Cystinosin is a Melanosomal Protein That Regulates Melanin Synthesis

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Running title: *CTNS* controls melanin synthesis

Abbreviation list:

4-AHP	4-amino-3-hydroxyphenylalanine
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumine
DAMP	*N*-(3-((2,4-dinitrophenyl)amino)propyl)-*N*-(3-aminopropyl)methylamine,
DCT	Dopa chrome tautomerase
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced Chemiluminescence
GFP	Green fluorescent protein
GSH	Glutathione
HPLC-EC	High pressure liquid chromatography/electrochemical detection
IC	Infantile Cystinosis
ITA	Individual typological angle
OCA	Oculocutaneous albinism
PBS	Phosphate buffered saline
PTCA	Pyrrole-2,3,5-tricarboxylic acid
RT-PCR	Real-time PCR
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tyrp1	Tyrosinase-related protein 1

Abstract

Cystinosis is a rare autosomal recessive disease characterised by cystine crystal accumulation leading to multi-organ dysfunctions and caused by mutation in *CTNS*. *CTNS* encodes cystinosin, a cystine/H+ symporter that exports cystine out of the lysosomes. Patients with cystinosis frequently exhibit blond hair and fair complexion, suggesting an alteration in melanogenesis. However, the pigmentation singularities of these patients had not been studied and the role of cystinosin in melanogenesis remained unknown.

In our study, a clinical evaluation of 27 cystinosis patients showed that 44% had a cutaneous pigmentation dilution compared to their relatives. Analysis of the hair melanin content in these patients by HPLC demonstrated a 50% decrease in eumelanin (4360 vs 9360 ng/mg), and a 2 fold increase in pheomelanin (53 vs 20 ng/mg), the yellow/red pigments. Cystinosis deficient mice also showed a 4 fold increase in hair pheomelanin content. In vitro studies showed that cystinosin was located at melanosomes. *CTNS* silencing led to a 75% reduction of melanin synthesis that was caused by a degradation of tyrosinase by lysosomal proteases. Our results objectify the pigmentation defect in patient with cystinosis. We also identify the role of *CTNS* in melanogenesis and add a new gene to the list of the genes involved in the control of skin and hair pigmentation.

Key words: Pigmentation, Melanocyte, Melanogenesis, Tyrosinase, Cystinosis

Introduction

Cystinosis, a rare autosomal recessive disease, is a lysosomal transport disorder characterised by the intra-lysosomal accumulation of cystine, the disulphide-linked dimer of the amino acid cysteine. Cystinosis leads to a progressive dysfunction in multiple organs, but renal failure remains the most sever and characteristic symptoms of the disorder (1, 2).

The causative gene, *CTNS*, was first mapped to the short arm of chromosome 17 (17p13) in 1998 (3). *CTNS* is composed of 12 exons that encode a 367-amino acid protein called cystinosin. Cystinosin is predicted to contain seven transmembrane domains, a 128-amino acid N-terminal region bearing seven *N*-glycosylation sites, and a 10-amino acid cytosolic C-terminal tail (3). Cystinosin is localised to the lysosomes (4, 5) and acts as a cystine/H^+-symporter (4, 5) to export cystine out of the lysosomes.

CTNS mutations have been detected in all forms of the disease, confirming the mutations' allelic status. The mutations associated with cystinosis are deletions, insertions, splice site mutations and nonsense mutations that cause premature termination of cystinosin (6, 7). The most common mutation associated with cystinosis is a 57-kilobase (kb) deletion that removes the 5' region of the *CTNS* gene (up to and including exon 10). This mutation is found in 76% of cystinotic patients of European origin (8).

Cystinosis has been classified as a lysosomal storage disorder on the basis of cytologic evidence that points to the intra-lysosomal accumulation of cystine (9). However, the molecular mechanisms involved in the evolution of the cystinotic phenotype are not fully understood. It has been proposed that cystine accumulation in the lysosomes impairs lysosomal and cellular functions, including ATP and GSH metabolisms (10). Two reports showed that cystinotic fibroblasts and renal proximal tubule epithelial cells are more sensitive to apoptosis due to activation of the pro-apoptotic protein kinase C delta by cystine released from the lysosomes (11, 12).

Interestingly, patients with cystinosis exhibit hypopigmentation; Caucasian subjects have often blond hair, blue eyes and light complexion (13). However, it seems that some patients, in particular, African American patients, have no hypopigmentation. A recent study (14) of 208 patients with cystinosis reported hypopigmentation of the retinal pigment epithelium in the periphery, with pigmentary stippling in 43% of cases. Because the pigments found in the retinal epithelium, hair and skin are melanins, cutaneous and retinal hypopigmentation may be caused by the same mechanism.

In humans, pigmentation results from the synthesis and distribution of melanin in the skin, hair bulbs, and eyes. Melanin synthesis, or melanogenesis, is an enzymatic process that is catalysed by tyrosinase, tyrosinase-related protein 1 (Tyrp1) and dopachrome tautomerase (DCT), which convert tyrosine to melanin pigments. This process takes place in melanocytes within the lysosome-related vesicles named melanosomes (15).

Two types of pigments are produced by melanocytes: eumelanins, which are black/brown pigments, and pheomelanins, which are yellow/red pigments. Eumelanins play a key photoprotective role against UV-induced DNA damage and skin cancer. Conversely, phaeomelanins, which are red/yellow sulphur-containing pigments, appear to be deleterious and may favour skin cancer development.

Although it has been shown that the levels of tyrosinase, Tyrp1 and DCT play key roles in pigment production, the content of cystine and its reduced form, cysteine, may also be a very important regulator of melanogenesis (16). Pheomelanins contain sulphur that may be provided by the cystine that is available in the melanosomes. Chintala et al. (17) have recently shown that the gene *SCL7a11*, which encodes a cystine-glutamate exchanger, is directly responsible for cystine transport into melanocytes and possibly into melanosomes. The subtle grey (*sut*) mouse, which produces less pheomelanin than a normal mouse, has a mutation in the *Slc7a11* gene, demonstrating the major regulatory roles of Slc7a11 and cystine in pheomelanin pigment synthesis.

In this study, we performed an objective evaluation of skin and hair pigmentation in patients suffering from cystinosis. Additionally, we studied the role of cystinosin in melanogenesis in vitro. Our results clearly show that cystinosis patients have a cutaneous pigmentation defect in comparison with their relatives. Analysis of the melanin content and the composition of the patients' hairs demonstrated a decrease in eumelanin and an increase in pheomelanin. In vitro analysis showed that *Ctns* silencing led to a loss of melanin synthesis that was caused by the lysosomal degradation of tyrosinase. Our results depict the role of cystinosin in melanogenesis and add *CTNS* to the list of the genes that are involved in the control of skin and hair pigmentation.

Materials and Methods

Patients

Twenty-seven patients (15 females and 12 males, middle age 13.75 years, from 2 to 34 years) suffering from cystinosis and their relatives (parents and/or siblings) were recruited for this study. Nephropathic cystinosis was diagnosed based on a typical clinical presentation and a leukocyte cystine concentration > 3 nmol half-cystine per milligram of protein. All patients but 2 had received systemic cysteamine (Cystagon, Orphan Europe, Paris, France) and cysteamine eye drops since diagnosis. Patients were examined at academic hospitals in Nice (patients 1-3), Paris (patients 4-9), Lille, Lyon, Caen and Bordeaux, France, by two examiners (C.C. and L.S.). The dermatological evaluation included a full body examination and evaluation of the phototype of the patient (18), parents, and when available, the siblings. *CTNS* mutations and their functional repercussions were available for 20 patients. Pictures were taken after approval of patients. Clinical features are summarized in supplementary table 1.

This study was approved by the Nice Hospital Research Ethics Committee and informed consent was provided by the legal representatives of all subjects.

Skin pigmentation evaluation

A clinical evaluation of skin pigmentation was conducted. Patients were considered to have hypopigmentation if their phototype was lower than that of family members. Quantitative skin colour was measured with a portable colorimeter (Spectrophotometer CR-200, Minolta Co. Ltd., Osaka, Japan) in photo-exposed (forehead) and non photo-exposed (inner arm) areas. Three consecutive readings were taken at each site, and their mean values were used in the statistical analysis. The results are displayed as colorimetric values in the L* (luminance or lightness) and b*(brown/yellow component reflecting melanin content) colour system. The individual typology angle (ITA°) is calculated by the following formula: $ITA° = ArcTangent ((L-50)/b) \times 180/\Pi$, where $\Pi = 3.14159$ and which provides a quantitative means for classifying individual skin colour. For instance, ITA°>55° corresponds to a very light skin, 55°>ITA>41° corresponds to a light skin.

Hair analysis

A lock of hair was cut from each patient and their relatives for melanin content assay. Hair samples were cut to about 5 mm in length, with scissors, and their weights were accurately measured. For the eumelanin assay, about 1-2 mg of hair was subjected to alkaline hydrogen peroxide oxidation (19). PTCA, a specific eumelanin marker, was analysed by HPLC-UV

detection. For the pheomelanin assay, about 1-2 mg of hair was subjected to reductive hydrolysis with hydroiodic acid (20). A specific pheomelanin marker, 4-AHP, was analysed by HPLC-EC detection. Eumelanin and pheomelanin contents were calculated by multiplying PTCA and 4-AHP contents with factors of 80 and 9, respectively (19, 20).

Statistical analysis

Wilcoxon tests were used to compare patients' skin and hair pigmentation with their relatives. Significance level was set to 5%. SPSS 11.0 software (Chicago, IL) was used for the statistical analyses.

Cell culture, transfection and infection

Mouse melanoma B16 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), 7% fetal bovine serum and penicillin/streptomycin (100 IU; 50µg/ml). B16 cells were transfected with lipofectamine (Invitrogen) according to the manufacturer's protocol. In brief, 3 x 10^6 cells, in 100 cm^2 culture dishes, were transfected with 70 µl of lipofectamine and 15 µg of plasmid or 100 nM of annealed siRNAs in Opti-MEM. After 6 h, the medium was replaced with DMEM supplemented with 7% fetal bovine serum.

Plasmids and siRNA

The green fluorescent protein (GFP)-CTNS and GFP-N1 plasmids were described previously (21). Small interfering RNAs against *Ctns* were designed using the Invitrogen™ tools; GGAGCTTCCTGATGAAGTT-siCTNS1 and GCAGTCGGTATCACCACAT-siCTNS2 were used in this study.

Antibodies

Rabbit polyclonal antibodies directed against Tyrp1 (pep1), tyrosinase (pep7) and DCT (pep8) were gifts from Dr. Hearing (Bethesda, MD) and were used at a 1/750 dilution for western-blot and a 1/50 dilution for immunofluorescence. Monoclonal antibodies against Tyrp1, B8G3 (provided by Dr. Parsons, Brisbane, Australia) and MITF (C5, Abcam) were used at a 1/100 dilution for western-blot and a 1/10 dilution for immunofluorescence. The monoclonal antibody against pMEL 17 (HMB45, TebuBIO) was used at 1/50 for immuno-fluorescence. The monoclonal anti-ERK2 (D-2) antibody was from Santa Cruz Biotechnology.

Immunofluorescence study

B16 cells were cultured on glass slides in 24 mm wells at 15 x 10^3 cells per slide and treated, or not, for 36 hours by 20 µM Forskolin. For determination of melanosome pH, cells were washed with serum-free culture medium and incubated for 20 minutes in the presence of

30 μM DAMP. Cells were fixed in 3% PFA for 20 minutes at 25 °C. Slides were washed with PBS, incubated 10 minutes in NH_4Cl and permeabilized in PBS with 0.1% Triton for two minutes. Slides were then incubated with a FITC-labeled rabbit anti-DNP antibody (1/50 in PBS plus 1% BSA). Melanosomes labeling with primary mAb (B8G3 anti-TYRP-1, 1/10, HMB45 anti-Pmel-17, 1/20) was performed in PBS plus 1% BSA. After three washes with PBS, slides were incubated with Texas-red-labeled anti-mouse antibody (1/500). Cell labeling was observed using a confocal microscope (LSM510, Zeiss).

Electron microscopy

B16 cells cultured with or without Forskolin 20μM and/or H89 5μM for 36 hours were then fixed in 2% glutaraldehyde for 3 hours at 4°C, followed by a post fixation in 2% osmic acid, for 1 hour at 4°C. Cells were subjected to successive dehydrations in baths of alcohol 30 °, 50 °, 75 °, 95 ° for 15 minutes each then in 100 ° over night. A replacement is realized in epon/alcohol (50/50) during 3 hours, then in epon/alcohol (75-25), and finally in 100% epon for 18 hours. Sections of 700 Å were finally labeled with uranyl acetate and lead citrate.

Immunopurification of CTNS-containing vesicles

B16 cells transfected with GFP or GFP-CNTS encoding plasmids were grown on 10 cm dishes. After three washes with cold PBS, cells were scraped in a buffer containing 50 mM Tris, pH 7.4, 250 mM sucrose, and 3% nonfat dry milk. Cells were lysed by 3 freeze-thaw cycles (liquid nitrogen/ 37°C), passed 10 times through a 26G needle, and centrifuged for 10 min at 400 *g* to remove the nuclei. The supernatants were incubated for 1 h at 4°C with the polyclonal anti-GFP antibody that had been previously fixed onto magnetic protein A-Sepharose beads (Dynal Biotech, Oslo, Norway). After incubation, the beads were washed three times with cold PBS and then resuspended in buffer containing 10 mM Tris, pH 7.4, 1% Triton X-100, and protease inhibitors. Solubilised proteins were analysed by SDS-PAGE and western blot.

Quantitative real-time one-step RT-PCR and western blot

RNA was extracted using TRIzol reagent (Invitrogen). Real-time one-step RT-PCR with SYBR-Green PCR Master Mix (Applied Biosystems) was used to quantify mRNA expression levels. The oligo sequences of murine *Ctns* were GTTCAACCAACGTCGACATCA (forward) and TTGAGCGAAACGTGACTTCAA (reverse). The reported results are the averages of three separate experiments. For western blot analysis, the samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The membrane was saturated for 1 hour at 25°C in 10mM Tris HCl pH 7.4, 150mM NaCl, 1mM EDTA, 0,1% Tween 20, 3% BSA (Weight/Volume, W/V), 5% gelatin (W/V) (blocking buffer) and

incubated with primary antibodies in blocking buffer. After three 10-min washes in saline buffer containing 1% Triton X-100, the blots were incubated for 1 h at room temperature with the corresponding peroxydase-conjugated secondary antibody and then washed again as previously described. Antigen/antibody complexes were detected with the ECL kit (Amersham Biosciences).

Mouse models

Two *Ctns* knockout mouse models was kindly provided by Pr Corinne Antignac (22) One is on the C57BL/6 background and the second one on a mixed (C57BL/6 X 129sV background), the latter having an agouti coat. Cystinosin deficient mice (*Ctns*-/-) did not develop the early onset of kidney failure that is characteristic of infantile cystinosis (22). However, *Ctns* knockout in a C57BL/6 background lead to progressive chronic renal failure (23).

Results

Objective evaluation of skin and hair pigmentation in patients with nephropathic infantile cystinosis

Even though pigmentation dilution has been frequently described in patients with cystinosis, no systemic study was available so far. Therefore, we performed an objective evaluation of the skin and hair pigmentation in these patients. We studied 27 patients and their close relatives. The frequencies of blue eyes, in patients and their relatives were not significantly different (25.9% vs 29.5%) and there was no difference between patients with or without hypopigmentation. Neither nystagmus nor vision impairment was noted. However, clinical phototype evaluation showed that 44.4% of patients had skin and hair hypopigmentation compared to their relatives. The clinical pigmentation features are summarised in Supplemental Table 1.

The clinical phototype evaluation results were confirmed by spectrophotometer analysis of patients and their relatives. Patients had an ITA median value 10% higher than that of their parents for the non-exposed area, which means a decrease in pigmentation (Table 1). This effect was even more pronounced in the photo-exposed area, which had an ITA median value that was 20% higher in patients. Of the 12 patients with clinical depigmentation, 11 were analysed by spectrometry, and 8 showed a clearly increased ITA (more than 10%). In this population, no predictive factor for hypopigmentation, such as severity of renal involvement, eye colour or intraleukocyte level, was found. However, both clinical evaluation and

spectrometric analysis showed no skin depigmentation in the most pigmented patients with phototype IV (light brown skin).

The median value of ITA in siblings was about 10% lower than in patients (exposed and non-exposed zone); this difference was not statistically significant because too small of a population sample was analysed.

Hair from 21 patients and their relatives was available for determination of the melanin contents (Table 2). The median value of the eumelanin content of patients' hair was 4,360 ng/mg and was 9,360 ng/mg for their relatives. In comparison with their relatives, 17 of the 21 patients had lower hair eumelanin content. The median value of eumelanin decrease in the hair of patients was 54%.

Conversely, the median of hair pheomelanin content was 53 ng/mg for patients, whereas it was 20 ng/mg for their relatives. 16 of the 21 patients had higher hair pheomelanin values compared to all their relatives. The median value of pheomelanin increase in the hair of patients compared with their relatives was 150%.

In the patients' hair, both eumelanin decrease and pheomelanin increase were highly statistically significant. It should be noted that the decrease in the hair eumelanin content was reflected by a lighter hair colour.

We conclude that *CTNS* mutations cause an objective skin pigment dilution in patients with infantile cystinosis (IC), as well as an alteration of hair pigmentation that is characterised by decreased eumelanin and increased pheomelanin.

Quantification of the melanin contents in the hair of cystinosin deficient mice

Ctns-/- mice in a C57BL/6 (non agouti coat color) or C57BL/6 X 129sV (agouti coat color) background did not show any hair pigmentation dilution (Fig. 1A). Quantification of the mouse hair melanin content showed no significant difference in the eumelanin content of WT and *Ctns*-/- mice from both C57BL/6 and a C57BL/6 X 129sV backgrounds (Fig. 1B). However, as is the case with IC patients, we observed a marked increase in the hair pheomelanin content in of *Ctns*-/- mice from both genetic backgrounds.

In conclusion, *Ctns*-/- mice have no perceptible pigmentation dilution but have increased pheomelanin content in the hair.

The above observation both in humans and mice demonstrates that cystinosin loss of function clearly affects melanin synthesis. Therefore, the role of *Ctns* in melanogenesis remains to be elucidated.

Expression and subcellular localisation of cystinosin

Real time PCR analysis of cystinosin mRNA expression in the mouse kidney, liver, and lung and in B16 mouse melanoma cells showed a strong expression of *Ctns* in mouse melanoma cells that was half of that found in the mouse kidney. *CTNS* is also expressed in normal human melanocytes (Fig. 2A). It should be noted that we have not been able to detect cystinosin protein by western blot or immunofluorescence using the commercially available antibodies.

To study the localisation of cystinosin within B16 mouse melanoma cells**,** we transfected B16 melanoma cells with a CTNS-GFP construct. The co-localisation of CTNS-GFP (green) with melanosome markers (red) Tyrp 1 (B8G3) and pMEL17 (HMB45) was then evaluated by confocal microscopy. CTNS-GFP showed a vesicular pattern that co-localised with Tyrp1 that is present in melanosomes stage II to IV. CTNS-GFP co-localised to a lesser extent, with HMB45 labelling that recognises pMEL17 mainly in stage II melanomes (Fig. 2B, upper panels). Higher magnification analysis of Tyrp1 labelling showed discrete co-localisation of CTNS-GFP with Tyrp1 at the level of the cell body (Fig. 2B, middle panels). Finally, superimposition of bright field images (pseudo coloured in red) with CTNS-GFP labelling showed that CTNS engulfed dark melanosomes. When transfected with a GFP construct, cells were uniformly marked, and no co-localisation with melanosome markers was observed (not shown).

To confirm the localisation of cystinosin in melanosomes, detergent-free cell lysate from B16 melanoma cells transfected with CTNS-GFP was immunoprecipitated with an anti-GFP antibody (24). We detected, by western blot, CTNS-GFP and tyrosinase in the immune complex, which indicated the presence of CTNS-GFP in the melanosomal membrane fraction. No Rab4 was found in these immune complexes (Fig. 2C, right panel). No tyrosinase could be found after immunoprecipitation of the detergent-free cell lysate from B16 cells transfected with GFP alone (Figure 2C, left panel). These data indicate that CTNS is a melanosomal protein.

***Ctns* silencing inhibits melanin synthesis**

To further investigate the role of *Ctns* in melanogenesis, we designed specific siRNA directed against murine *Ctns* and then evaluated, by RT-PCR, its efficiency in B16 melanoma cells. In these cells, transfection of increasing doses of siCtns1 showed an 85% extinction of cystinosin messenger at 20µM of siCtns1. Scrambled siRNA (siSCR) had no effect on cystinosin expression (Fig. 3A). We used 20µM of siCtns1 in all the subsequent experiments.

Next, we evaluated the effect of *Ctns* silencing on melanin synthesis in B16 melanoma cells. B16 melanoma cells transfected with siSCR or siCtns1 were treated or not treated by forskolin (20µM) for 48 h. As shown by direct light microscopy (Fig. 3B), visualisation of the cells pellets (Figure 3C) and spectrophotometric measurement of the melanin content (Fig. 3D), *Ctns* silencing inhibited cell pigmentation and reduced melanin synthesis by more than 50% in comparison with control cells. Identical results were found with a second siCtns (Sup. Fig.1).

Ctns silencing inhibits tyrosinase expression, but does not affect melanosome structure.

We evaluated the effect of *Ctns* silencing on the expression of the enzymes of melanogenesis. Western blot analysis showed that *Ctns* silencing led to a striking inhibition of tyrosinase expression, whereas the levels of Tyrp1 and DCT were not significantly affected (Fig. 4A). Furthermore, analysis of *Ctns* and tyrosinase messenger levels (Fig. 4B) showed a two-fold increase in *Ctns* mRNA upon forskolin treatment, indicating that cystinosin is up-regulated during the differentiation program in melanocytes. In cells transfected with siCtns, we observed an inhibition of *Ctns* mRNA expression but no effect on tyrosinase mRNA expression. These data indicate that *Ctns* silencing does not affect tyrosinase gene expression, but suggest that cystinosin loss of function affects tyrosinase expression at a post-transcriptional level. Electron microscopy analysis of B16 melanoma transfected with siSCR and exposed to forskolin (Fig.4.C) showed the presence of stage II melanosomes (black arrow head), stage III (white arrow head) and IV (*) pigmented melanosomes. After transfection with siCtns1, no stage IV melanosomes can be observed; stage II and III melanosomes seems to have a normal structure but do not contain melanin. Ctns loss of function does not seems to affect drastically the structure of melanosomes.

***Ctns* silencing affects melanosome pH**

Cystinosin is a lysosomal cystine/H^+ co-transporter. In melanocytes, cystinosin inhibition is supposed to increase the melanosomal H^+ levels. Because melanosome pH controls melanin synthesis (25), we evaluated the effect of *Ctns* silencing on this parameter.

B16 cells transfected with control (siSCR) or *Ctns* siRNA (siCtns1) were treated with forskolin for 24h. We then used DAMP, a weak base that accumulates in acidic compartments, and an antibody against Tyrp1 to label melanosomes and to evaluate their pH. In the control condition, we observed a weak DAMP labelling (green). Most of the melanosomes, labeled in red, showed no green labelling (Fig. 5A), indicating that the

melanosomes in forskolin treated cells did not have an acidic pH. We observed that transfection with siCtns increased the DAMP labelling (green). In merged images, the melanosomes appeared yellow, indicating a decrease in the melanosome pH (Fig. 5B). We conclude that *Ctns* silencing affects melanosome pH by rendering it more acidic.

Ctns silencing increases tyrosinase degradation

Melanosome pH has been shown to regulate tyrosinase stability (26). Therefore, the loss of tyrosinase expression may be caused by melanosome acidification and consequent tyrosinase degradation. To test this hypothesis, we studied the effect of a proteasome inhibitor (MG132) or lysosome protease inhibitors (E64+leupeptin) on tyrosinase expression in siCtns-transfected cells. In our hands, MG132 did not block the degradation of tyrosinase in siCtns-transfected cells (not shown). A cocktail of E64 and leupeptin restored tyrosinase expression after *Ctns* silencing (Fig. 6A), but did not allow recovery of melanin synthesis, which was shown by cell pellet observation (Fig. 6B) and melanin quantification (Fig. 6C). Bafilomycin, a vacuolar ATPase inhibitor that prevents acidification of intracellular vesicles, restored both tyrosinase expression (Fig. 6A) and melanin synthesis (Fig. 6B, C). These results indicate that *Ctns* silencing causes an acidification of melanosome pH that favors tyrosinase degradation by lysosome proteases. Recovery of tyrosinase expression is not sufficient to restore melanin synthesis, probably because the acidic pH can impair tyrosinase function.

Discussion

For the first time, we have described an objective evaluation of skin, eyes and hair pigmentation in patients with nephropathic infantile cystinosis. We clearly demonstrated a statistically significant decrease in both skin and hair pigmentation of patients in comparison with their close relatives. Clinical and colorimetric analysis showed that more that 40% of patients showed an objective skin hypopigmentation. However, analysis of hair melanin content showed that 85% of the patients displayed lower eumelanin and higher pheomelanin contents in the hair. It should be noted that higher hair pheomelanin contents can be observed in patients without any other signs of pigmentation dilution. Patients with a phototype IV parent did not show any clinical skin or hair depigmentation, but they frequently had increased hair pheomelanin content. It is interesting to note that the same observations were made in regard to the *Ctns* knockout mice, of either C57bl6 or agouti background, in which there was no observable pigment dilution but there was a higher amount of pheomelanin in the hair.

The above data demonstrate a key involvement of *Ctns* in pheomelanin synthesis, probably through the control of the melanosome cysteine content, which may be the main source of the sulphur compounds required for pheomelanogenesis (16).

Taking into account that cystinosin transports cystine out of the lysosome and that melanosomes are lysosome-related vesicles, it is tempting to propose that cystinosin is involved in the active melanosomal efflux of cystine and, therefore, regulates melanogenesis. To gain further insight into the role of *Ctns* in melanogenesis, we studied the expression, localisation and function of *Ctns* in melanin synthesis in B16 melanoma cell.

First, we showed that cystinosin is expressed in B16 melanoma cells and in human melanocytes. Both morphological and molecular approaches demonstrated that cystinosin was a melanosomal protein. Interestingly, *Ctns* expression was increased during forskolin induced differentiation of B16 melanoma cells. These data suggest that *Ctns* belongs to the molecular program that is implemented during melanocyte differentiation.

Further, we showed that *Ctns* silencing inhibits the forskolin induced pigmentation and specifically decreases tyrosinase activity and expression in B16 melanoma cells. In cystinosin-depleted cells, the level of tyrosinase messenger was not affected in comparison with that of the control cells, which indicates a post-transcriptional or post-translational regulation of tyrosinase expression by cystinosin.

Indeed, tyrosinase is subjected to numerous post-translational modifications/regulations that control the activity, targeting and stability of the protein. Alterations in targeting and stability have already been demonstrated for two oculocutaneous albinisms, OCA2 (27) and OCA4 (28), that are caused by mutations in the genes encoding P protein and SLC45A2, respectively. These proteins are melanosomal transmembrane proteins with unknown function, but defects in such proteins lead to a mislocalisation of tyrosinase and an increase in its degradation. Similar processes can explain the loss of tyrosinase in cystinosin-depleted cells.

Furthermore, melanosome pH is a key parameter of melanogenesis (25, 26, 29). As a cystine/H^+ cotransporter activity has been ascribed to cystinosin (21), a cystinosin loss of function could affect melanosome H^+ efflux and therefore, pigment synthesis.

Evaluation of melanosome pH demonstrated that *Ctns* silencing increased the DAMP labelling of Tyrp1 containing melanosomes, which indicated an acidification of the melanosome environment. It was previously demonstrated that abnormal acidification disrupted normal tyrosinase trafficking and caused its retention in the early secretory pathway (29) and/or its degradation through the proteasome (26). In cystinosin-deficient cells, no

apparent alteration of tyrosinase glycosylation was observed, which suggests that the misrouting of tyrosinase may occur after the medial Golgi network. Further experiments with specific glycosidase should be performed to confirm this hypothesis. In contrast with the data from Watabe et al. (26), we did not observed stabilisation of tyrosinase by MG132, a proteasome inhibitor, while in the same conditions we observed an increase in Hif1α expression (data not shown). However, leupeptin and E64 almost completely restored tyrosinase expression in cystinosin-depleted cells, which suggest that lysosomal proteases mediate the degradation of tyrosinase. This observation is in agreement with a recent report (30) demonstrating that the inhibition of melanogenesis by inulavosin was due to the degradation of tyrosinase by lysosomal proteases.

Even though lysosome protease inhibitors prevented tyrosinase degradation in cystinosin-depleted cells, they were not able to allow recovery of melanin synthesis. However, bafilomycine A1, a vacuolar ATPase inhibitor that allows alkalinisation of the endosomal compartment, was able to restore both tyrosinase and pigmentation levels in cystinosin-depleted cells. In OCA2, it has also been shown that bafilomycine A1 restores normal targeting of tyrosinase and normal pigmentation (31).

Ctns silencing inhibited pigmentation in B16 melanoma cells, whereas *Ctns* KO in C57bl6 mice (from which B16 cells are derived) did not affect pigmentation. This apparent discrepancy may be ascribed to the difference between the levels of cystine in mouse blood (around 50 µM) (32) and in B16 cell culture medium (380 µM). The higher amount of cystine in culture medium may be more detrimental for melanogenesis in case of a cystinosin loss of function.

The absence of pigmentation dilution, in dark-skinned IC patients, remains puzzling. In humans, the genetic basis for the differences in the skin pigmentation traits has been extensively studied. Recently, genome-wide association studies have led to the identification of single nucleotide polymorphisms (SNPs) in pigmentation genes that could account for the variation from light to dark skin (33). Among the 11 gene alleles playing a key role in pigmentation traits, four (SLC45A2, OCA2, SLC24A5, and SLC24A4) encode proteins with transporter or exchanger activity, which suggests that ionic equilibrium and probably the pH in melanosomes are key determinants of human pigmentation diversity (34). Indeed, it was reported that melanocytes from Africans have less acidic melanosome than melanocytes from Caucasians (35).

In light of the above observations, the melanosome pH and the subsequent degradation of tyrosinase are the key parameters of the control of skin pigmentation by *CTNS*. Melanocytes

of dark-skinned patients probably express transporter gene alleles favouring a neutral or basic pH in melanosomes. In these melanocytes, the ionic exchange machinery might overcome the loss of *CTNS* to maintain the pH in melanosomes.

In summary (Fig. 7), in addition to its role in the control cystine content in melanosomes, *CTNS* plays a key role in the control of melanosome pH, which seems to be a pivotal parameter in proper tyrosinase targeting to the melanosomal compartment. In the absence of functional cystinosin, melanosomes are more acidic, but keep an apparent normal structure. In this condition, it can be proposed that tyrosinase is misrouted through the lysosomal compartment, where it is degraded by lysosomal proteases. V-ATPases were reported to influence vesicular trafficking by controlling and sensing luminal pH (36). It is unlikely that tyrosinase is degraded following the reactivation, by the acidic pH, of the lysosomal proteases located in melanosomes (37); if this were the case, we would have observed a degradation of Tyrp1 and DCT. However, we cannot rule out that Tyrp1 and DCT are more resistant to proteolytic degradation.

Beyond identifying a new player in melanosome functioning and melanin synthesis, our data also stimulate the evaluation of intracellular protein mistargeting and processing, in affected kidney, as a possible molecular cause of the cystinosis phenotype.

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Figure Legends

Figure 1. *Ctns* knock-out affects pheomelanogenesis. A) Wild type (*Ctns*+/+) and *Ctns*-deficient (*Ctns*-/-) mice, on a C57bl6 (left) or an agouti (right) background. B) Eumelanin (left) and pheomelanin (right) quantification in mouse hair. The histograms show the means ±SEM of three determinations.

Figure 2. *Ctns* is expressed in melanocytes and located in melanosomes. A) QPCR analysis of *Ctns* expression in mouse tissues, B16 mouse melanoma cells, A293 human embryonic kidney cells and normal human melanocytes. Expression was normalised to actin. B) B16 mouse melanoma cells were transfected with the GFP-CTNS expressing vector. Forty-eight hours later, the cells were fixed and permeabilised. Melanosomes were identified with antibodies against Tyrp1 (B8G3) or pMEL17 (HMB45) (red) and cystinosin by the green fluorescence (upper panels). The middle panels show a higher magnification of Tyrp1 labelling, CTNS-GFP fluorescence and a merged image. The lower panels show a bright field image of a pigmented cells, CTNS-GFP fluorescence and a merged image with the bright field image pseudo coloured in red. C) B16 mouse melanoma cells were transfected with GFP (left) or GFP-CTNS (right) expressing vectors. Forty-eight hours later, the cells were mechanically disrupted and, after nucleus removal, the membrane fraction was immunoprecipitated with a polyclonal anti-GFP antibody. The immune complexes were analysed by SDS-PAGE and western blot with anti-GFP, anti-Tyrosinase or anti-Rab4 (negative control) antibodies.

Figure 3. *Ctns* silencing inhibits melanin synthesis. A) B16 mouse melanoma cells were transfected with increasing amounts of either siRNA against *Ctns* (siCtns1) or scrambled siRNA (siSCR). Forty-eight hours later, RNA was extracted and *Ctns* expression was evaluated by QPCR. B) Phase contrast and direct light images of B16 cells after transfection with siSCR or siCtns1 (20µM). C) Pictures of the pellets of B16 cells that were transfected with either siSCR or siCtns (20µM), as described above, and exposed or not exposed to forskolin. D) Quantification of the melanin content in B16 cells that were treated as described in C.

Figure 4. *Ctns* silencing inhibits tyrosinase at the protein level. B16 mouse melanoma cells that were transfected with 20µM of either siRNA against *Ctns* (siCtns1) or scrambled siRNA

(siSCR) were exposed or not exposed to forskolin. Forty-eight hours later, A) proteins were extracted, then tyrosinase, Tyrp1 and DCT expression were evaluated by western blot with pep7, pep1 and pep8, respectively; ERK2 was used as a loading control. B) RNA was extracted and then *Ctns* and *tyrosinase* expression were evaluated by QPCR. C) Electron microscopy of B16 melanoma cells transfected with siSCR (upper panels) or with siCtns1 (lower panels) and exposed to forskolin. ▷ Melanosme stade II, ▶ melanosome stade III, * melanosome stade IV.

Figure 5. *Ctns* silencing induces melanosome acidification. Forskolin treated B16 mouse melanoma cells were transfected with 20µM of A) scrambled siRNA (siSCR) or B) siRNA against *Ctns* (siCtns1). Forty-eight hours later, the cells were incubated with DAMP and then fixed and permeabilised. DAMP was visualised with a FITC coupled anti-DNP antibody and melanosomes were visualized with an anti-Tyrp1 primary antibody (B8G3) and a Texas-red coupled anti-mouse secondary antibody. The nuclei were labeled with DAPI.

Figure 6. Bafilomycine prevents the inhibition of tyrosinase expression and the decrease inmelanin synthesis that are induced by *Ctns* silencing. B16 mouse melanoma cells that were transfected with 20µM of either siRNA against *Ctns* (siCtns1) or scrambled siRNA (siSCR) were exposed or not exposed to forskolin in the following conditions: control, in the presence of E64 (µM) plus leupeptin (µM), or in the presence of bafilomycin (µM). A) Proteins were extracted and tyrosinase expression was evaluated by western blot with pep7 antibody; ERK2 was used as a loading control. B) Pictures of the pellets of the B16 cell that were treated as described above. C) Quantification of the melanin content in the B16 cells that were treated as described in B.

Figure 7. A schematic representation of cystinosin functioning in melanocytes. The greater degree of co-localisation of *cystinosin* with Tyrp1 than with HMB45 (pMEL17, Silv) suggests that cystinosin is targeted to the mature melanosome. This hypothesis is consistent with the observation that the early melanosome is more acidic than the mature melanosome (38). cystinosin may favor the neutralised pH of the mature melanosome. The neutral pH may be a signal for tyrosinase targeting to the mature melanosome and may also allow proper tyrosinase functioning and melanin synthesis to occur. In the absence of functional cystinosin, mature melanosomes may remain acidic; this may lead to mistargeting of tyrosinase to the

lysosome compartment and subsequent tyrosinase degradation by lysosomal proteases. Of course cystinosin plays a key role in the control of cystine content in melanosome and could thereby influence the eumelanin/pheomelanin ratio.

Supplemental Figure 1. Two different siRNAs against *Ctns* inhibit tyrosinase expression and melanine synthesis. B16 mouse melanoma cells that were transfected with 20µM of either siRNA against *Ctns* (siCtns1 or siCtns2) or scrambled siRNA (siSCR) were exposed or not exposed to forskolin. A) RNA was extracted, and then *Ctns* expression was evaluated by QPCR. B) Quantification of the melanin content in the B16 cells that were treated as described above. C) Proteins were extracted, and then tyrosinase expression was evaluated by western blot with pep7; ERK2 was used as a loading control.

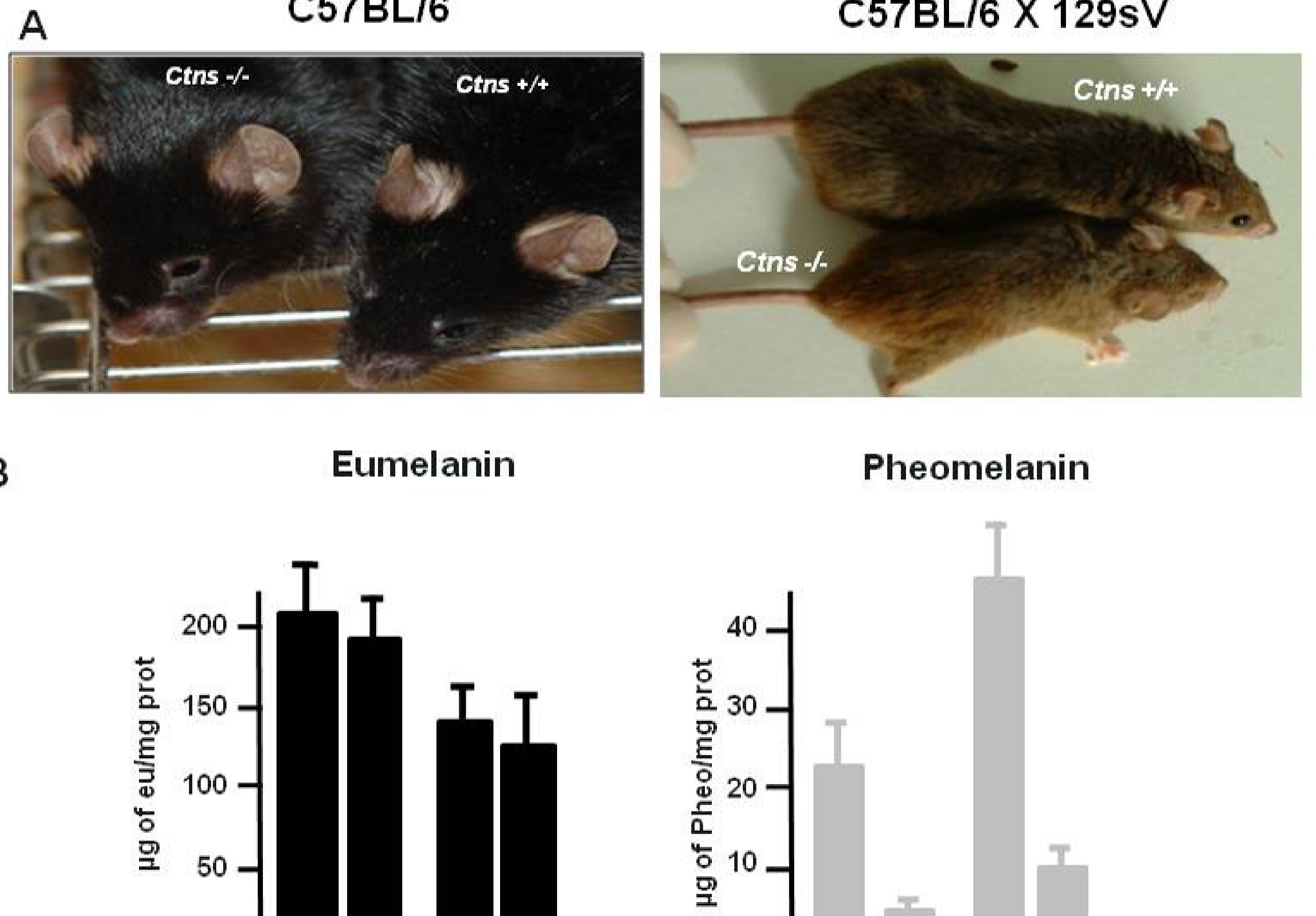


Fig.1

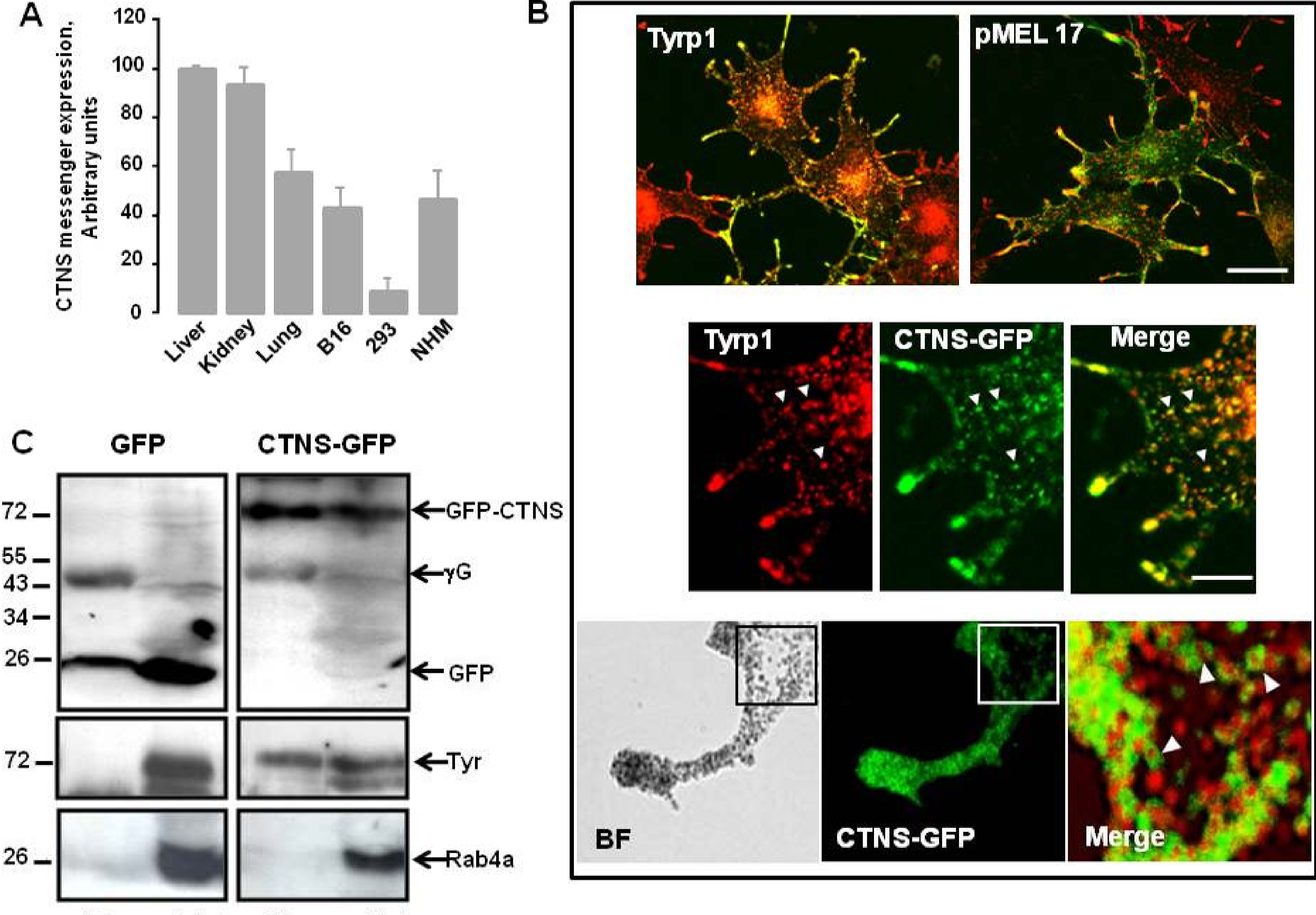
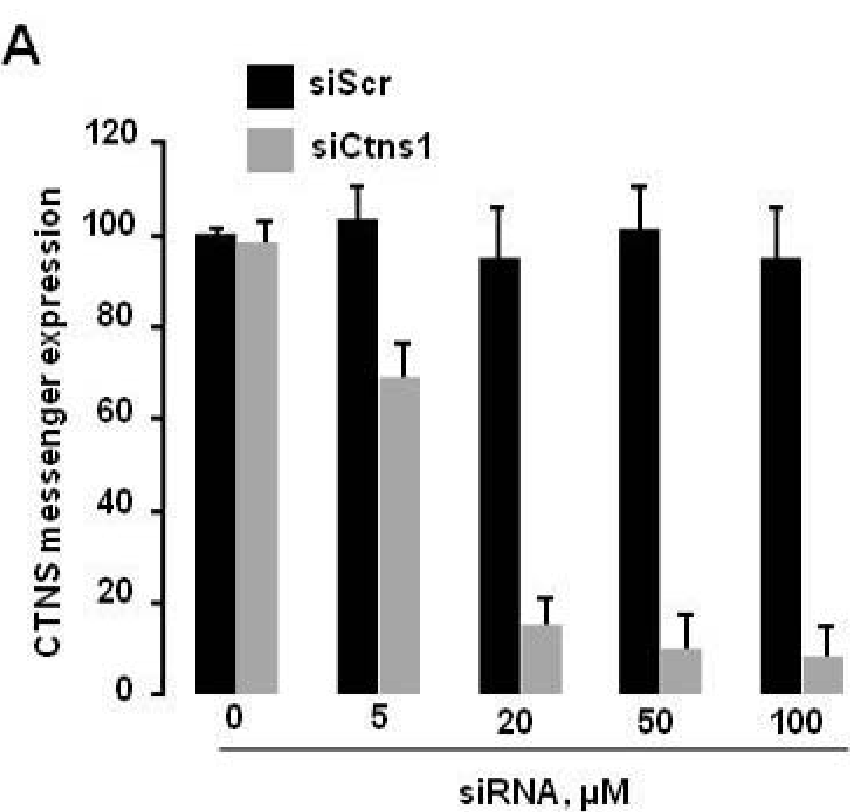
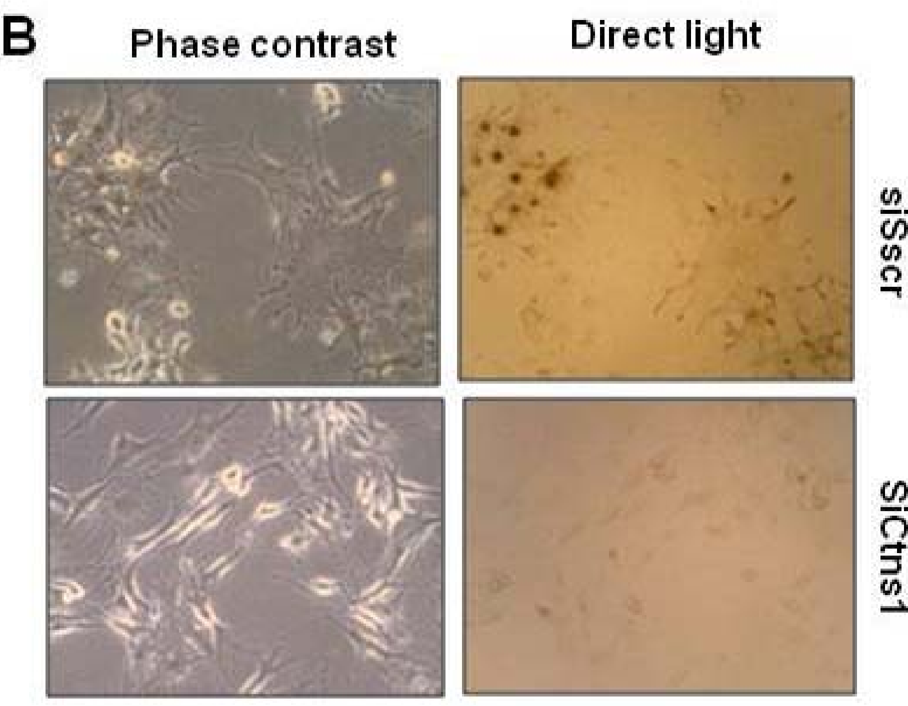
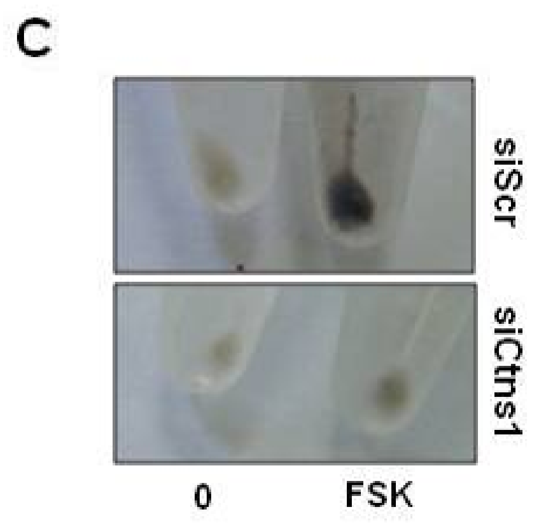


Fig.2







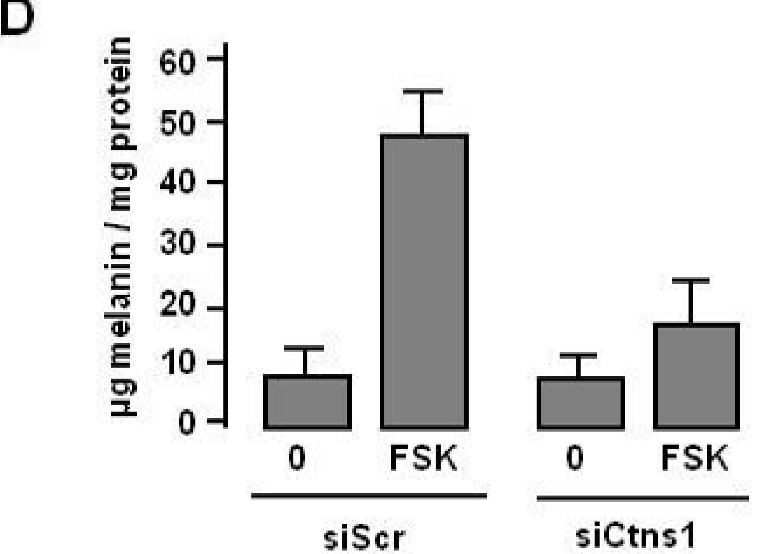


Fig.3

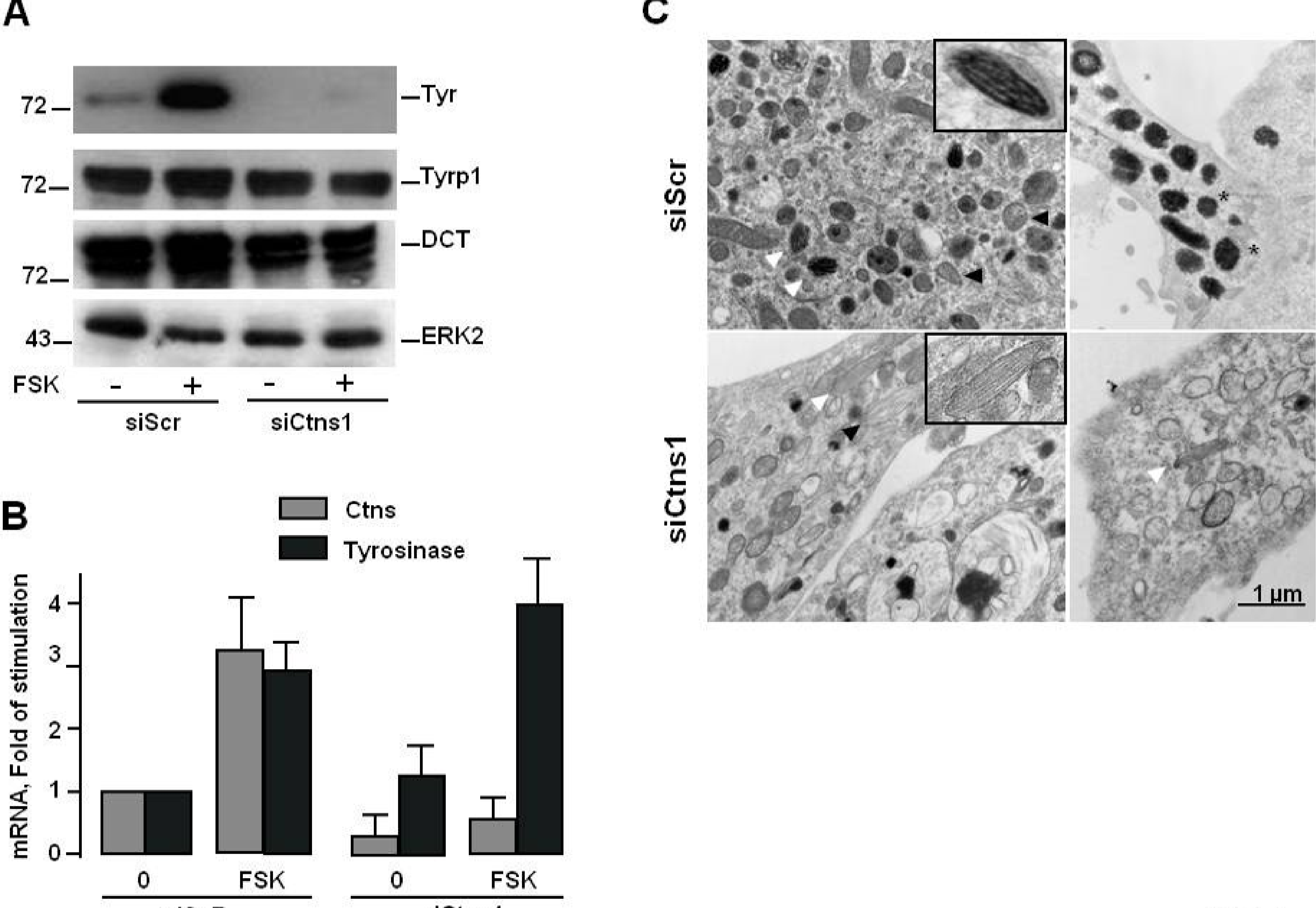
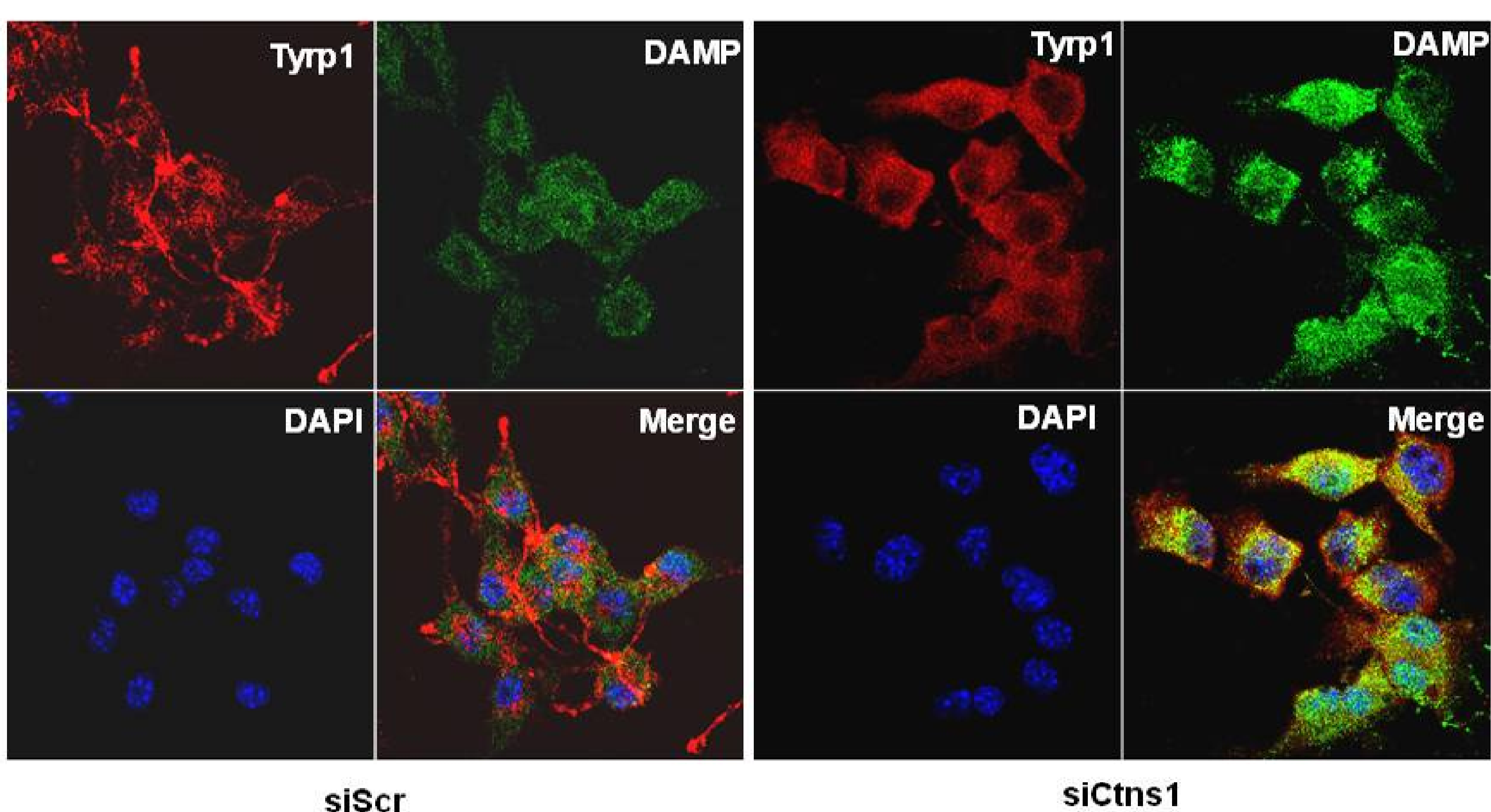


Fig.4



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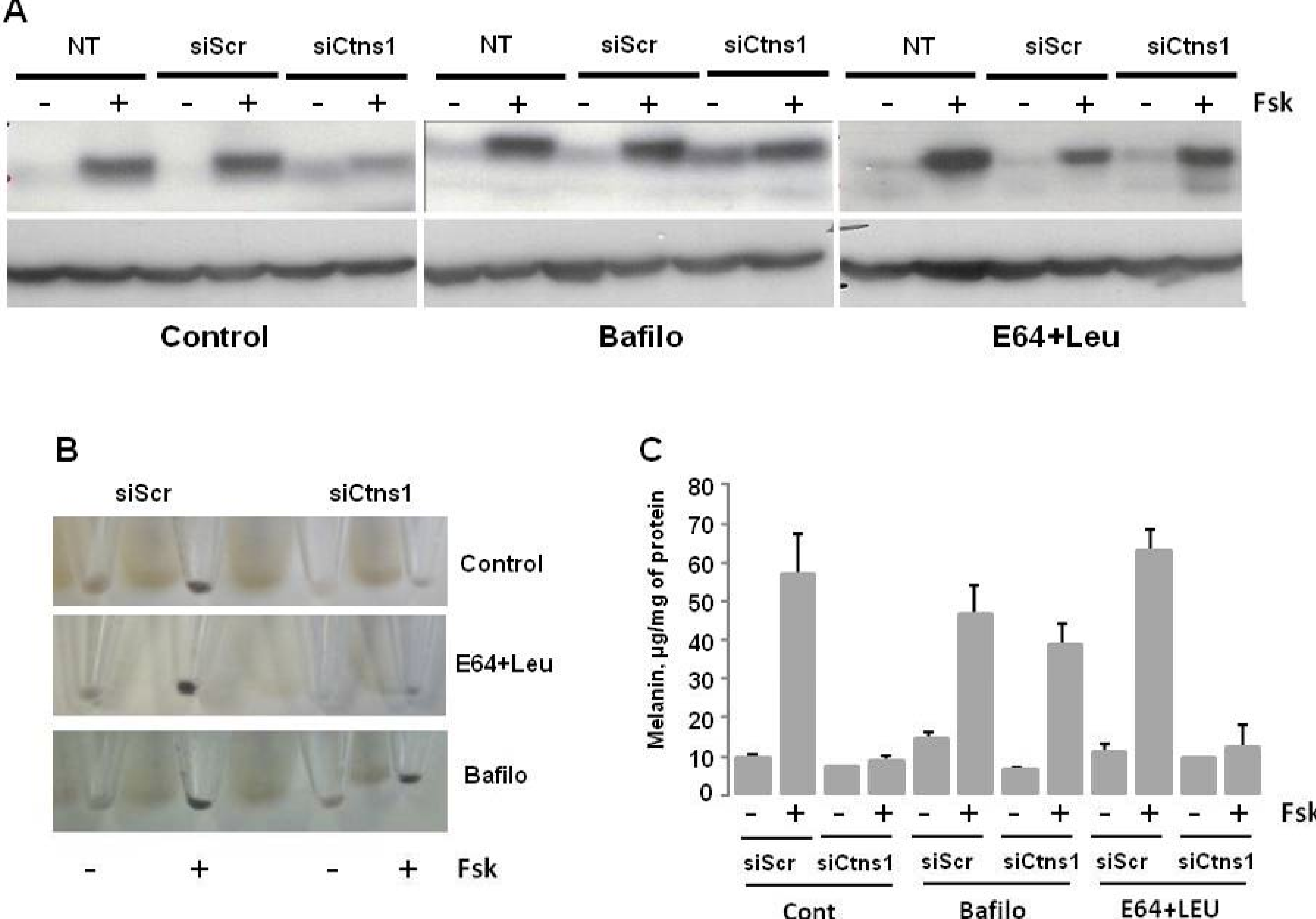
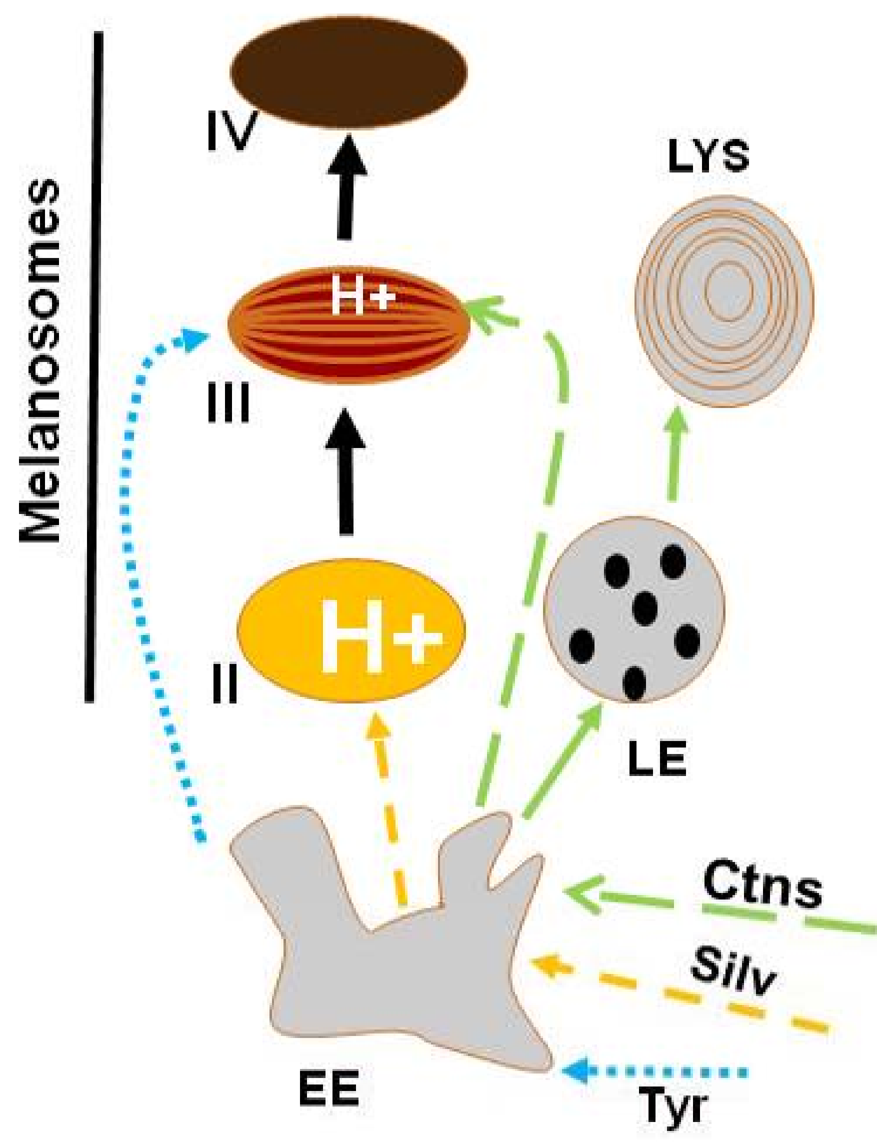


Fig.6



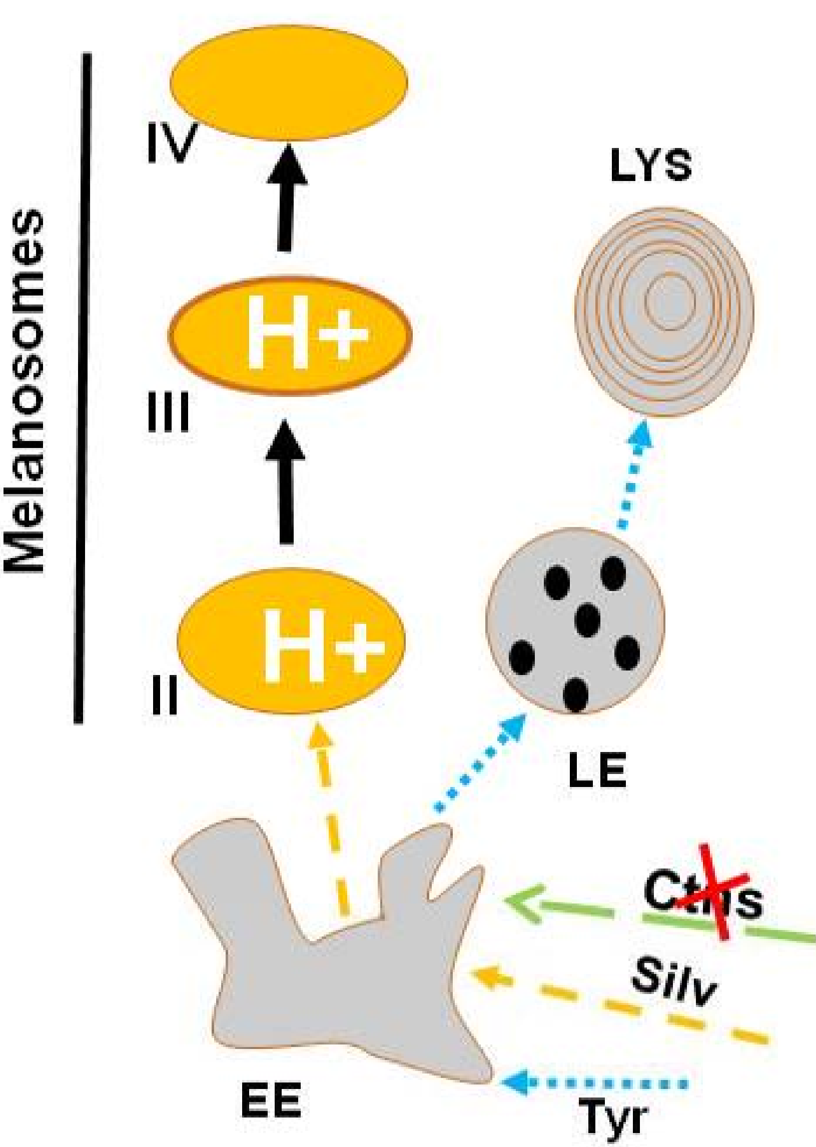
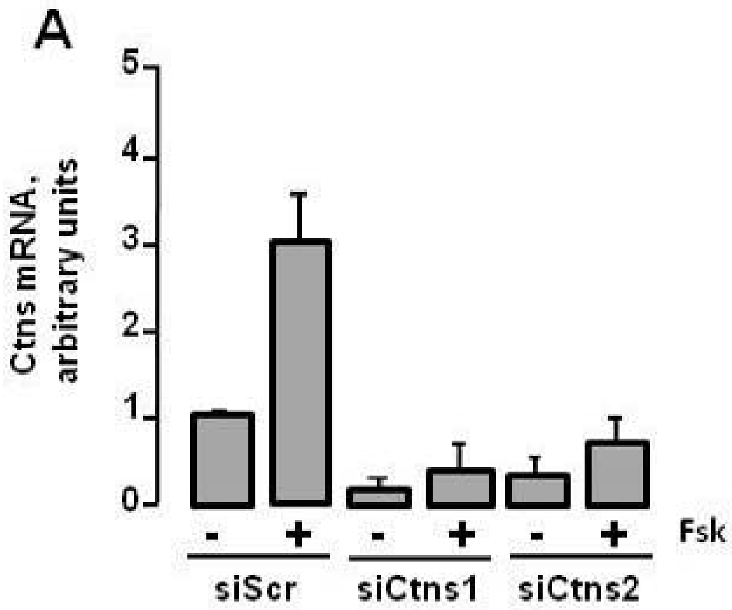
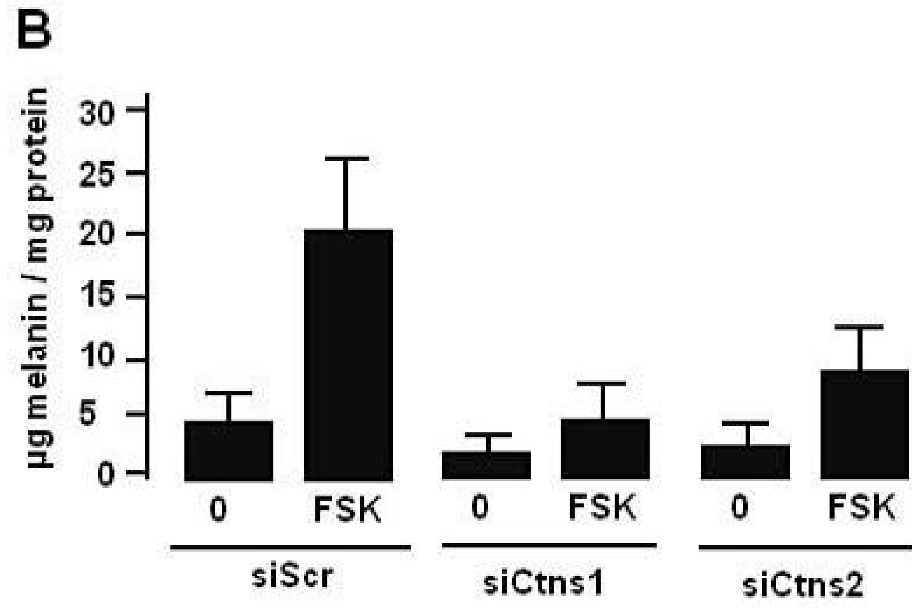
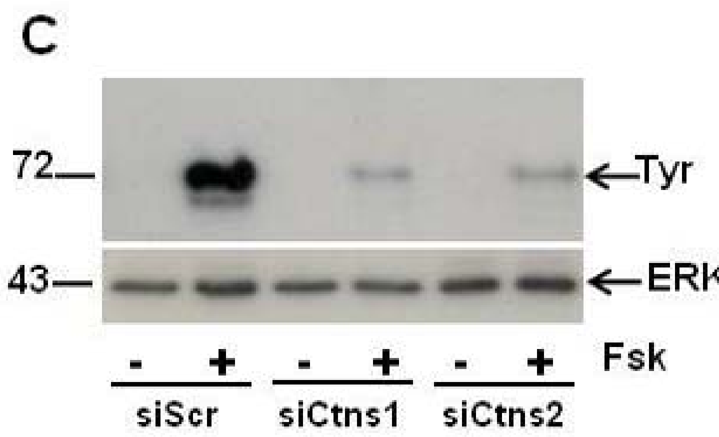


Fig.7







Sup Fig.1

	Patient phototype	Skin color	Hair color	Eye color	Lighter parent phototype
1	IV	Light brown	Brown	Brown	IV
2	III	Darker white	Brown	Brown	III
3	II	Fair	Blond	Blue	II
*4	II	Fair	Blond	Brown	III
5	III	Darker white	Chestnut	Brown	III
6	IV	Light brown	Brown	Brown	IV
*7	II	Fair	Blond	Brown	III
*8	II	Fair	Blond	Brown	III
*9	II	Fair	Blond	Brown	III
10	III	Fair	Chestnut	Brown	III
11	III	Fair	Chestnut	Brown	III
12	IV	Light brown	Brown	Brown	IV
*13	II	Fair	Blond	Blue	III
*14	II	Fair	Blond	Blue	III
*15	I	Pale	Red	Blue	III
*16	II	Fair	Blond	Brown	III
*17	II	Darker white	Blond	Brown	III
18	III	Darker white	Chestnut	Green	III
19	III	Darker white	Chestnut	Blue	III
*20	II	Fair	Blond	Brown	III
*21	II	Fair	Chestnut	Brown	III
22	III	Darker white	Chestnut	Brown	III
23	III	Light brown	Chestnut	Brown	III
*24	II	Fair	Blond	Blue	III
25	IV	Light brown	Brown	Brown	IV
26	IV	Light brown	Brown	Brown	IV
27	III	Fair	Chestnut	Brown	III

Supplemental Table 1: Clinical evaluation of skin, hair and eye pigmentation in cystinosis patients and their relatives. * indicates the patient with a clinical hypopigmentation.