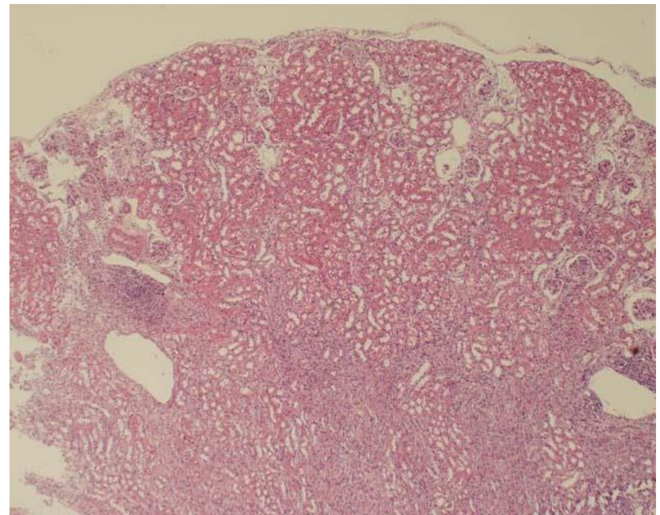
Impact of HSC transplantation on the kidney pathology in *Ctns*$^{-/-}$ mice

Kidney histology in 15-17 month old mice after over 1 year post-transplantation

Wild-type

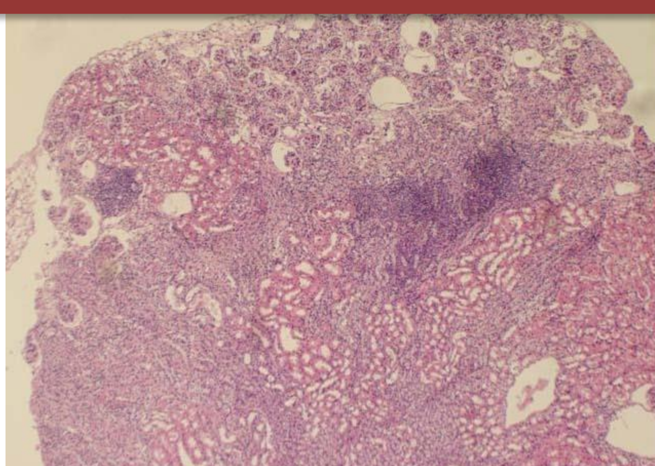


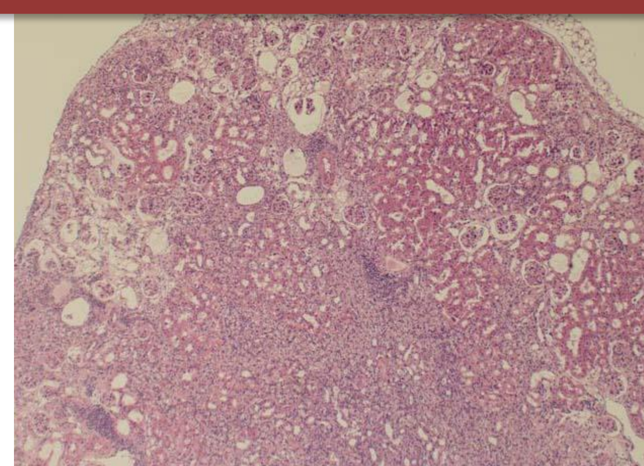
Treated *Ctns-/-*



High level of donor-derived blood cell engraftment expressing *Ctns* (>50%)

The higher the quantity of bone marrow cells expressing *Ctns* the better the preservation of the kidney

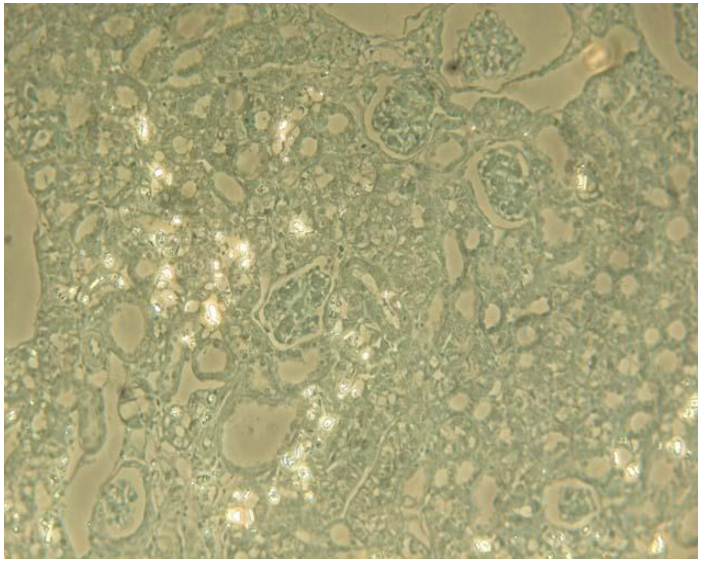




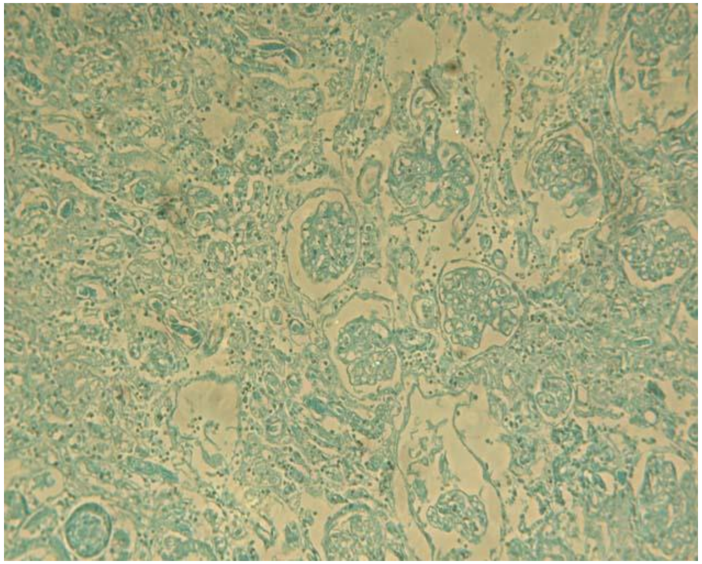
Low level of donor-derived blood cell engraftment expressing *Ctns* (<50%)

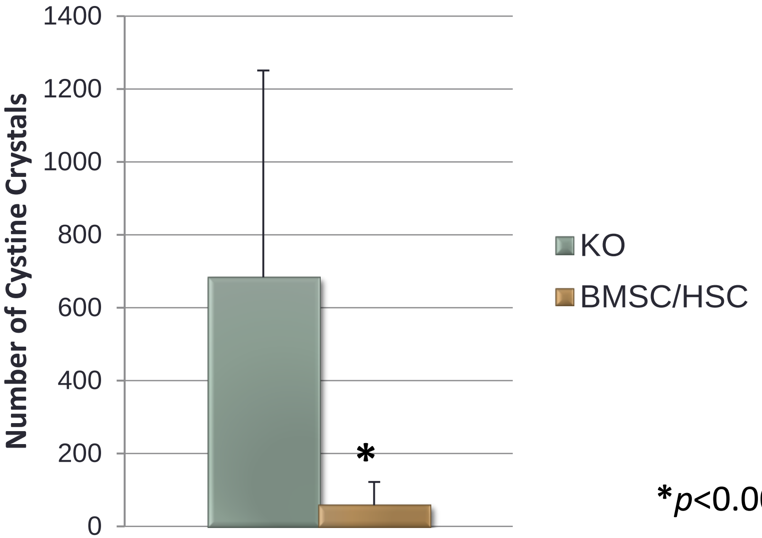
Impact of HSC transplant on cystine crystals in the kidney

Ctns-/-



Treated *Ctns-/-* (low engraftment)





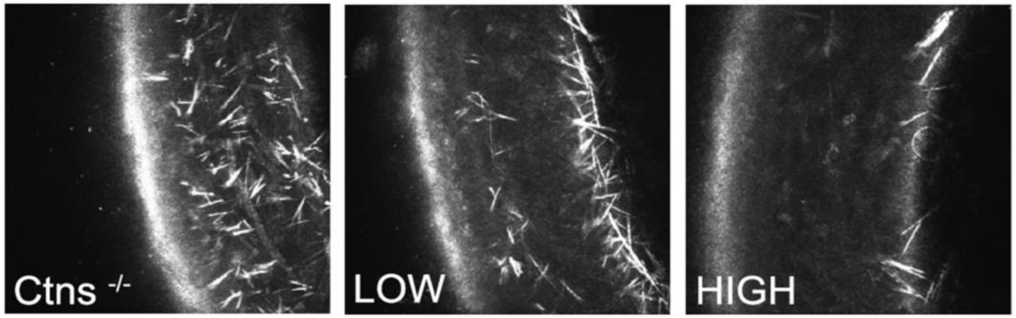
Yeagy et al., *Kidney Int.*, 2011

*p<0.00001

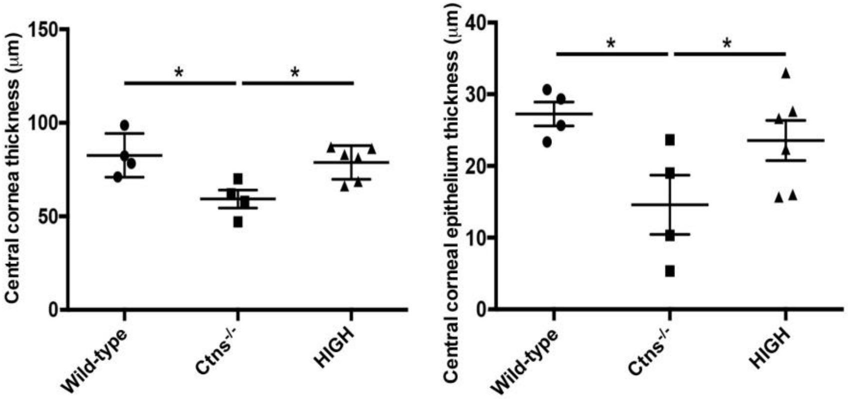
Impact of HSC transplant on the eye defects in *Ctns*$^{-/-}$ mice

Eye study after over 1 year post-transplantation

In Vivo **Confocal Microscopy (IVCM)**



Histology and central cornea thickness (CCT) measurement



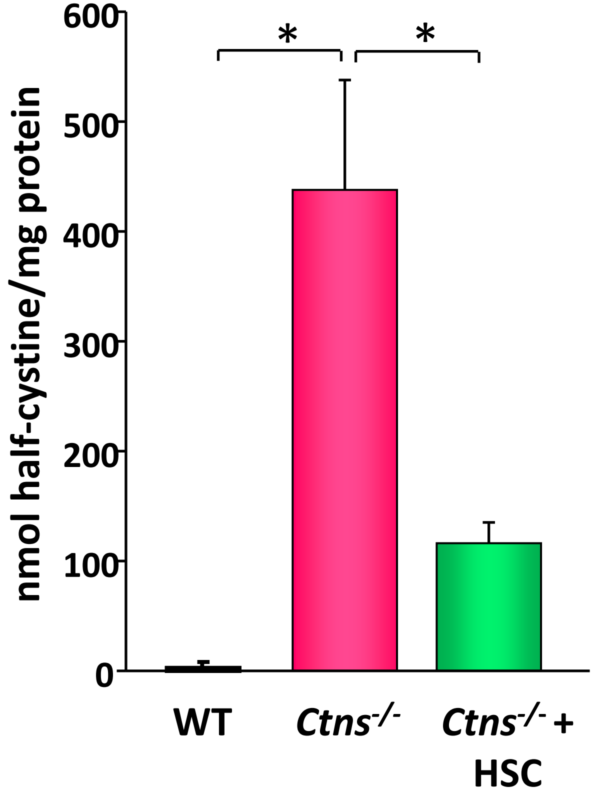
Rescue of corneal defects by HSC transplantation

Rocca *et al.*, *IOVS.*, 2016

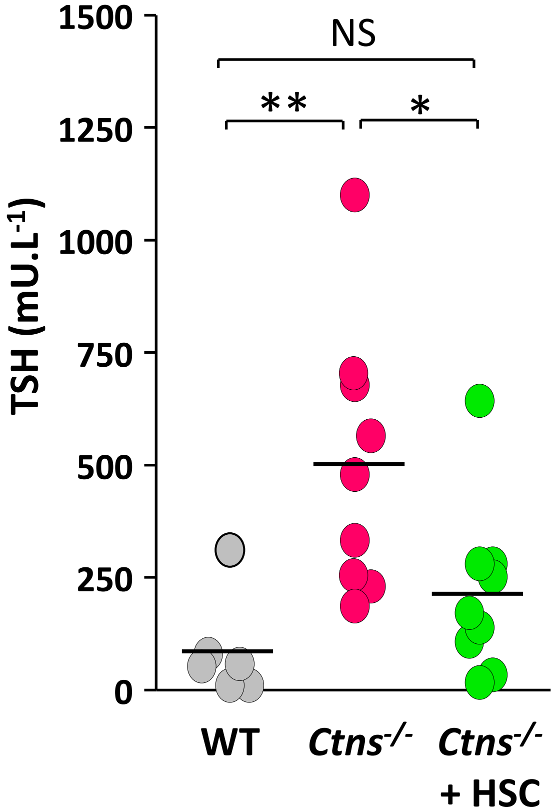
Thyroid pathology in *Ctns*$^{-/-}$ mice and impact of HSC transplantation

Most frequent and earliest endocrine complication of cystinosis

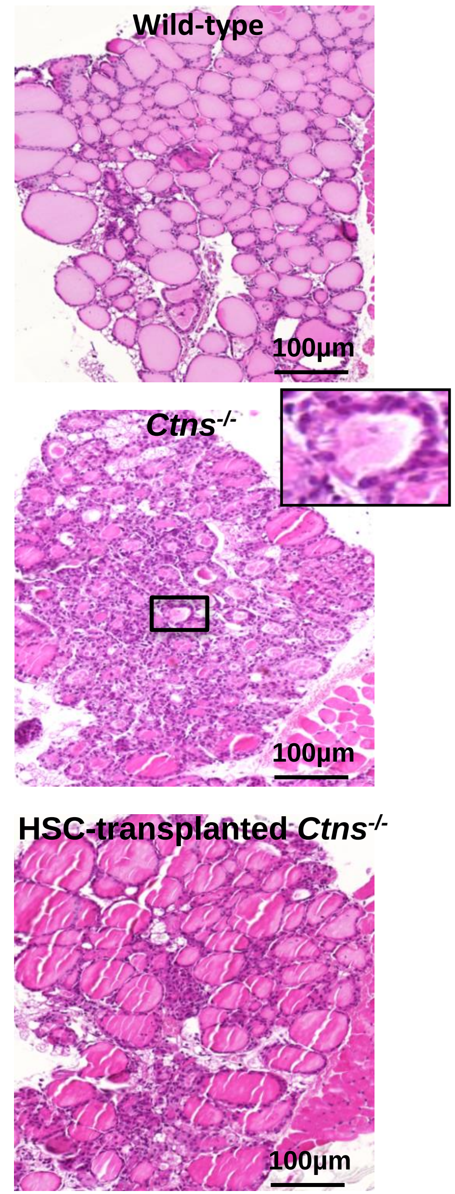
Cystine measurement in the thyroid



Mesure of Thyroid Stimulating Hormone (TSH) in serum



Drs X.H. Liao & S. Refetoff, UChicago

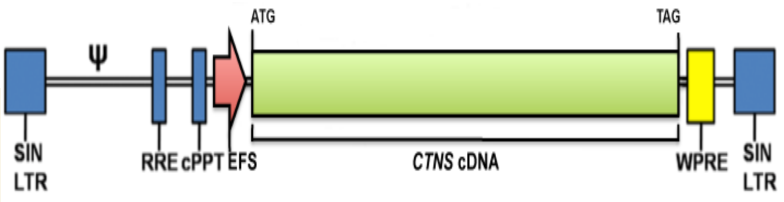


Drs H.P. Gaide Chevronnay & P.J. Courtoy, UCL-Brussels, BE

Gaide Chevronnay et al., *Endocrinology,* 2016

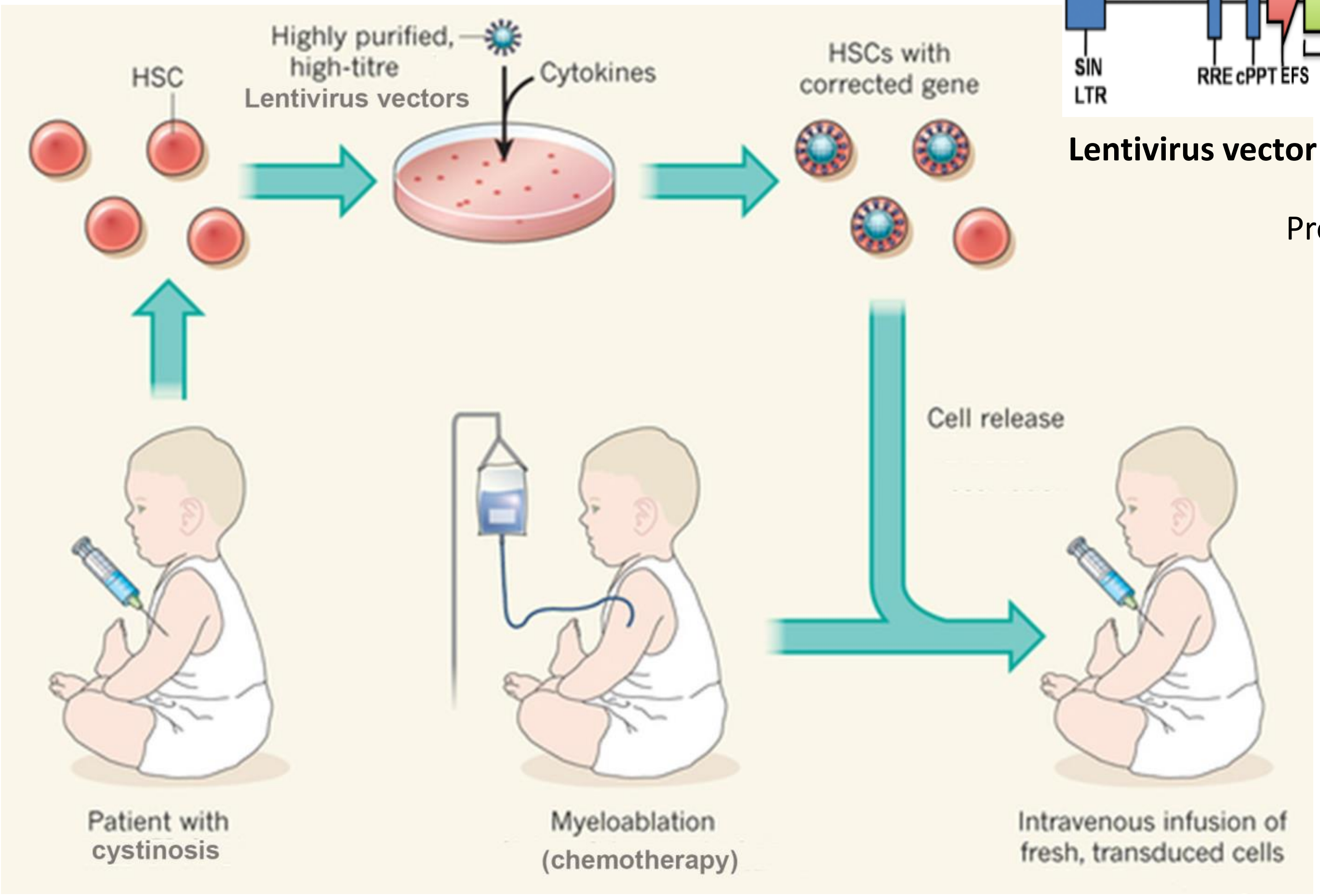
Clinical translation: autologous gene-modified HSC transplantation

pCCL-EFS-CTNS-WPRE



Lentivirus vector (safe version of HIV)

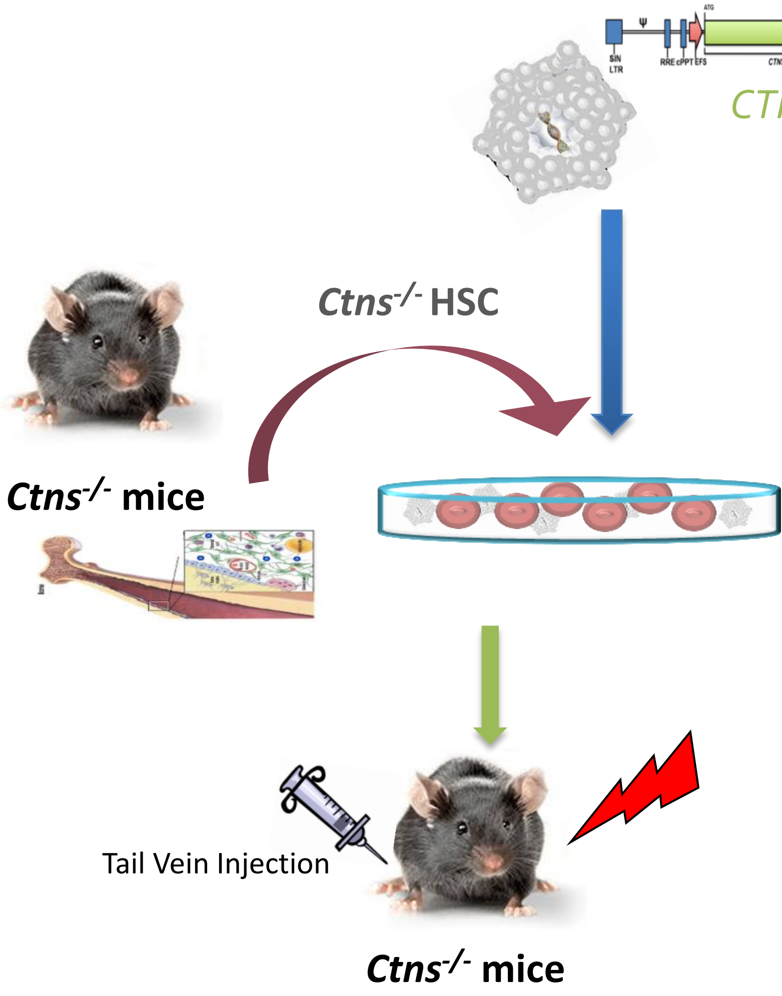
Provided by Dr. Donald Kohn (UCLA)



- Safety
- Gene frequency
- Risk of integration mutagenesis

Adapted from Leboulch, Nature 2013

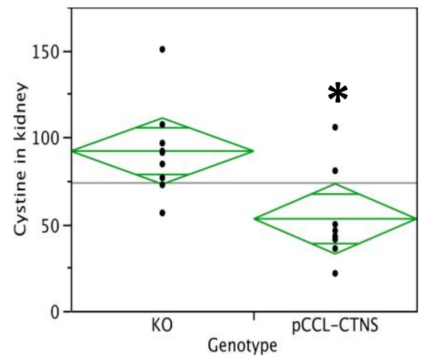
Preclinical studies for genetically-modified HSC transplantation



- Decrease cystine levels in all tested tissues
- Long term transgene expression

8 months post-transplant

Kidney cystine content



Renal function

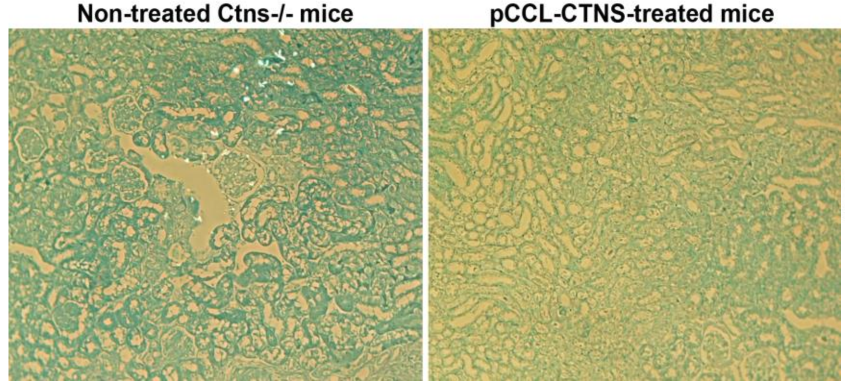
Table 1. Serum and urine analyses for renal function

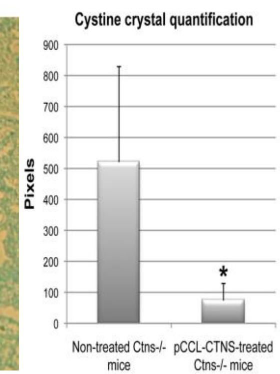
	Wildtype n=6	Control Ctns-/- n=9	pCCL-CTNS Treated Ctns-/- n=8
serum			
Creatinine (mg/dL)	0.27 ± 0.03	0.31 ± 0.08	0.22 ± 0.11[b]
Creatinine clearance (ml/mi/kg)	4.44 ± 0.39	3.86 ± 1.42	4.89 ± 5.56
Urea (mg/dL)	14.55 ± 1.87	28.29 ± 16.11[a]	24.10 ± 7.32[a]
Phosphate (mg/dL)	12.25 ± 2.38	13.20 ± 2.90	13.16 ± 2.21
urine			
Phosphate (mmol/24h)	6.82 ± 2.90	8.84 ± 4.60	4.78 ± 3.87[b]
Volume (ml)	1.05 ± 0.51	1.26 ± 0.54	0.70 ± 0.60[b]

[a] $P<0.05$ compared to wildtype mice
[b] $P<0.05$ compared to Ctns-/-

Cystine crystals quantification

Non-treated Ctns-/- mice | pCCL-CTNS-treated mice



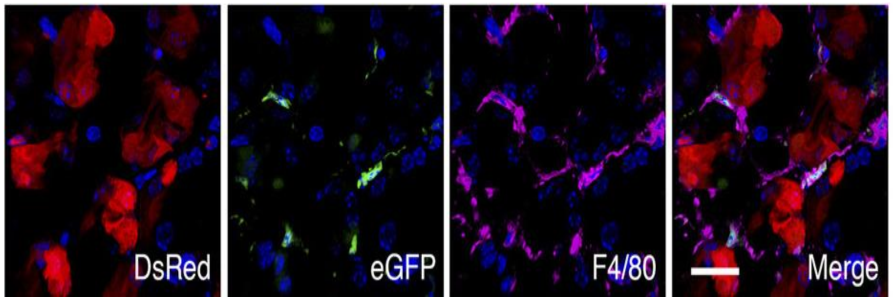


Harisson et al., *Mol. Ther.*, 2013

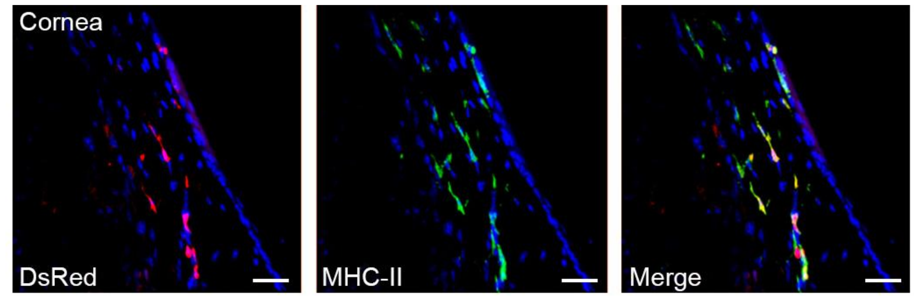
Characterization of the transplanted HSCs within the kidney

Differentiation, fusion or transdifferentiation ?

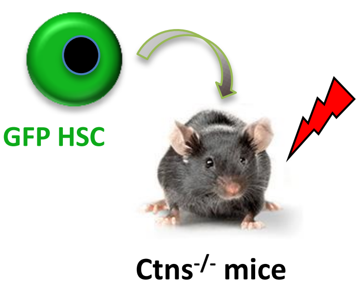
Kidney

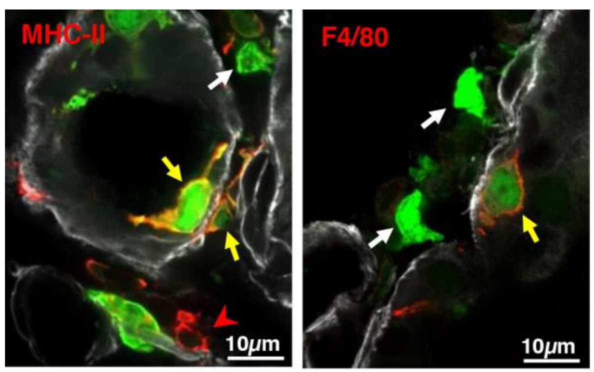


Eye



Thyroid





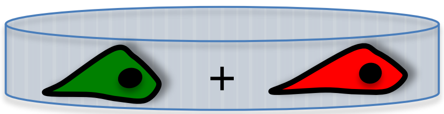
Transplanted HSCs differentiate into macrophages within tissues in *Ctns*$^{-/-}$ mice

How do transplanted HSCs mediate tissue repair in cystinosis ?

- Phagocytic functions
- Cross-correction i.e. transfer of cystinosin from the transplanted cells to the adjacent *Ctns*$^{-/-}$ cells

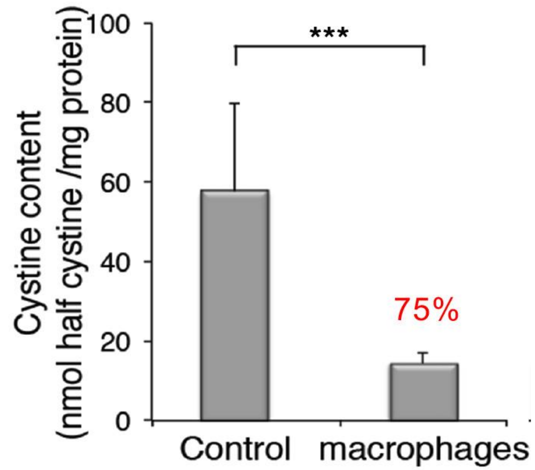
Cross-correction : *in vitro* studies

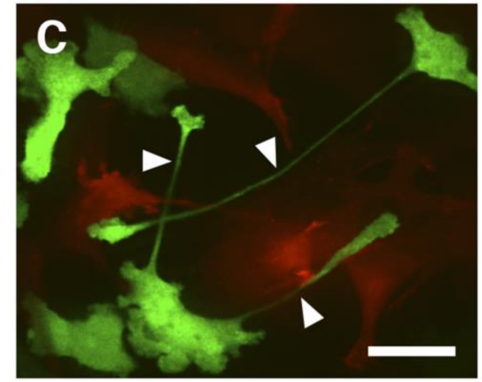
Cystinosin transfer via cell-cell contact



GFP^{+} macrophages

DsRed Ctns$^{-/-}$ Fibroblasts

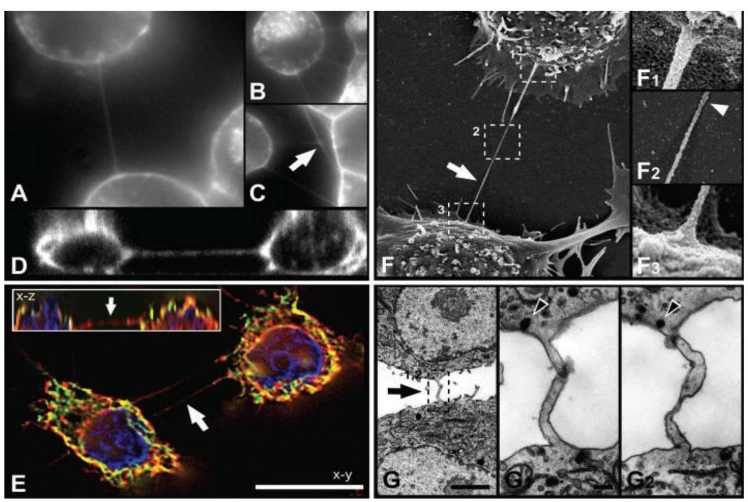


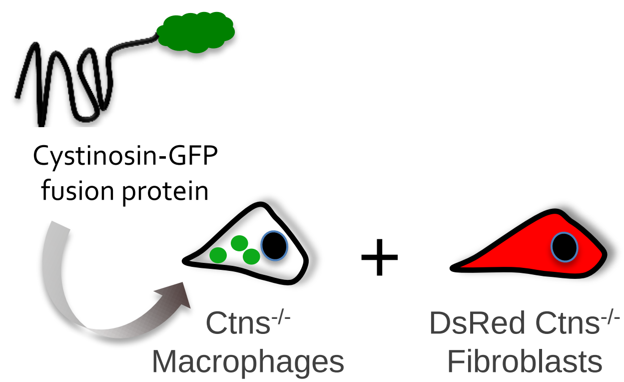


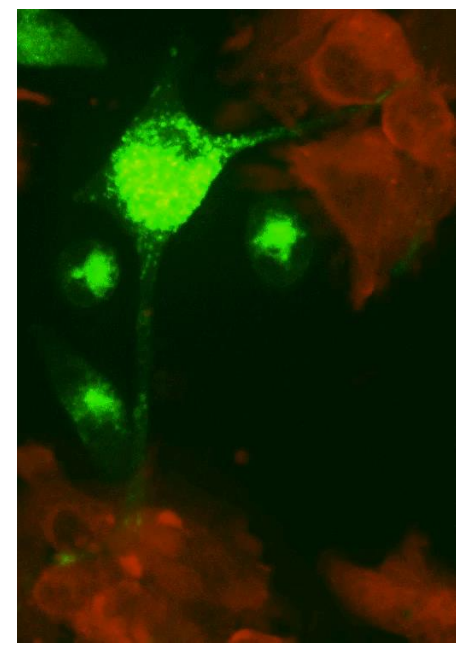
Nanotubular Highways for Intercellular Organelle Transport

Amin Rustom, Rainer Saffrich, Ivanka Markovic, Paul Walther, Hans-Hermann Gerdes

Science, 2004

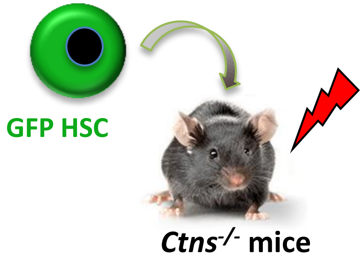


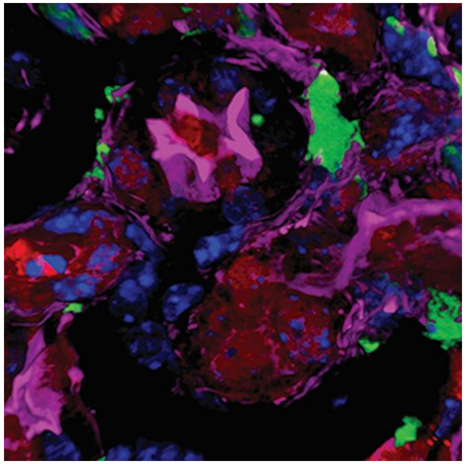




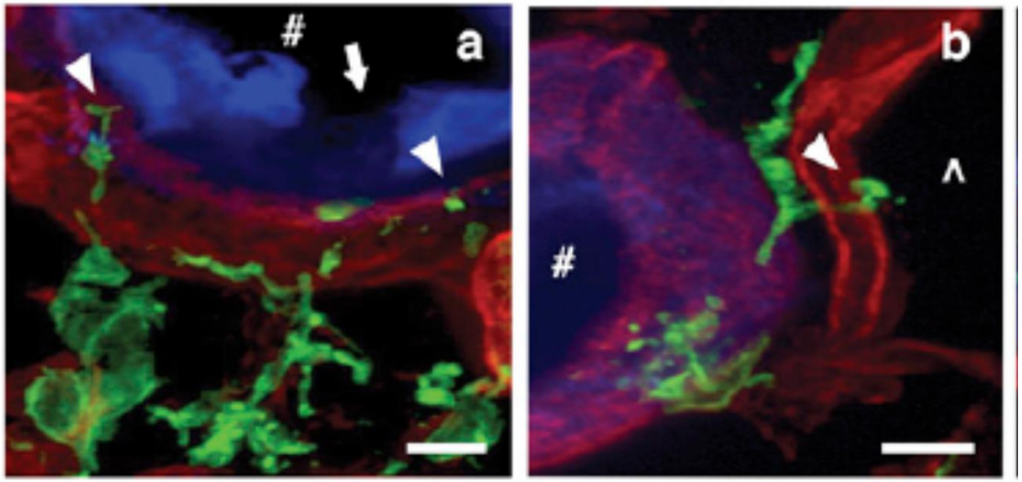
Naphade et al., *Stem Cells*, 2015

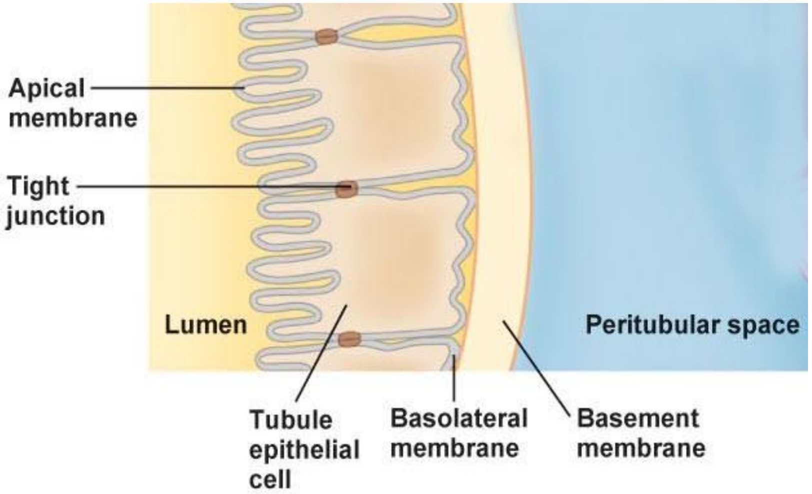
Cross-correction : *in vivo* studies





Kidney





- Vesicular cross-correction in kidney
- Also demonstrated in cornea and thyroid

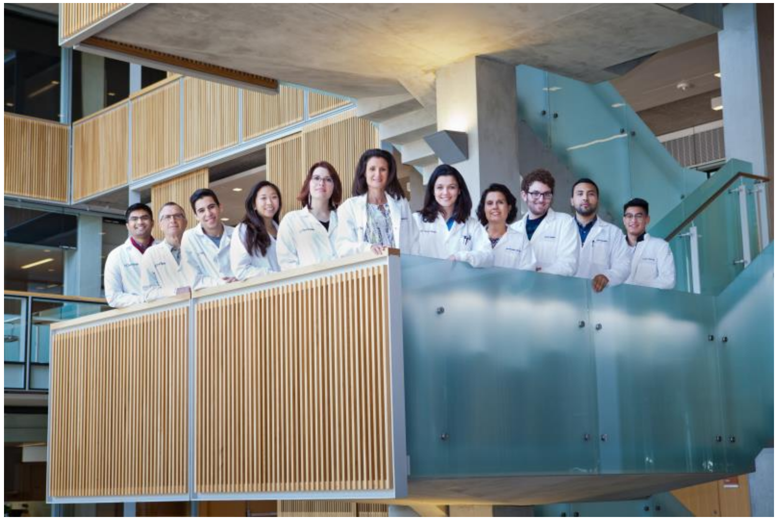
Naphade et al., *Stem Cells*, 2015
Rocca et al., *IOVS,* 2015
Gaide Chevronnay et al., *Endocrinology*, 2017

Conclusions

- Several key cellular dysfunctions are observed in cystinosis linked to the lysosomal cystine accumulation and/or additional roles of cystinosin beyond cystine transport

- Several new lines of treatment are being developed:
  - In addition to cysteamine therapy, drugs targeting the various pathways altered in cystinosis
  - Stem cell therapy
    - Long term significant reduction of cystine levels in all organs by hematopoietic stem cells in a *Ctns-/-* mouse model
    - Differentiation of HSC in macrophages
      - Phagocytic function
      - Cross-correction through nanotubes
    - Clinical trials being set up in the US (autologous stem cell transplantation)
  - Novel additional eye treatments (corneal nanowafers)

ACKNOWLEDGEMENTS



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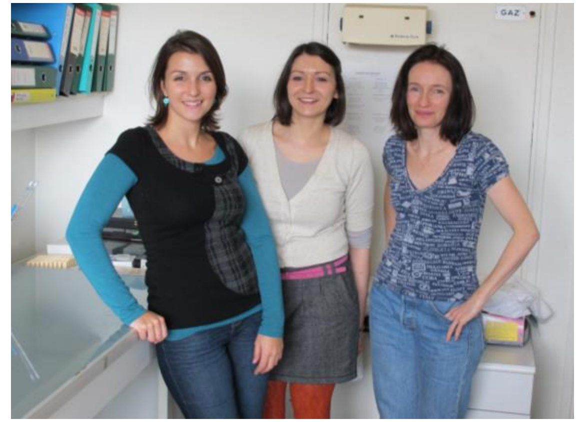
Pierre Courtoy (UCL, Brussels)
Stéphanie Cherqui (Scripps, La Jolla)



imagine INSTITUT DES MALADIES GÉNÉTIQUES

CYSTINOSIS RESEARCH FOUNDATION NATALIE'S WISH

AIRG Association pour l'Information et la Recherche sur les maladies

VML VAINCRE LES MALADIES LYSOSOMALES

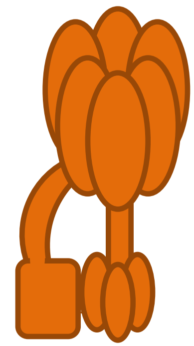
searching for a cure Cystinosis Research Network

EUNEFRON

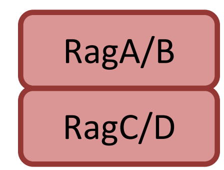
Cystinosin interacting partners

Proteins interacting with cystinosin (by mass spectrometry)

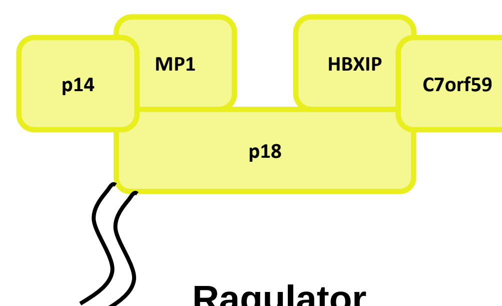
Protein Description	CD63-GFP		Fibroblast cystinosin-GFP				MDCK cystinosin-GFP			
	n=2		n=4				n=4			
Human Cystinosin-GFP			32	57	22	45	40	48	45	47
GFP	40	39								
V-type proton ATPase catalytic subunit A			19	33	25	39	11	24	40	28
V-type proton ATPase subunit B, brain isoform			21	23	23	33	11	19	35	28
V-type proton ATPase subunit C 1			6	12	8	19	3	3	19	9
V-type proton ATPase subunit D			2	6	4	7		4	5	5
V-type proton ATPase subunit E 1			6	12	6	12	4	8	14	8
V-type proton ATPase subunit F				8	2			4		
V-type proton ATPase subunit G 1			2	2	2		2	4		
V-type proton ATPase subunit H			4	8	5	14	4	10	16	9
V-type proton ATPase subunit S1			1			3	2	2	9	3
V-type proton ATPase subunit d 1			10	10	9	18	11	12	18	14
V-type proton ATPase 16 kDa proteolipid subunit			6	13				4		
V-type proton ATPase 116 kDa subunit a isoform 1			17	20	10	22	14	14	18	13
V-type proton ATPase 116 kDa subunit a isoform 2			2	1			4	15	22	14
Ragulator complex protein LAMTOR1			4	3	5		1	1	1	
Ragulator complex protein LAMTOR2			3	1						
Ragulator complex protein LAMTOR3			2	1				1		
Ragulator complex protein LAMTOR5			1							
Ras-related GTP-binding protein C			3	4	5	10			5	
Ras-related GTP-binding protein A				2	1	6			3	1



v-ATPase



Rag GTPases

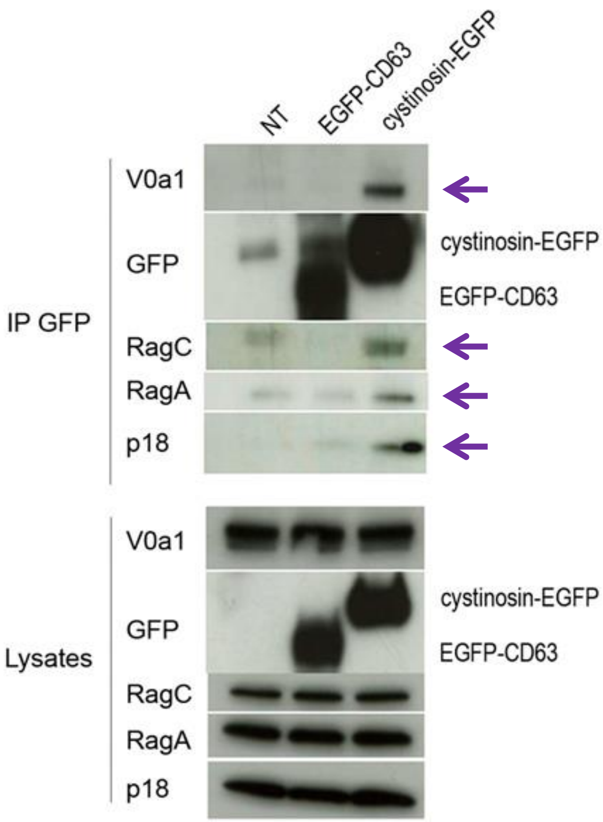


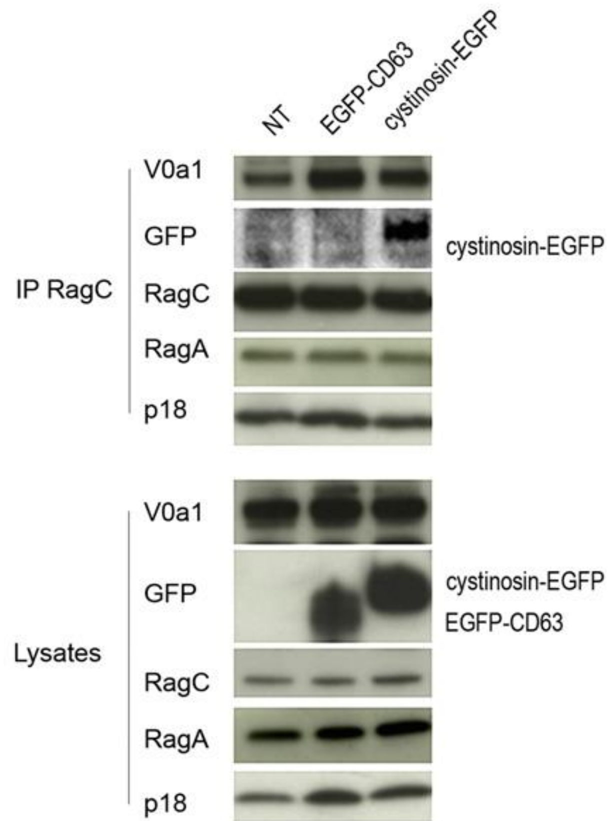
Ragulator

- Additional control: Lamp1-GFP
- Ers1 (homolog of *CTNS*) in yeast also involved in the TOR pathway

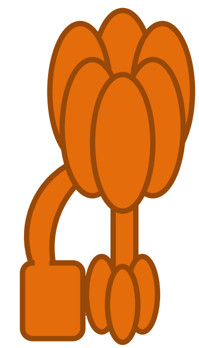
(Andrzejewska *et al.*, JASN, 2015)

Cystinosin binding partners

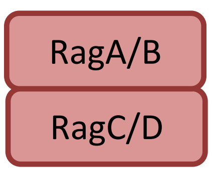




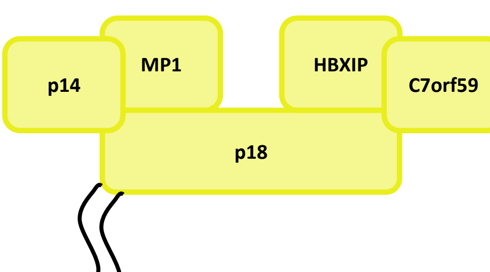
v-ATPase



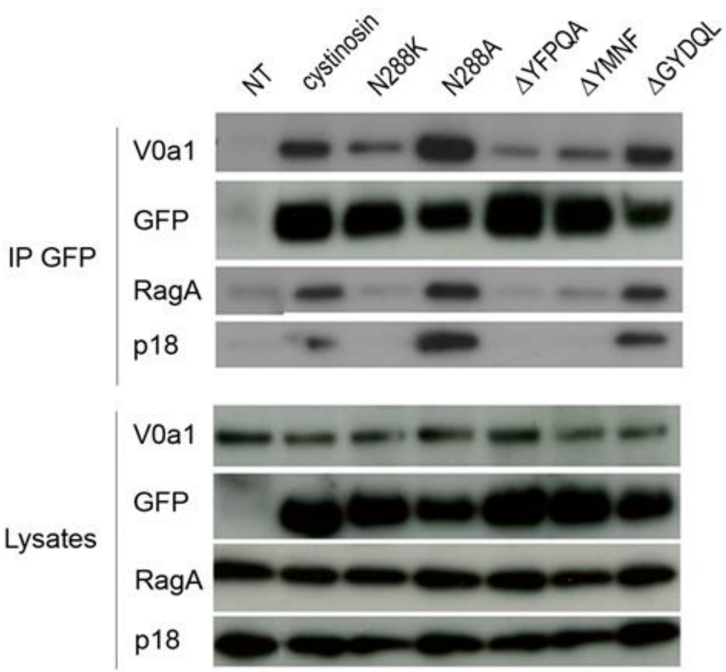
Rag GTPases

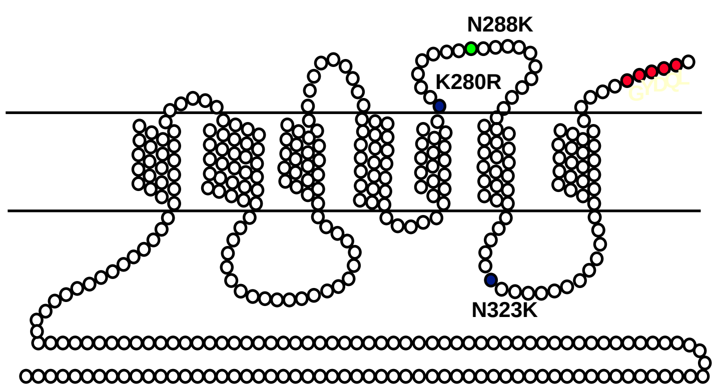


Ragulator

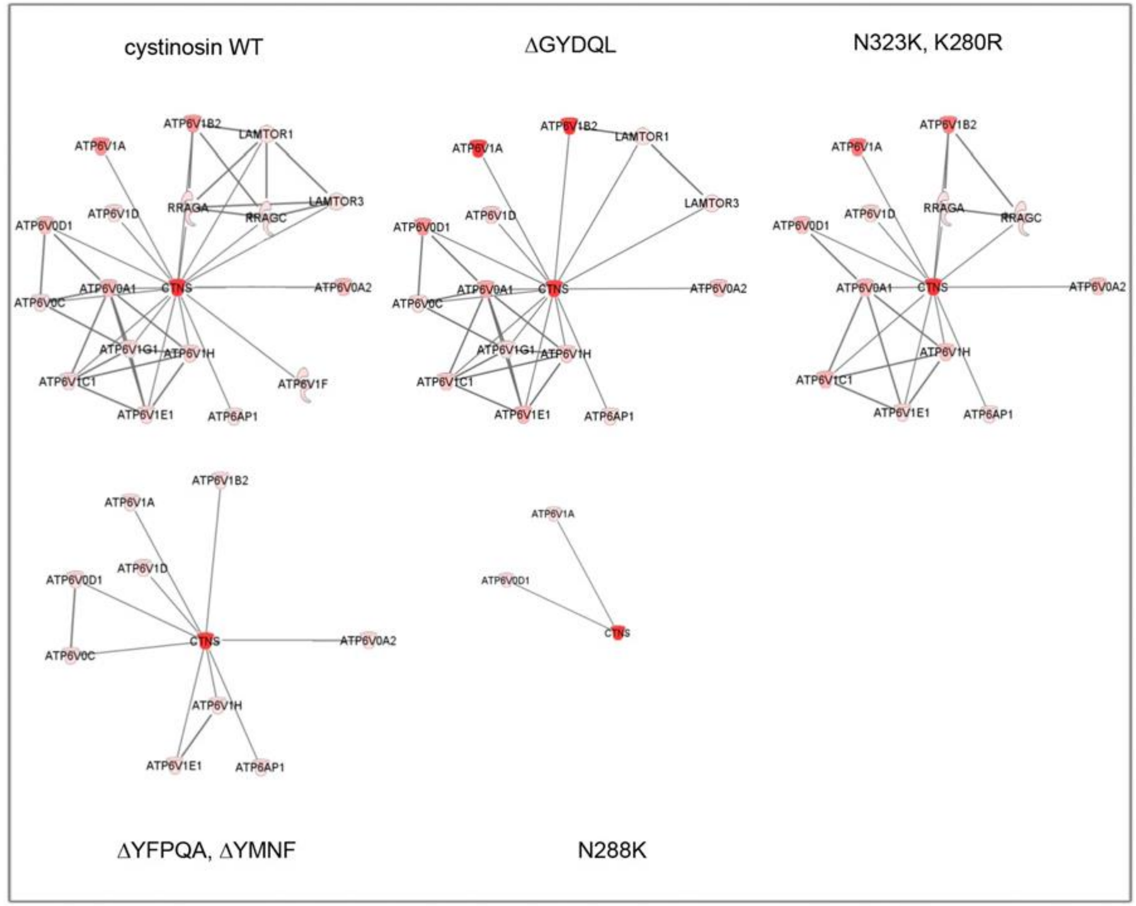


Interaction networks for mutants of cystinosin



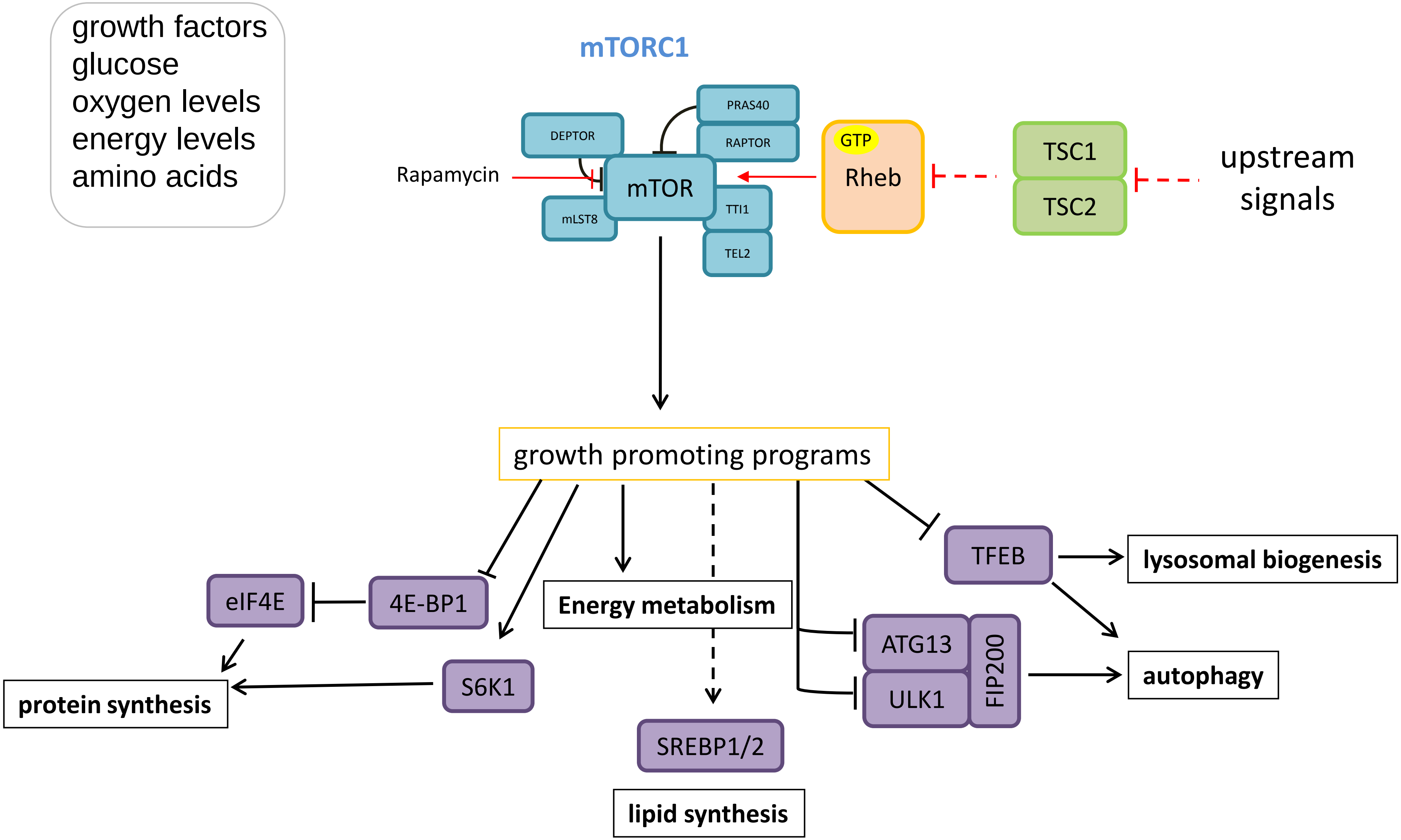


- Mutation observed in infantile cystinosis
- Mutations observed in juvenile, ocular or atypical cystinosis

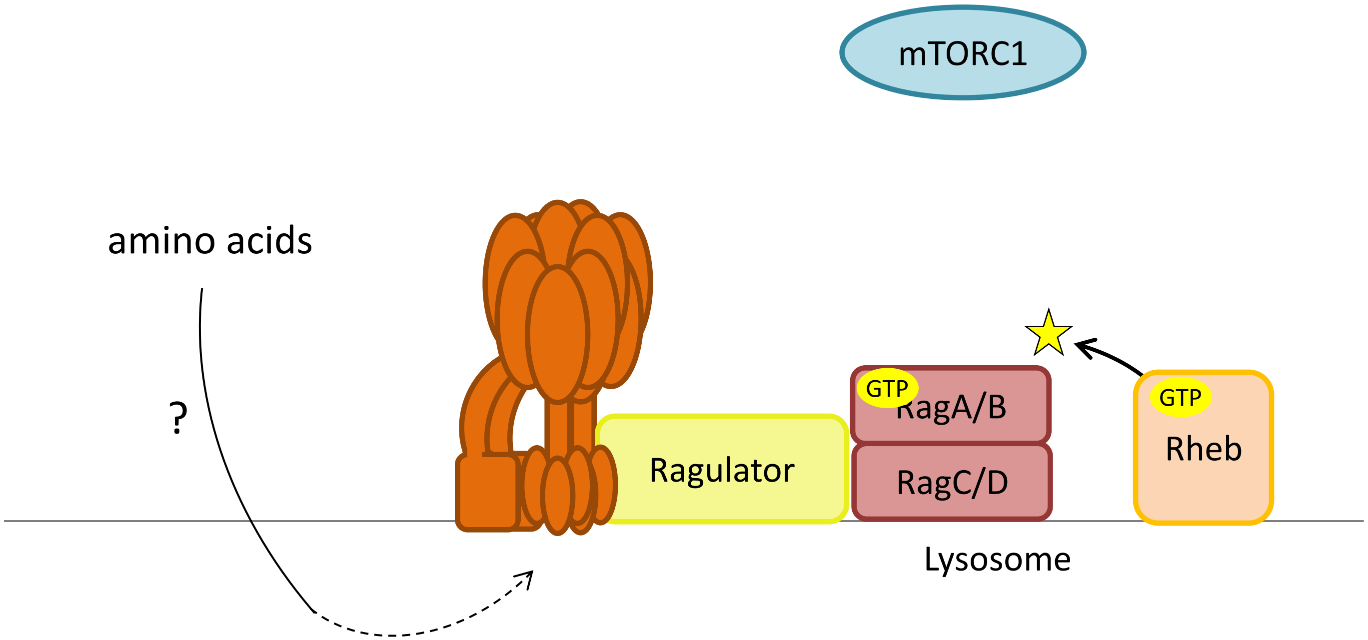


Role of the 5th inter-TM loop +++

mTORC1 signaling complex



Amino acid-dependent activation of mTORC1 pathway

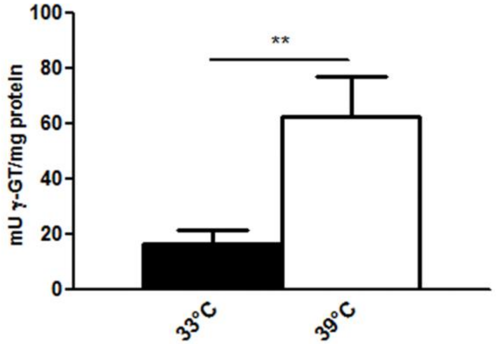


lysosomal «inside-out» amino acid sensing mechanism

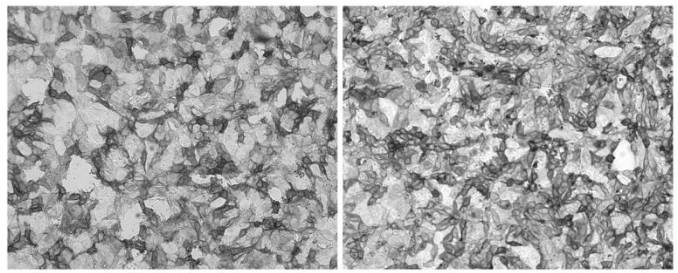
Zoncu et al. 2011
Bar-Peled et al. 2012

Characterization of mouse proximal tubular cell lines

Activity of brush border enzymes

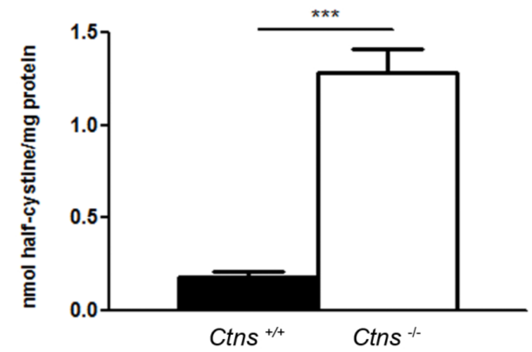


γ- glutamyl transferase



alkaline phosphatase

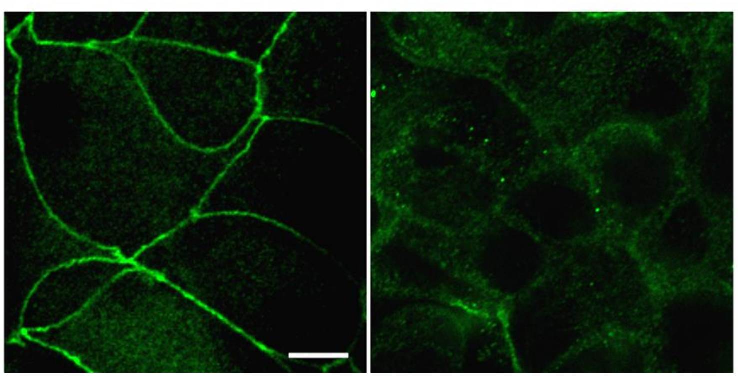
Cystine accumulation



Expression of markers of polarized epithelia

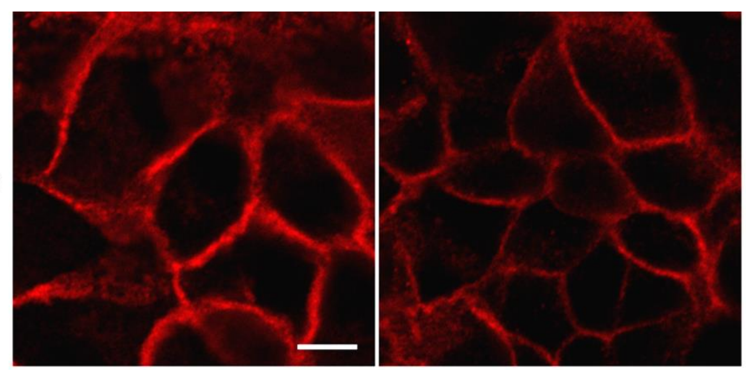
Ctns +/+ *Ctns* -/-

ZO-1

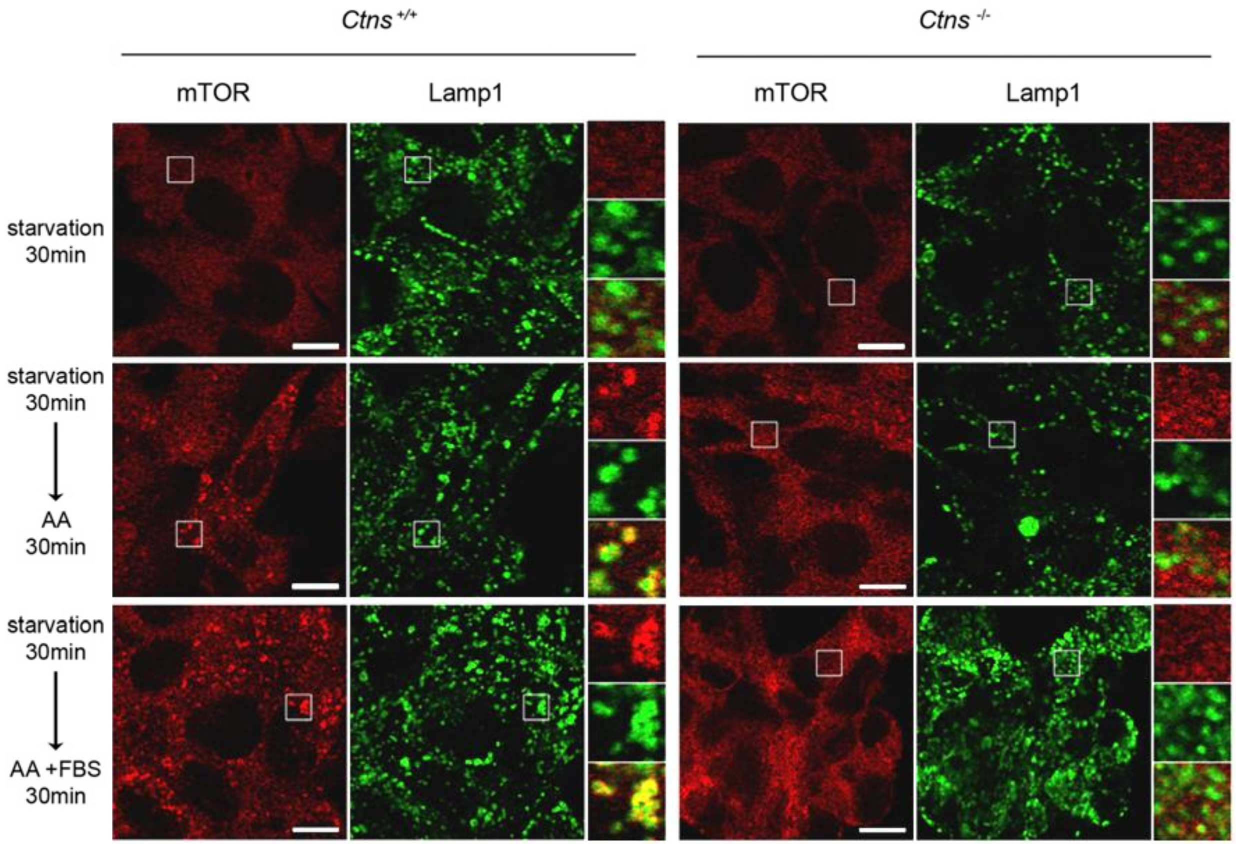


Ctns +/+ *Ctns* -/-

β-catenin

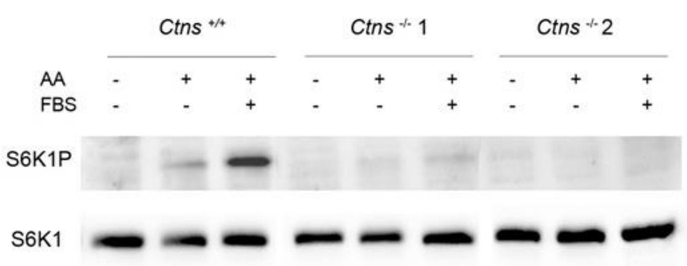


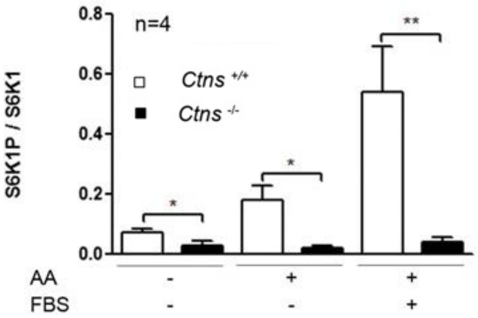
Cellular repartition of mTOR and Lamp-1 in response to nutrients



Lack of cystinosin alters mTOR localization

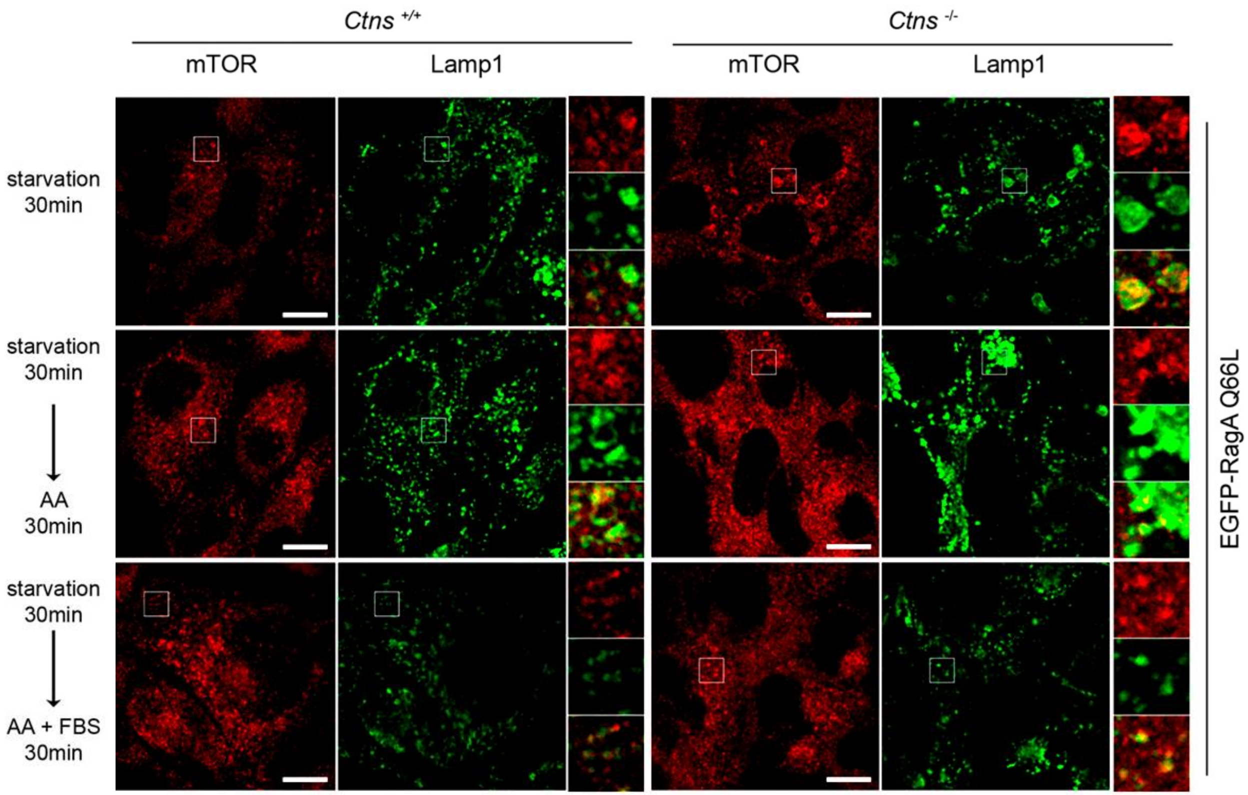
Cystinosin essential for mTOR regulation by nutrients in MPT cells



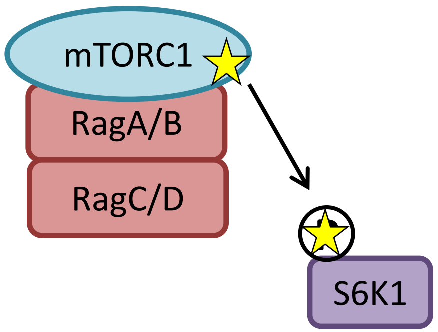


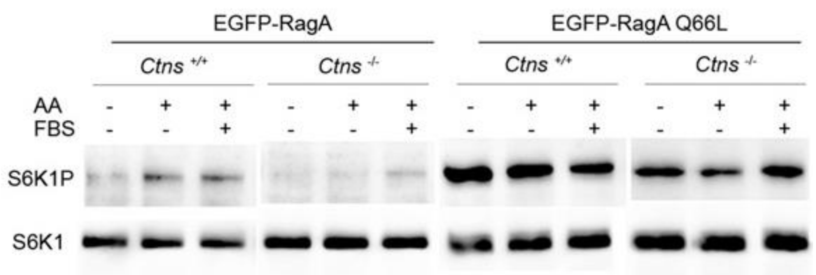
Defective mTOR relocalization in *Ctns* $^{-/-}$ cells correlates with impaired downstream signaling

Rescue of mTOR signaling by RagA Q66L



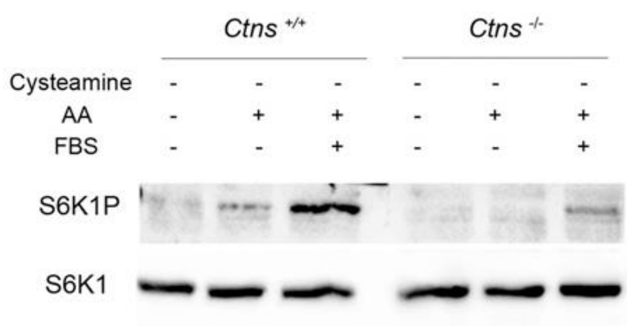
RagA Q66L - dominant active mutant mimicking GTP-bound state of RagA

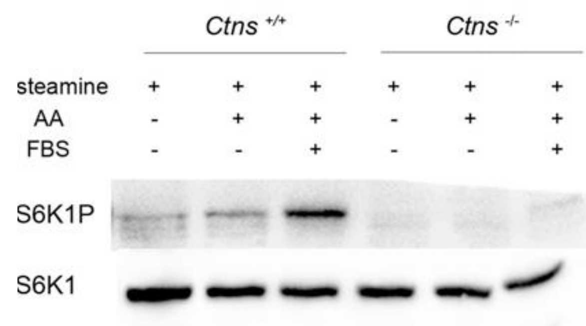


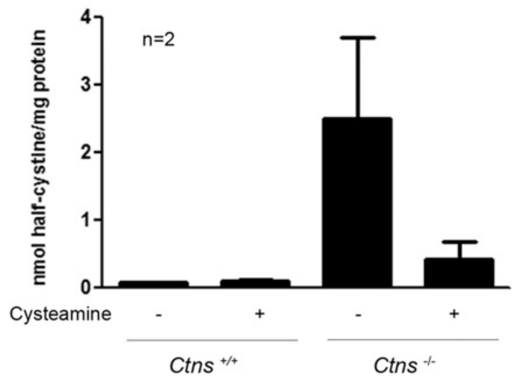


Cystinosin acts upstream of Rags

No effect of cysteamine on mTOR signaling in *Ctns* $^{-/-}$ cells



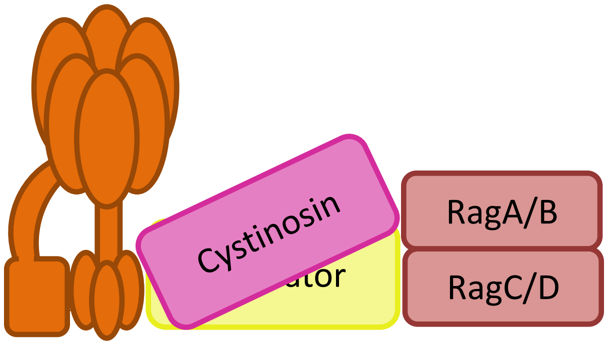




Dysregulation of mTOR signaling in *Ctns* $^{-/-}$ cells due to the absence of cystinosin and not the lysosomal cystine accumulation

Conclusions (I)

- Dual role of cystinosin
  - lysosomal cystine/proton symporter
  - part of the nutrient-sensing machinery involved in mTORC1 signaling – aminoacid sensor for the mTOR pathway?



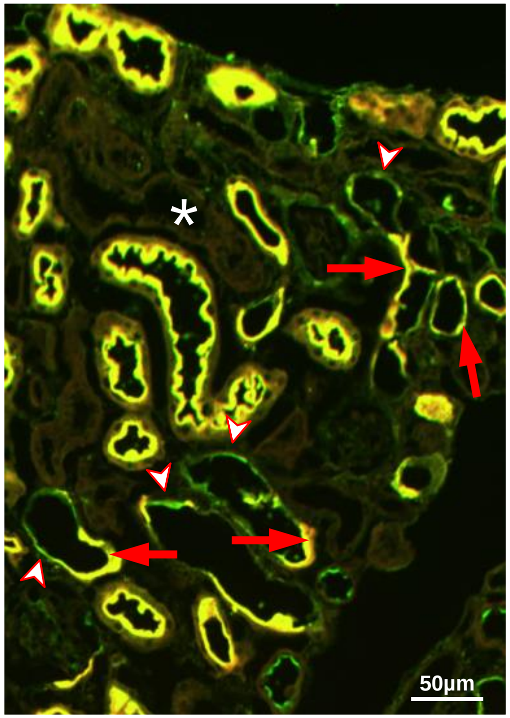
- Mechanism for the development of Fanconi syndrome
  - mTOR-vATPase controls megalin expression in Drosophila epithelial cells and PTC in mouse (Gleixner et al., 2014)
  - Gradual loss of cubilin and megalin in *Ctns-/-* PT (Gaide Chevronnay et al., 2014)

↓

Low molecular weight proteinuria

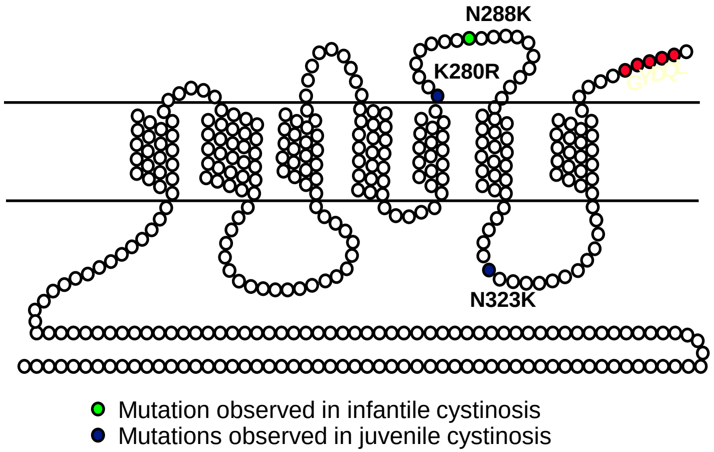
Ctns-/- prox tubules

megalin - cubilin



(courtesy of P. Courtoy)

Conclusions (II)



- Rationale to explain the apparent discrepancies between phenotype-genotype correlations in patients with juvenile phenotype and no cystine transport

- No effect of cysteamine on mTOR signaling: Need for developing new treatments besides lysosomal cystine depletion

- Other lysosomal amino acid transporters involved in the nutrient-sensing machinery [**PAT1** (Ögmundsdóttir et al. 2012), **SLC38A9** (Wang et al. 2015; Rebsamen et al 2015, Jung et al. 2015), **PQLC2 / LAAT-1** (?)] – Is there a cumulative role of the defects?

What's ongoing

- Analysis of mTORC1 activity and autophagy in cystinotic mice
- Characterization of the mTORC1 pathway in cell lines bearing the N288K vs. K280R, N323K mutations (CRISPR/Cas9 technology)
- Characterization of the strength of the interactions under aminoacid starvation
- Phenotype of the double KO *Ctns/Tsc ?*

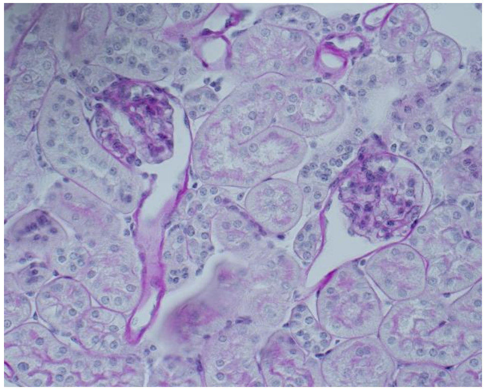
- Search for modifier genes responsible for the absence of renal disease in the FVB background.

Development of an animal model

Ctns$^{-/-}$ knock-out mice

- Sex ratio = 1
- No embryonic letality
- Normal development and fertility
- No phenotype in the first months of life

- Widespread cystine accumulation increasing with age
- Ocular, muscular and bone abnormalities
  - Osteoporosis
    - ↘ bone mineralization
    - ↘ cortical width
    - bone deformity
- Renal phenotype dependant upon the genetic background

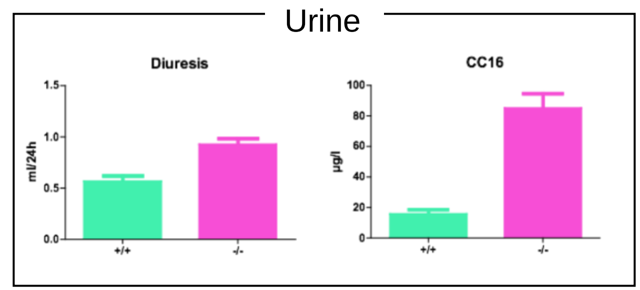


(Cherqui et al., 2002; Nevo et al., 2010)

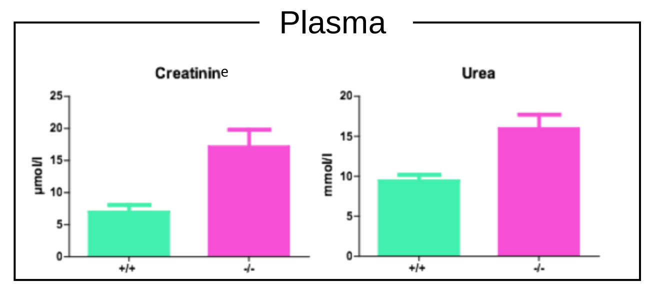
Ctns$^{-/-}$ knock-out mice: renal phenotype dependent upon genetic background

- Proximal tubulopathy and progressive renal failure in C57BL/6 *Ctns*$^{-/-}$ mice
  - Failure to thrive
  - Polyuria (from 2 months) with decreased urinary osmolarity
  - Marked increased CC16 excretion (LMW proteinuria)
  - Increased daily urinary excretion of glucose, phosphate and potassium
  - No hyper aminoaciduria
  - Chronic renal failure from 9 - 10 months
  - Great variability between mice even from the same litter

- No renal symptoms in FVB/N *Ctns*$^{-/-}$ mice



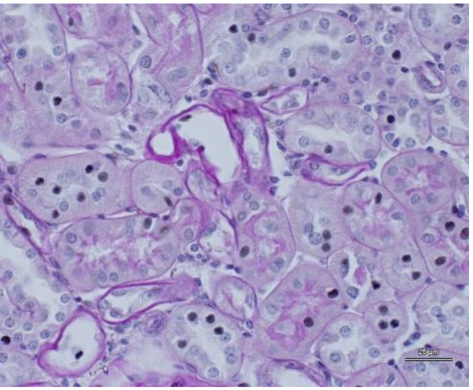
C57BL/6 2-9 mths

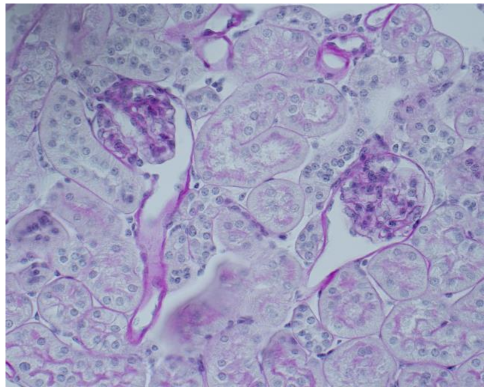


C57BL/6 10-18 mths

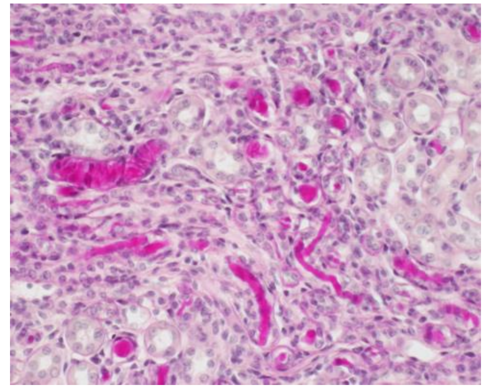
(Nevo et al., 2009)

Proximal tubular lesions in kidneys of C57BL/6 *Ctns* -/- mice

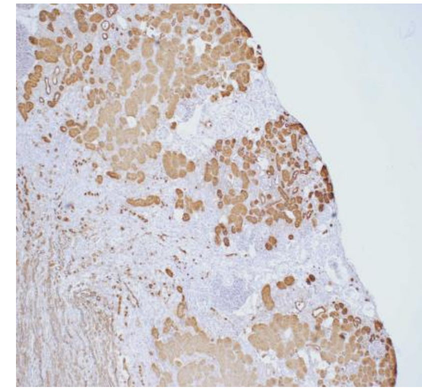




6 months



12 months

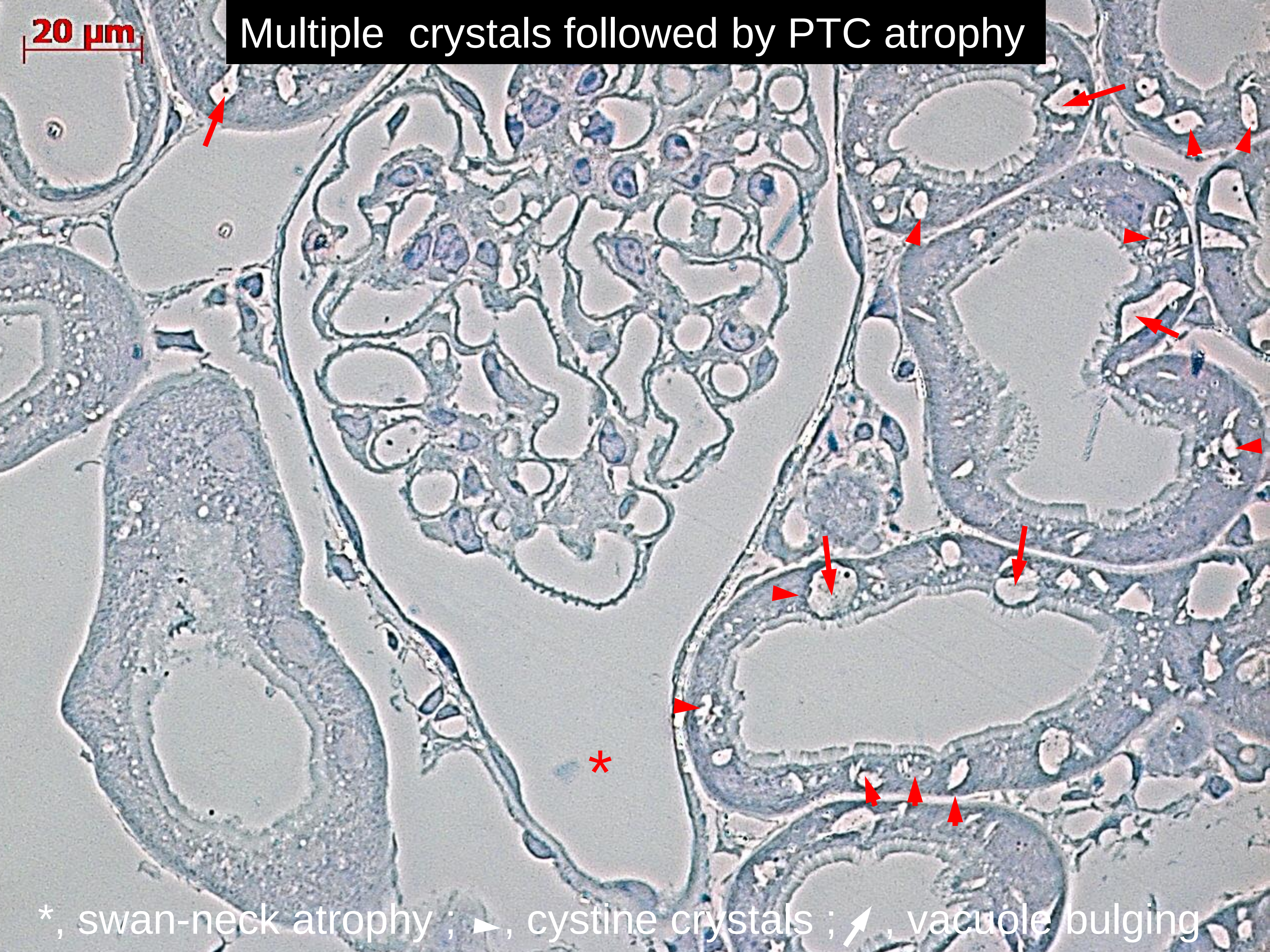


LT lectin labeling

- From 6 months, development of focal lesions of proximal tubules mainly in the superficial cortex
- Atrophy with complete disappearance of the epithelial cell layer and thickening of the BM leading to focal disappearance of proximal tubules
- More extensive lesions at 9-12 months

- No tubular lesions up to 18 months in FVB/N *Ctns*$^{-/-}$ mice

Multiple crystals followed by PTC atrophy



*, swan-neck atrophy ; ►, cystine crystals ; ↗, vacuole bulging