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« Physiopathology of apical receptor-mediated endocytosis in untreated cystinotic mice »
Third progress report (as of October, 2011)

A. Background and objectives

Infantile cystinosis manifests itself by an early Fanconi syndrome characterized by loss of solutes – reflecting a defect of corresponding apical channels and transporters - combined with urinary excretion of low molecular weight proteins (LMWP) – likely explained by a deficit of their endocytic apical recapture. The working hypothesis of the research project is that apical receptor-mediated endocytosis (ARME) plays a pivotal causal role in (i) early proximal tubular cell (PTC) atrophy observed in cystinotic kidney and (ii) its subsequent progression along the proximal tubular segment, by displacing the load of protein uptake to more distal parts of the PTC. In accordance with this model, we observed that LMW proteinuria was associated with decreased expression of megalin and cubilin in PTC of congenic C57BL/6 *Ctns*^{-/-} mice.

In this third report, three directions have been successfully followed. The **first objective** was to analyse whether ARME was also defective in cystinotic patients. We thus looked for LMW proteinuria and analyzed the expression of megalin and cubilin in kidney of cystinotic patients. The **second objective** was to clarify the relation between defective ARME and apical PTC dedifferentiation in congenic C57BL/6 *Ctns*^{-/-} mice. To this aim, the overall apical functional differentiation state of PTC was characterized by multiplex immunofluorescence confocal microscopy analysis. The **third objective** was to identify local markers/actors of disease progression by comparing transcriptomes of altered vs preserved PTC. To this aim laser capture microdissection of altered and preserved PTC have been initiated.

B. Progress report

Objective #1. Expression level of megalin/cubilin in kidneys and assessment of LMW proteinuria in cystinotic patients: completed.

We first analysed the expression of megalin and cubilin by immunohistochemistry in kidneys of 4 cystinotic patients (3 to 12 years-old). Analysis by conventional light microscopy of paraffine sections after periodic acid/Schiff (PAS) staining disclosed the characteristic atrophy of proximal tubular cells (not showed). Whereas megalin and cubilin were homogeneously expressed in proximal tubular cells of an age-matched control kidney (Figs 1 A, C), their expression was systematically decreased in cystinotic patients, with a marked heterogenous pattern (Figs 1 B, D).

We also screened by western blotting for LMW proteins in the urine of 5 cystinotic patients (5 to 15 years-old; 3 males, 2 females; kindly provided by Dr. E. Levchenko) compared to 5 normal volunteers of similar age (5 to 16 years-old; 1 males, 4 females). All cystinotic patients except patient #4 exhibited a strong urinary excretion of both transferrin and retinol-binding protein, none of which was detected in control samples with our assay conditions (Fig 1 E). Patient #4 is particularly interesting since this was the only one of this series in which a Fanconi syndrome was not diagnosed : whereas his urinary transferrin was elevated, indicating a specific cubilin defect, RBP was not, in agreement with our previous proposal that dedifferentiation programs (here induced by cystinosis) first hits cubilin before megalin (see also Lima et al, 2010).

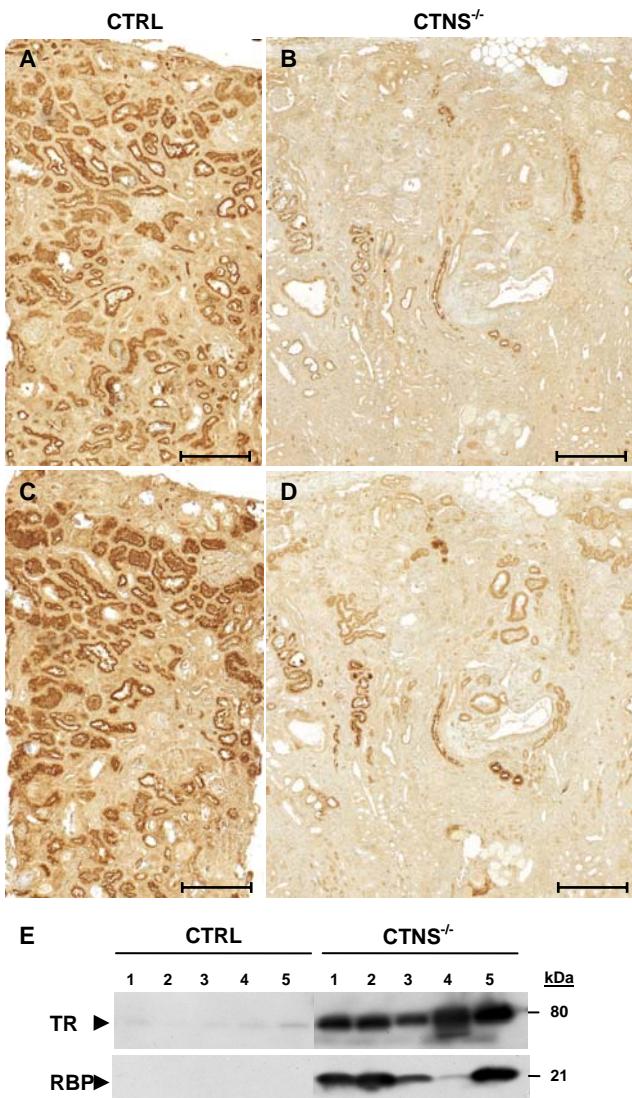


Fig 1. Decreased megalin and cubilin expression in kidneys of cystinotic patients (upper panel) and LMW proteinuria (lower panel). A-D. Immunoperoxidase detection of megalin (A, B) and cubilin (C, D) on serial kidney paraffine sections. The control biopsy (CTRL, 3 years-old patient with oxalosis) shows homogenous apical labelling of the tandem apical endocytic receptors in all PTC. This markedly contrasts with atrophy and heterogeneous loss of their expression in a surgical sample from a 8 years-old cystinotic girl (CTNS^{-/-}). These lesions are representative of 4 cystinotic patients, all showing a major heterogenous loss of megalin and cubilin PTC expression. Bars: 200 μ m. E. Urinalysis by western blotting. Loads were normalized to 0.5 μ g of creatinine and blots were processed strictly in parallel. Increased urine excretion of transferrin (TR) and retinol binding protein (RBP) is obvious when compared to age-matched controls. A Fanconi syndrome was diagnosed in all cystinotic patients except patient #4, where RBP is the lowest. Bars: 200 μ m

Whereas these urinary results are in perfect agreement with the report of Wilmer et al. (2008) our immunoperoxidase analysis contradicts their conclusion based on a single case, and shows instead that extensive loss of megalin and cubilin in human cystinotic kidneys provides a straightforward explanation for their LMW proteinuria.

These new observations are also in agreement with our *in vivo* data in C57BL/6 *Ctns*^{-/-} mice (see our two previous reports), thus further validate this experimental model, and altogether strongly support a role of defective ARME in the physiopathology of cystinosis in human patients.

Objective #2. Relation between defective ARME and apical PTC dedifferentiation: completed.

The second objective was to test whether an early ARME defect, initiated in the most proximal segment of *Ctns*^{-/-} PTC, would act as an aggravating event, responsible for disease extension by inducing secondary, more distal PTC atrophy or would rather occur concomitantly with (and be part of) a global apical dedifferentiation process.

To test whether defective uptake directly correlated with apical PTC dedifferentiation, texas-red ovalbumin, used as endocytic tracer, was injected to 9 months-old mice at 20 min before whole-body fixation. Immunolocalisation of megalin and cubilin was performed on

kidney sections to monitor PTC differentiation. In wild-type mice, uptake of Texas-red ovalbumin was restricted to proximal tubular cells expressing megalin and cubilin, with the highest load in the first segment of the tubule (Fig 2 A), and a modest complementary modest uptake of Texas-red ovalbumin by the straight (distal) PTC, which also express megalin and cubilin (the latter at a lower level than in the convoluted segments) (Fig 2 C). In contrast, in *Ctns*^{-/-} mice, defective uptake of Texas-red ovalbumin was observed selectively in cortical foci of PTC that no longer expressed megalin and cubilin (Fig 2 B), reminiscent of the mosaic pattern of megalin or cubilin KO mice. Of particular interest, these foci included cells that had apparently lost cubilin expression but still expressed megalin; the converse (preservation of cubilin despite loss of megalin) was never observed. Again, this observation is consistent with the view that cubilin expression is more vulnerable to dedifferentiation; in other words, urinary loss of a specific cubilin ligand such as transferrin could be considered a more sensitive (earlier) sign of cystinosis progression in the kidneys. As in our previous report based on ¹²⁵I-beta2-microglobulin injection followed by autoradiography, we noted some “compensatory” increased uptake of Texas-red ovalbumin by PTC of the outer stripe of the outer medulla in *Ctns*^{-/-} mice, when compared to wild-type mice, supporting our proposal of displacement of protein supply (thus cystine source) towards more distal PTC segments, preceding their subsequent dedifferentiation/atrophy.

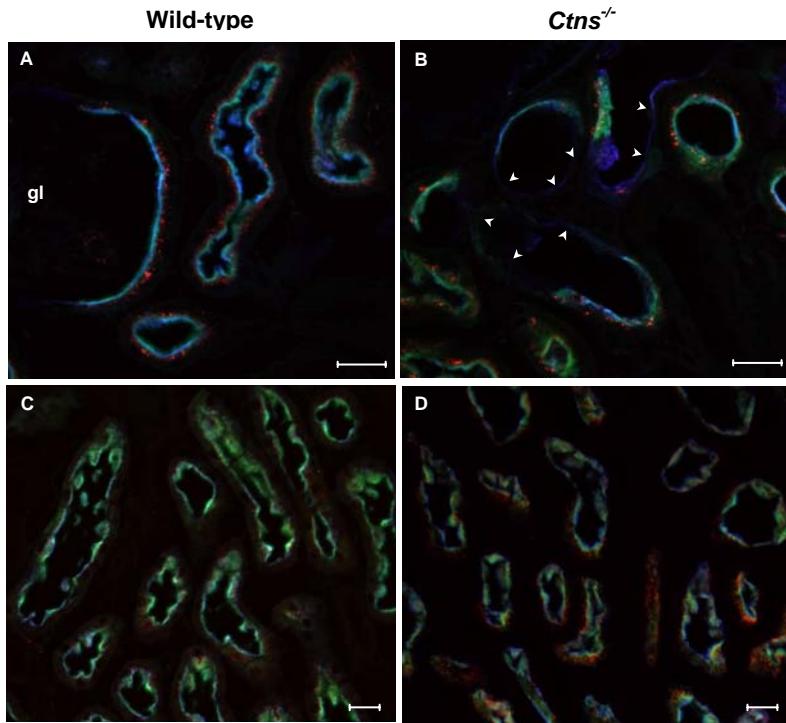


Fig 2. Functional *in situ* study of ARME. Focal cortical loss of megalin and cubilin expression is associated with defective ARME in *Ctns*^{-/-} mice kidneys. 300ng Texas red-ovalbumin was injected into 9 months-old wild-type (A, C) and *Ctns*^{-/-} mice littermates (B, D). Twenty minutes after injection, mice were flushed with PBS perfusion-fixed with 4% formaldehyde and kidneys were processed into paraffine sections. The uptake of Texas red-ovalbumin was visualized by confocal microscopy and correlated to megalin (blue) and cubilin (green) expression, as detected by duplex immunofluorescence. WT mice show a strong homogenous tracer uptake by all PTC in convoluted proximal (cortical) segments (A), including those extending at the urinary pole of Bowman's capsule and in some non-specified glomerular cells (gl : podocytes ?); lesser uptake is also detected in distal PTC of the straight segment located in the outer stripe of the outer medulla (C). In *Ctns*^{-/-} mice, defective uptake of Texas red-ovalbumin is observed in foci of early cortical atrophic PTC that selectively lost cubilin expression (blue signal of megalin only) or stopped expressing both receptors (loss of blue signal) (B, arrowheads). In contrast, somewhat higher (compensatory?) uptake is noted in distal PTC of the straight segment located in the outer stripe of the outer medulla in *Ctns*^{-/-} mice, where expression of both receptors is still preserved (D, compare with C)

The overall apical functional differentiation of PTC was further characterized by triplex immunofluorescence. Wild-type and *Ctns*^{-/-} mice were compared at 6-months (first lesions) as well as 9- and 12-months (progression toward kidney insufficiency). In this report, we selected 6-months kidneys to focus on the early stage of the dedifferentiation process. We previously described decreased focal expression of the tandem apical endocytic receptors, megalin and cubilin, in 6 months *Ctns*^{-/-} mice kidneys (see second report as April 2011 and Fig 3, please compare panels A, C with B, D). More careful studies revealed that the loss of megalin and cubilin was not necessarily synchronous, with some cells having lost the cubilin signal still expressed megalin, while the reverse was never found, as shown by Fig 3E, this was reflected in the urine of *Ctns*^{-/-} mice by the earlier increased excretion of transferrin, a cubilin ligand (at 6 months), as compared to albumin, which is ligand of both megalin and cubilin (peak at 9 months).

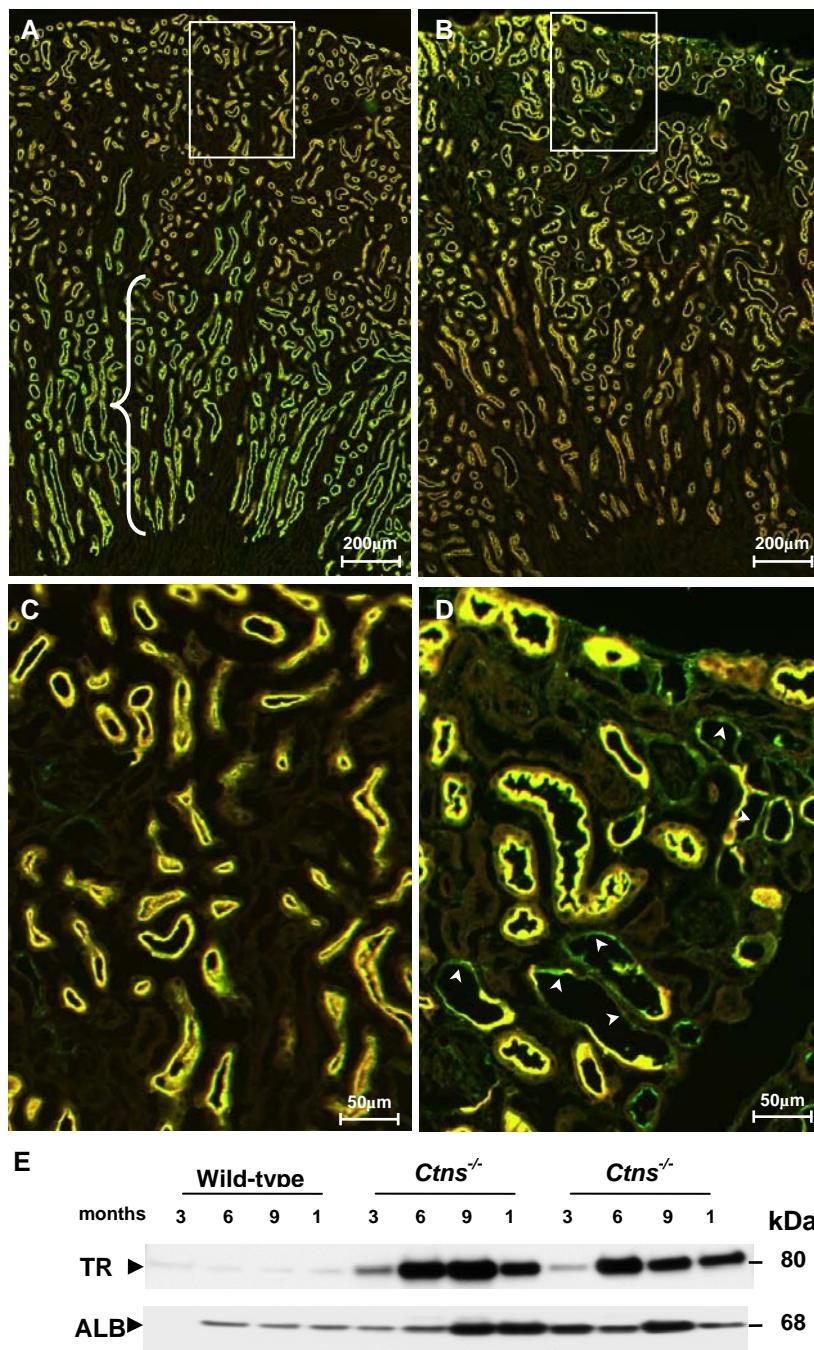


Fig 3. Decreased expression of megalin and cubilin in atrophic PTC of *Ctns*^{-/-} mice is asynchronous. A-D. Double immunofluorescence of megalin (green) and cubilin (red) in 9 months-old wild type (A, C) and *Ctns*^{-/-} mice kidney (B, D). In the cortex of WT mice (A,C), combined homogenous expression of megalin and cubilin generates a yellow-to-orange signal. Notice in passing the decreased labelling for cubilin in straight segments of proximal tubules (OSOM) that still well-express megalin, which results in a clearcut green signal (bracket). *CTNS*^{-/-} mice are shown at right. In the boxed area at B enlarged at D, the key information is the focal cortical disappearance of cubilin contrasting with some preservation of megalin expression (pure green signal) (arrowheads). E. time course of LMW proteinuria. Strong urinary excretion of transferrin (TR), a selective cubilin ligand, is observed as early as 6 months in *Ctns*^{-/-} mice and precedes that of albumin, a mixed megalin and cubilin ligand (peak at 9 months).

To correlate loss of megalin and cubilin expression intensity with apical differentiation state, megalin and cubilin were immunolocalized together with ezrin, a structural marker for the brush border cytoskeleton. In wild-type mice, brush border was well developed at the apical surface of PTC, as shown by homogenous and intense signalling for ezrin (Fig 4 A). In contrast, in *Ctns*^{-/-} mice, altered expression/localization of ezrin was observed in proximal tubules segments showing decreased expression of megalin and cubilin, pointing to focal loss of apical microvilli differentiation (Fig 4 B). Apical functional alteration of PTC in *Ctns*^{-/-} mice was further characterized by looking at solute channels or transporters expression pattern. Phosphaturia being a major feature of kidney Fanconi syndromes, we first focused on the sodium/phosphate co-transporter-IIa (NaPi2a), for which excellent antibodies were kindly provided by Drs C. Wagner and J. Biber (Zürich). In our diet conditions, we confirmed that labelling for NaPi2a in wild-type mice is localized at the apical surface as well as in endocytic vesicle of PTC (Fig 4C). In *Ctns*^{-/-} mice, apical and vesicular labelling for NaPi2a was decreased in atrophic segments of proximal tubules even when still expressing megalin (Fig 4 D). We conclude that NaPi2a could also be a sensitive early biomarker of apical dedifferentiation, like cubilin, and that phosphaturia (like transferrinuria) should be a sensitive early clinical marker (and cheaper).

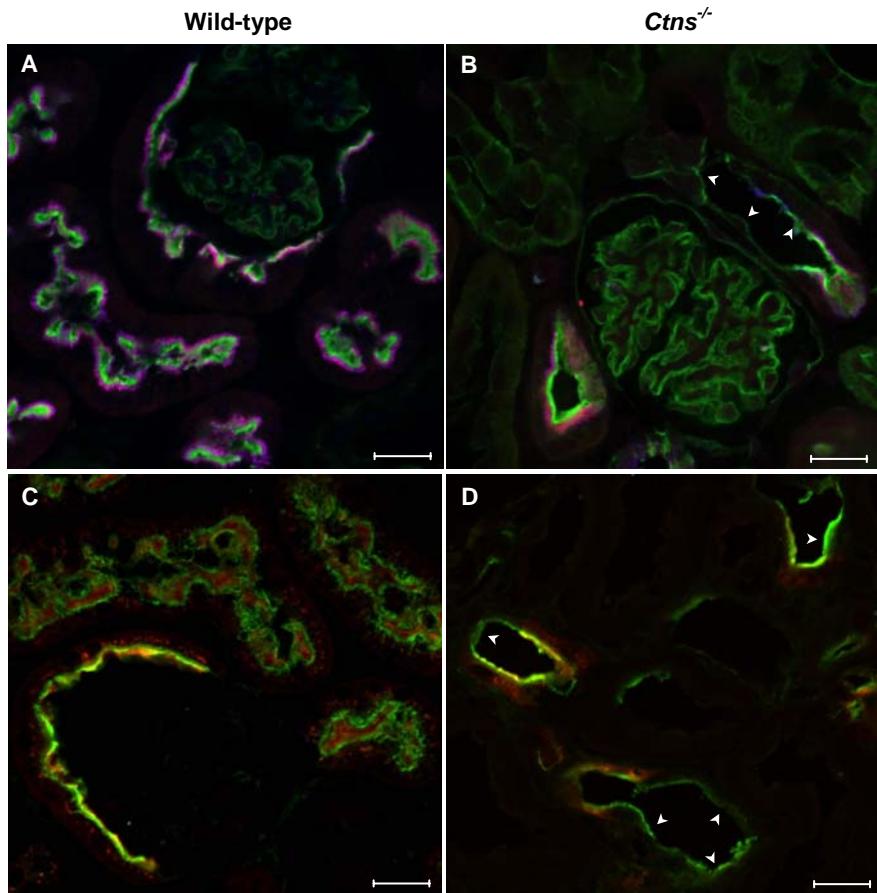


Fig 4. Altered apical functional differentiation of PTC in *Ctns*^{-/-} mice. A-B. Triple immunofluorescence of ezrin (green), cubilin (red) and megalin (blue). Decreased labelling and altered localisation of ezrin is observed in proximal tubule segments that no longer express megalin/cubilin (B, arrowheads). C-D. Double immunofluorescence of megalin (green) and NaPi2a (red). Apical and vesicular labelling of NaPi2a is decreased in atrophic proximal tubular segment that exhibit decreased labelling for megalin (D, arrowheads). Bars, 20 μ m.

Altogether, these results lead us to conclude on altered structural (ezrin) and functional (NaPi2a) apical differentiation of PT in cystinotic kidneys. The time-course of the dedifferentiation process indicates that NaPi2a and cubilin are more sensitive to atrophy compared to megalin. Furthermore, we noted in the same tubule segment asynchronous dedifferentiation between adjacent cells, consistent with a “cell-autonomous response” to cystine overload.

Objective #3. A global screen for local markers/actors of disease progression: in progress

Based on our experience in the focal control of human endometrium remodelling (Gaide Chevronnay et al, 2010), we are convinced that focal gene profiling after labelling-targeted laser capture microdissection of preserved *vs* atrophic tubules could shed invaluable light on the mechanisms by which lysosomal cystine accumulation causes tissue injury. To allow targetted microdissection, Texas red- ovalbumin (for validation, see above, Objective #2) was injected in 2 pairs of 9 months-old wild-type and *Ctns*^{-/-} mice. Twenty minutes after injection, mice were flushed with fresh cooled PBS and kidneys were snap frozen in liquid nitrogen-cooled isopentane. Laser capture microdissection of preserved (Texas red-positive PTC profiles) and atrophic (Texas red-negative profiles) was then performed on 5- μ m thick frozen sections. Typically 15 slides were prepared for each sample and 40 to 65 microdissected foci were pooled separately. In wild-type mice, Texas red-ovalbumin-labelled proximal tubules were microdissected (Fig 5 A-C) whereas in *Ctns*^{-/-} mice proximal tubules were microdissected both in foci with and without Texas red-ovalbumin uptake (Fig 5 D-E).

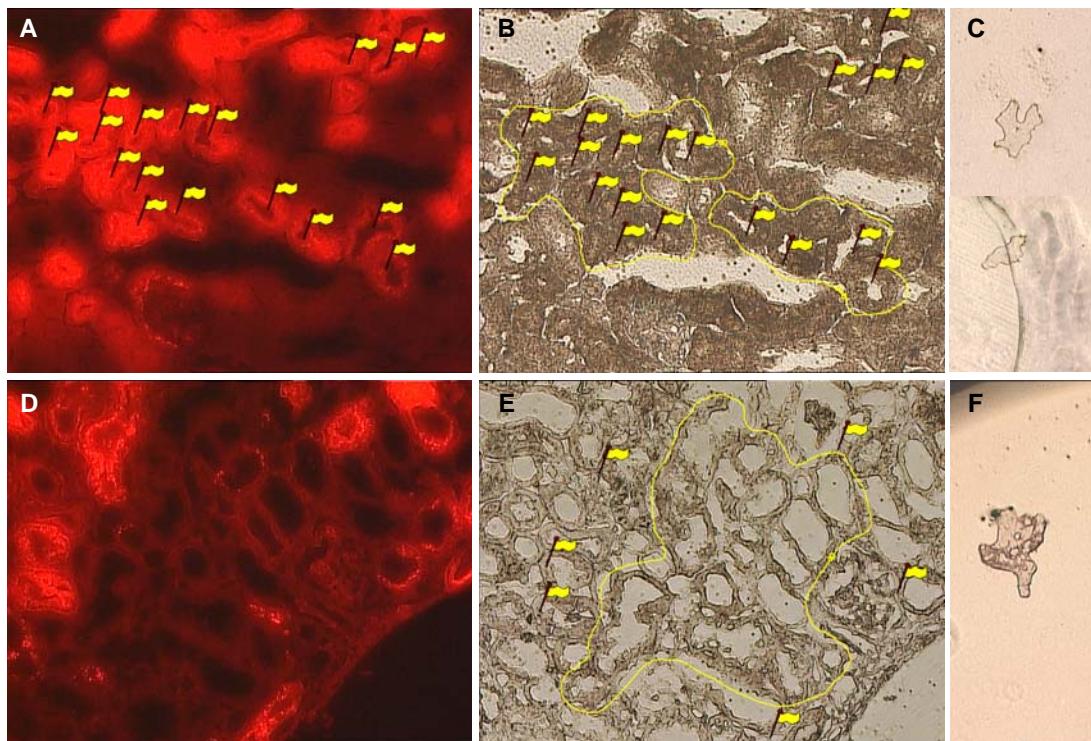


Fig 5. Endocytosis-targeted laser capture microdissection of proximal tubules in wild-type (A-C) and *Ctns*^{-/-} mice (D-F). Three hundred μ g of Texas red-ovalbumin were injected to 9 months-old mice for vital labelling of endocytosis-competent PTC. After 20 minutes, mice were flushed with ice-cold phosphate buffered saline and exsanguinated kidneys were immediately frozen in embedding medium using liquid nitrogen-cooled isopentane. This procedure is the preferred one for laser capture microdissection aiming at mRNA extraction, but kidney morphology is not optimal since the formaldehyde-fixation step is purposely omitted. Five μ m-thick cryosections were fixed in 75% ethanol for 15 sec, embedding medium was removed in water, then sections were further dehydrated in graded ethanol (75%, 95% and 100%, for 15 sec each) and finally in xylene for 3 min. Yellow flags are examples of PCT intensely labelled by Texas red-ovalbumin. Fields of interest were defined in the laser microdissection equipment thanks to fluorescence settings (A,D), excised as shown by yellow lines at B,E, collected as shown at C,F and propelled into RNase-free Eppendorf tubes. This figure documents the selectivity of laser capture microdissection of Texas-red ovalbumin labelled proximal tubules in wild-type mice (A-C) vs proximal tubules with defective uptake in *Ctns*^{-/-} mice (D-F).

C. Next Plans

We feel we have now collected sufficient data supporting the implication of ARME in the progression of cystinosis lesions along the proximal tubule and its relation with apical functional dedifferentiation. These observations can thus be assembled **for a first publication**, starting in the coming weeks.

As to the experimental work on untreated (non-grafted) *CTNS^{-/-}* mice kidneys, we will give priority in the next 6 months period to gene expression profiling analysis, exactly as originally planned in the first proposal. We already extracted RNA from the microdissected foci for the 2 pairs of mice. Microdissection in a third pair will complete the material collection to allow us to start **transcriptome analysis**.

Although the study of **thyroid defects** is developed in a second project (centred on cystinosis correction by stem cell grafting in the mouse model), we think it is fair to briefly update the CRF on this new line of investigations. It is well-recognized that lysosomal cystine accumulation in cystinotic patients induces progressive thyroid functional defect, reflected by hypothyroidism which justifies substitution by thyroid hormones. Since apical-receptor mediated endocytosis of the disulfide-rich thyroglobulin is necessary for thyroid hormone processing prior to secretion, we predicted that (i) like in kidney PTC, **ARME would explain cystine storage in thyrocytes**; and (ii) hypothyroidism would trigger a high TSH response, resulting into colloid exhaustion and **sustained thyrocyte proliferation, which could eventually lead to malignant transformation**, as is well-known for sporadic activating mutations of the TSH receptor leading to autonomous adenomas, luminal papillary expansions and cancer. Spectacular colloid exhaustion and replacement by proliferative thyrocytes forming papillae is exactly what our preliminary morphological analyses have disclosed already in 9-months old *Ctns^{-/-}* mice thyroids. We are particularly grateful to Dr. Bill Gahl for having shipped us this week an extremely valuable thyroid sample from a deceased non-compliant cystinotic patient, to test whether similar changes could occur in cystinotic patients. If this would be true, a closer follow-up of the thyroid gland itself could be recommended in the regular check-up of the patients.