Romanian Academy

Institute of Biochemistry

Ph.D. Thesis SUMMARY

-The NPC1 role in endosomes biogenesis-

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2023

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1. Purpose of the study

The biogenesis of endosomal and endosome-related organelles is an active process with different stages and multiple maturation pathways. Both melanosomes and endosomes originate from a common organelle, the early endosomes, but exhibit different maturation pathways depending on the target organelle. The Niemann-Pick type C1 (NPC1) protein is also involved in this maturation process of the endo-lysosomal system. This glycoprotein is a transporter of cholesterol and lipids in the endo-lysosomal system and influences the biogenesis, but also the activity of the endo-lysosomal system.

The main purpose of this doctoral thesis was to investigate the role of the NPC1 protein in the biogenesis of the endo-lysosomal system, especially in the development of organelles related to lysosomes. The chosen study model was melanosomes, within which we investigated the process of melanogenesis and biogenesis.

The synthesis process of melanin is of great interest because melanin is involved in certain hypopigmentation diseases, but also in skin cancer. Therefore, it is essential to identify the proteins that play a major role and influence this process.

The implications of NPC1 protein in melanogenesis were studied in a pigmented human melanoma line, MNT-1. This line was genetically modified by deleting the gene encoding the NPC1 protein and we investigated the changes that occur at the cellular level and that influence the biogenesis of melanosomes. We studied the role of NPC1 protein, a lysosomal protein, in the biogenesis of melanosomes, subcellular organelles structurally and proteomically related to lysosomes, but with completely different functions.

This thesis offers a new perspective in understanding the biogenesis of endo-lysosomal organelles and the transport of target proteins to these organelles.

2. Introduction

The NPC1 protein, being described in early studies as a cholesterol transporter in the lysosome, continued to be the target of research that led to the discovery of several of their functions and the characterization of Niemann-Pick type C1 disease (NPCD) (Pfeffer 2019). The NPC1 protein is not a simple transporter in the lysosome, but plays an essential role in lipid metabolism in the endo-lysosomal system. Together with the NPC2 protein, it contributes to the efflux of cholesterol from the lysosome, being also involved in the formation of multiple contact sites between the ER and lysosomes (Schultz et al. 2018). Mutations in the gene encoding the NPC1 protein lead to Niemann-Pick disease type C1, a lysosomal storage disease characterized by hepato-splenomegaly and neurodegeneration (Wheeler and Sillence 2020). At the cellular level, cholesterol and glycosphingolipids are being accumulated in the endo-lysosomal system, metal homeostasis is disturbed, and autophagy and intracellular traffic are affected. Moreover, some studies have demonstrated the influence and role of NPC1 protein in the biogenesis of lysosome-related organelles (LROs) in specialized cells. Thus, it has been shown that in the absence of NPC1 protein the biogenesis of alpha granules and dense granules in blood platelets is impaired (Platt et al. 2016).

Given these findings, our hypothesis was directed towards another category of LRO, which is required for the pigmentation process that occurs in melanocytes. Therefore, the influence of the NPC1 protein on the biogenesis of melanosomes was the target of the present research. The biogenesis of melanosomes involves a complex process including proteins with a specific role in melanogenesis such as the premelanosomal structural protein (PMEL17) or enzymes involved in the melanin biosynthesis pathway: tyrosinase (TYR), TYR-1-related protein (TYRP-1) and dopa-chromium tautomerase (DCT) (Seiji, Fitzpatrick, and Birbeck 1961).

Four stages of melanosome maturation have been described and characterized according to their morphology and degree of pigmentation (Raposo and Marks 2007). Stage I melanosomes, also called premelanosomes, originate from early endosomes and correspond to multivesicular bodies (MVB)/multiendosomal vesicles (MVE), which also contain some intraluminal vesicles (ILV). These premelanosomes are round, clathrin-coated organelles. The evolution of melanosomes from stage I to stage II involves the formation of fibrils, which are parallel

arranged and give the melanosomes an ellipsoidal shape. Melanosomes in the first two stages are immature and devoid of pigmentation. The delivery of the melanogenic enzymes, TYR, TYRP-1 and TYRP-2, to melanosomes facilitates the initiation of the melanin biosynthesis process (D'Alba and Shawkey 2019). Therefore, melanosomes in which melanin is deposited on the fibrils, but in which these fibrils are still observed, are classified in stage III, and melanosomes in which the fibrils were completely covered by melanin are part of stage IV. Stage IV melanosomes are then transferred to keratinocytes to perform their specific protective functions.

In the biogenesis process of melanosomes participate proteins with an enzymatic, structural or transport role. Among these, PMEL17 is the most important structural protein, which is involved in the formation of fibrils necessary for the deposition of melanin, and TYR, TYRP-1 and TYRP2 are among the most important enzymatic proteins. Also, an important role in the process of melanogenesis is attributed to the proteins involved in the trafficking of melanogenic proteins, for example the AP-3 protein and the AP-1 protein (Theos et al. 2005).

The PMEL17 protein is directly involved in the formation of melanosomes, because it actively participates in the formation of the amyloid fibrillar matrix, necessary for the process of melanogenesis, for organizing the architecture of melanin vesicles and for protecting melanin against degradation (Watt et al. 2013). To generate the fibrils required for melanosome formation, the PMEL17 protein undergoes post-translational processing and cleavage in the premelanosome/MVB (Hee et al. 2017). Thus, after the insertion of the protein into the ER (P1 form), it is transported to the Golgi apparatus in COPII vesicles, which recognize the valine residue in the C-terminus. In this compartment N-glycans are matured and O-glycans are added, forming the P2 form of the protein, which has a molecular mass 20 kDa higher than the P1 form (Bissig, Rochin, and Van Niel 2016). The P2 form is processed by a convertase and a large luminal fibrillogenic Mα fragment (composed of NTR, PKD and RPT) is formed, which remains linked by a disulfide bridge to the Mβ fragment (composed of KLD, transmembrane and cytoplasmic domain). In the MαS-S Mβ form, PMEL17 reaches early melanosomes (stage I) and is transferred by a CD63 protein-dependent process to the ILV. Next, PMEL17 is processed by various proteases, including BACE-2 and ADAM17, resulting in a soluble luminal form - Mα. This form is cleaved into two fragments MαN and MαC, from which the NTR and PKD and RPT domains are formed, respectively. The main domains contributing to fibril formation are PKD

and RPT, but the NTR domain has a major role in regulating melanosomal fibril assembly. As a protein that exhibits multiple forms, various antibodies have been developed that recognize specific forms of the PMEL17 protein. The main antibodies used in the present study are:

- αPEP-13, which recognizes the immature, uncleaved forms P1 and P2.

- αHMB-45, which recognizes the insoluble RPT domain with role in the formation of the fibrils (Watt et al. 2013).

Therefore, we can conclude that the PMEL17 protein plays an essential role in the biogenesis of melanosomes, being directly involved in the formation of fibrils on which melanin is deposited.

In the process of melanin biosynthesis, the enzymatic action of some proteins is necessary, of which TYR is indispensable (Hearing and Jiménez 1987). This protein, monophenol monooxygenase, was first described in 1895 in clam extracts, demonstrating its ability to oxidize tyrosine (Fitzpatrick 1949). TYR is a type I membrane glycoprotein that exhibits 6/7 N-glycosylation sites exposed on the lumenal side of melanosomes and 17 cysteine residues clustered in two cysteine-rich domains. To perform its enzymatic function, two copper ions are incorporated into the enzyme structure. The synthesis process of TYR takes place at the level of the ER, from where it is then transported to the Golgi apparatus and through the secretory pathway finally reaches stage II melanosomes. N-glycans covalently attached in the ER are necessary for protein folding and maturation, and the calnexin-calreticulin chaperone cycle intervenes in these processes (Petrescu et al. 2000). If TYR is misfolded, it is recognized by the EDEM1 protein and targeted for degradation via the proteasomal pathway (Marin et al. 2012). N-glycosylation of TYR is essential for its maturation and activity. If TYR is correctly folded and matured, it will be transported to the Golgi apparatus, where it will undergo further glycosylation modifications and then be directed to target organelles. TYR is transported directly or via the PM to early endosomes, and will then be directed to stage II melanosomes, where it participates in the process of melanogenesis.

In conclusion, melanogenesis is an extremely important process for the human body, many proteins being actively or passively involved. It is important to know closely and as precisely as possible the proteins that participate in these processes in order to be able to provide

the required medicine and to help people suffering from various pigmentation problems, but also to prevent and identify skin cancer in time.

3. Results

3.1 Generation and characterization of the NPC1-KO cell line

In the present study we chose to investigate the role of the NPC1 protein in the biogenesis of lysosome-related organelles (LROs), which are specialized in melanin production. We used a pigmented human melanoma cell line, MNT-1, that was depleted of the NPC1 protein by the CRISPR/CAS9 knock-out (KO) method. Cells were co-transfected with two commercial NPC1 KO specific plasmids and cultured in puromycin medium in order to select the transfected cells. A second selection was performed based on red fluorescence by flow cytometry and cells that showed fluorescence were individually plated in 96-well plates. In the obtained clones, NPC1 protein expression was tested by Western blot, and those that did not express NPC1 protein were kept (Figure 1).

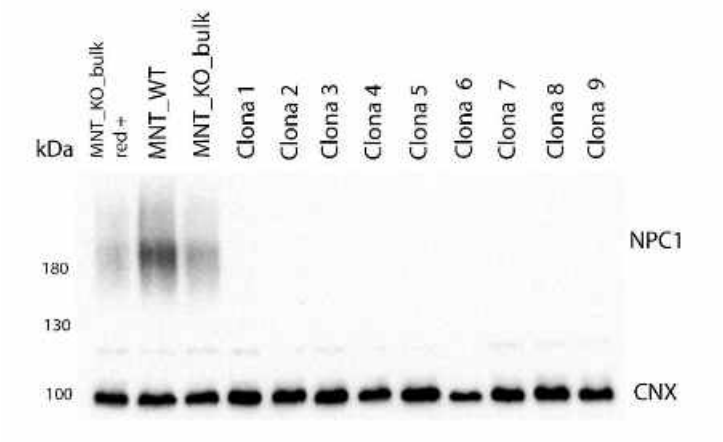

Figure 1. Verification of NPC1-KO clones by Western blotting.

NPC1 protein expression was determined by Western blot from lysates of cells co-transfected with the plasmids for CRISPR/Cas9 and MNT-WT. Calnexin (CNX) was used as a loading control.

According to the specialized literature, in the absence of the NPC1 protein, cholesterol and glycosphingolipids accumulate in the endo-lysosomal system (Bräuer et al., 2019). To characterize the obtained clones, a first experiment was performed in which we quantified the lysosomal volume with LysoTracker Green by flow cytometry. In Figure 2, a significant increase in lysosomal volume can be seen in certain clones, but a few clones show no significant variation in the lysosomal compartment. Interestingly, only the clones with reduced pigmentation (those represented in gray) also have an increased lysosomal volume, i.e. they also show the NPCD phenotype.

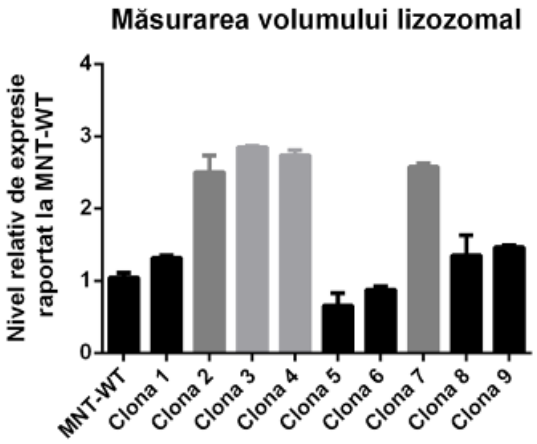

Figure 2. Determination of lysosomal volume by LysoTracker Green.

Lysosomal volume was measured in WT cells and in several NPC1-KO clones. Cells were incubated with LysoTracker Green, and their fluorescence was determined by flow cytometry.

Next, we chose a clone representing the NPCD phenotype and we determined several lipids species. The cholesterol level was assessed by filipin staining and by a biochemical method with Amplex red, and the glycosphingolipid level was determined by HPLC. In the absence of NPC1 protein, the level of cholesterol increased, but in the case of glycosphingolipids an approximately equal level of GlcCer was noted in both cell lines (Figure 3A, B and C). However, the total glycosphingolipid level was slightly higher in the NPC1-KO line (Figure 3D and E). Thus, in the new generated cell line, the NPCD phenotype including increased lysosomal volume, cholesterol and glycosphingolipid levels was observed.

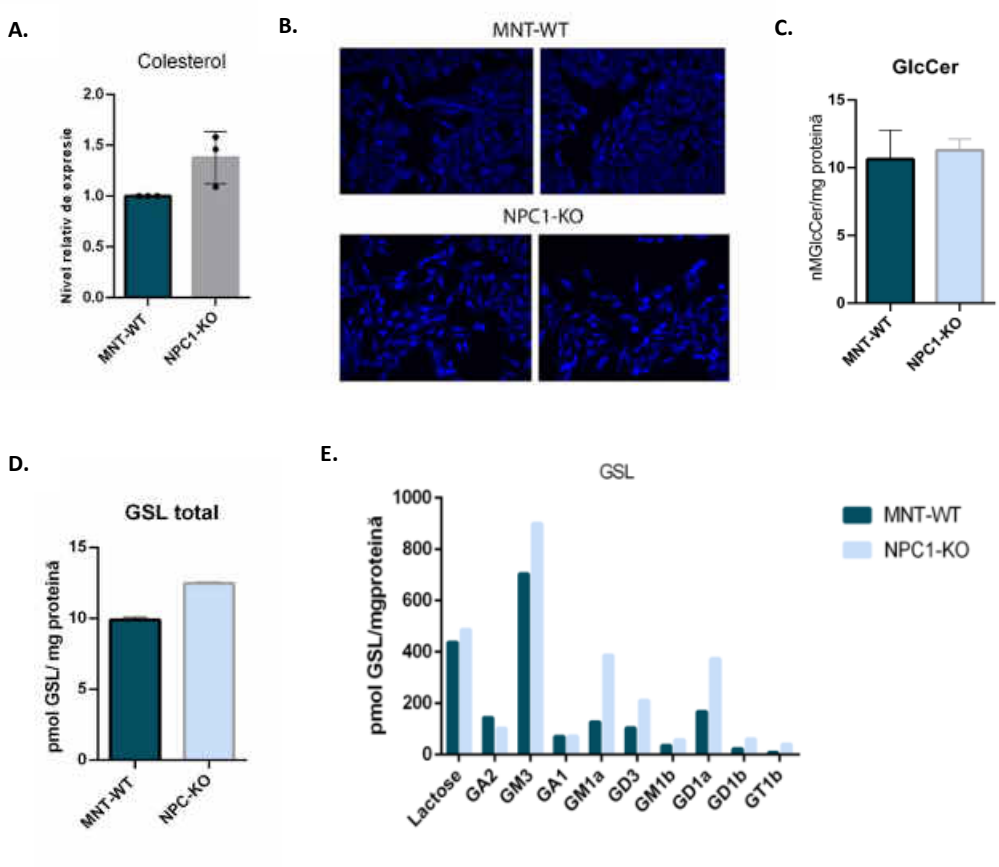

Figure 3. Cholesterol and glycosphingolipid level in MNT-WT vs NPC1-KO.

(A) Cholesterol measurement by Amplex-red method (B) Cholesterol staining with filipin and fluorescence microscopy visualization. (C) Determination of GlcCer level by HPLC. (D) Determination of the total amount of glucosphingolipids. (E) Determination of the level of different glycosphingolipid species.

3.2 The role of the NPC1 protein in the biogenesis of melanosomes

In the second part of the study, we focused on investigating the role of the NPC1 protein in the biogenesis of melanosomes and in the pigmentation process. From the cell harvesting we can notice significant differences in the pigmentation state of the MNT-WT vs NPC1-KO cell lines (Figure 4A). In the absence of the NPC1 protein, cell pigmentation is impaired, with cells being light brown. Knowing that TYR is an essential enzyme in melanogenesis, we determined its activity in the gel and its expression by Western blotting (Figure 4B, C and D). We observed

that in cells deficient in NPC1 protein significantly decreased expression of TYR is correlated with decreased activity. Not only the expression of TYR is decreased in the NPC1-KO cells, but also the expression of TYR-related proteins, TYRP-1 and DCT (Figure 4E and F). Therefore, in the absence of the NPC1 protein, the process of melanin biosynthesis is impaired.

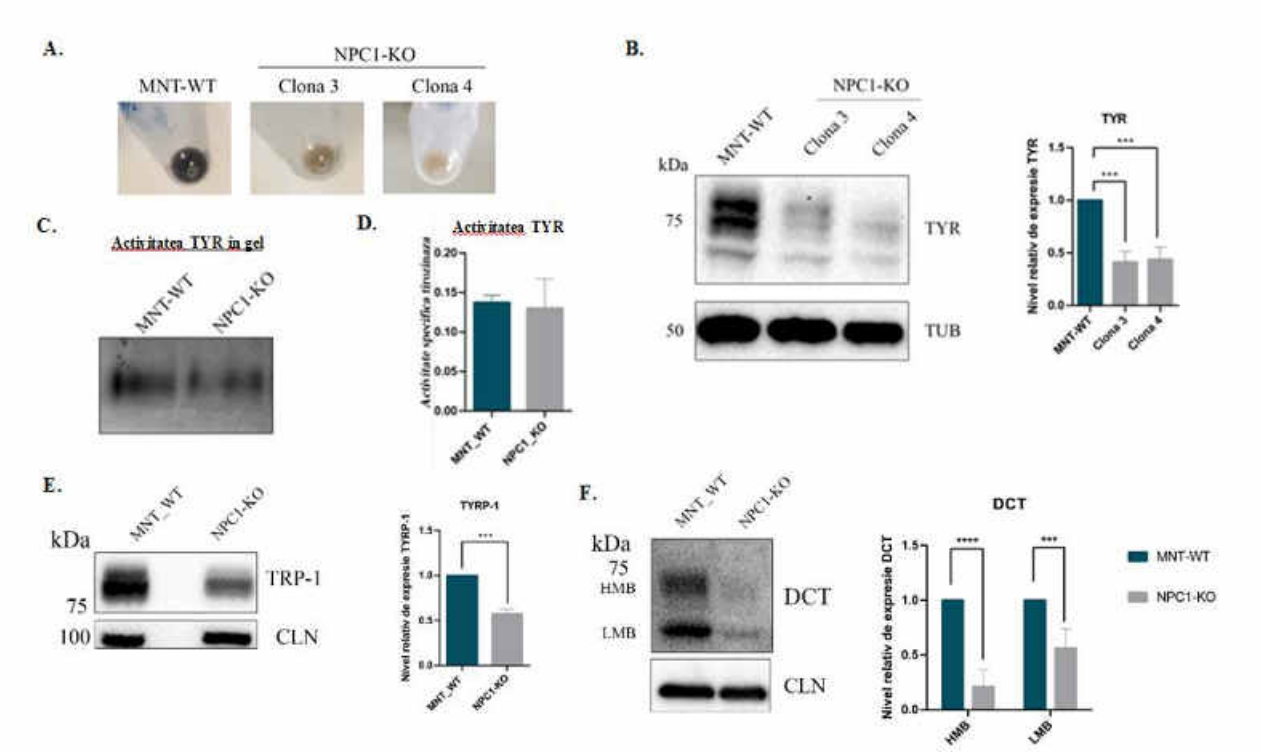

Figure 4. Melanogenic enzymes in NPC1-KO cells.

(A) Visual comparison of the pellets of MNT-WT cells and NPC1-KO clones. (B) The expression level of TYR was determined by Western blotting. TUB was used as loading control. (C) Image of the gel incubated with L-Dopa. (D) Specific TYR activity calculated from determination of TYR activity in the plate. (E, F) The protein level of TYRP-1 and DCT was determined by Western blot, and the protein expression was normalized to the level of CNX. Alongside is the graphical representation of the expression of the two proteins.

In the following experiments we wanted to find how the NPC1 protein influences TYR expression. To elucidate the cause of the decrease in the level of TYR in NPC1-KO cells, we analyzed by RT-PCR whether there are changes at the transcriptional level of TYR. TYR RNA

was determined in the two cell lines and according to Figure 5A there are no statistically significant differences at the transcriptional level. In addition, we analyzed by Western blot the level of microphthalmia-associated transcription factor (MITF), which is the main transcription factor of TYR (Wang et al. 2014). According to the data presented in figure 5B, it can be stated that there are no significant differences at the level of protein expression. So, in the absence of the NPC1 protein, TYR expression is affected, but no changes are identified at the transcriptional level.

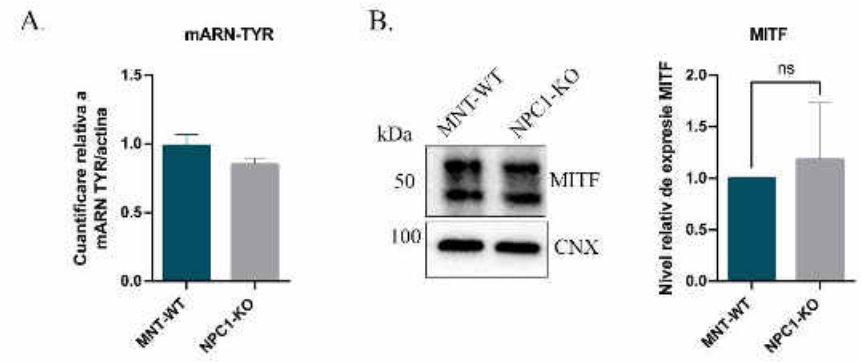

Figure 5. Transcriptional study of TYR.

(A) Quantification of TYR mRNA by RT-PCR in MNT-WT and NPC1-KO cells. (B) Determination of MITF protein by Western blot and its quantification. CNX was used as a loading control.

Next, we studied the processing and glycosylation of TYR in the absence of NPC1 protein with the enzymes EndoH, PNGaseF and NeuA. The PNGaseF enzyme removes most of the N-glycans attached to the protein, resulting in a deglycosylated form (Chu 1986). In contrast, EndoH glycosidase only cleaves mannose-rich or hybrid oligosaccharides that have not been cleaved by mannosidase II in the Golgi apparatus. By subjecting the proteins to digestion with EndoH, it is possible to follow their processing pathway and whether they have transited the Golgi apparatus. Another enzyme that helps us to find out the differences in the processing of glycans with a complex structure belonging to TYR is NeuA, a sialidase, which cleaves sialic acid residues from the complex structure of glycoproteins.

The cell lysate from the MNT-WT and NPC1-KO cells was incubated separately with the three enzymes and the expression and processing of TYR was analyzed by Western blot. The effect of the NeuA enzyme can be noted by the presence of a small change in the molecular mass within the TYR. Between the two cell lines the expression of TYR is different, but there are no changes regarding the processing of sialic acid (Figure 6A). The expression of the deglycosylated TYR polypeptide was determined by PNGase enzyme digestion, and the decrease in TYR expression in the absence of NPC1 protein was revealed (Figure 6B). Following the action of the EndoH enzyme on TYR by Western blot, two bands belonging to TYR were visualized, one EndoH sensitive (EndoH sens) and one EndoH resistant (EndoH rez). We observed that the ratio between the two bands (EndoH rez/EndoH sens) is different in the two cell lines (Figure 6B). Thus, in cells depleted of NPC1 protein the ratio is low, indicating an increase in the EndoH sens protein form, the immature form of TYR. In NPC1-KO cells there is an alteration of TYR maturation, the complex glycosylation process at the level of the Golgi apparatus being affected. These observations indicate that in cells depleted of NPC1 protein, it is possible that the processing at the Golgi apparatus is delayed or impaired, or that mature TYR is more rapidly degraded.

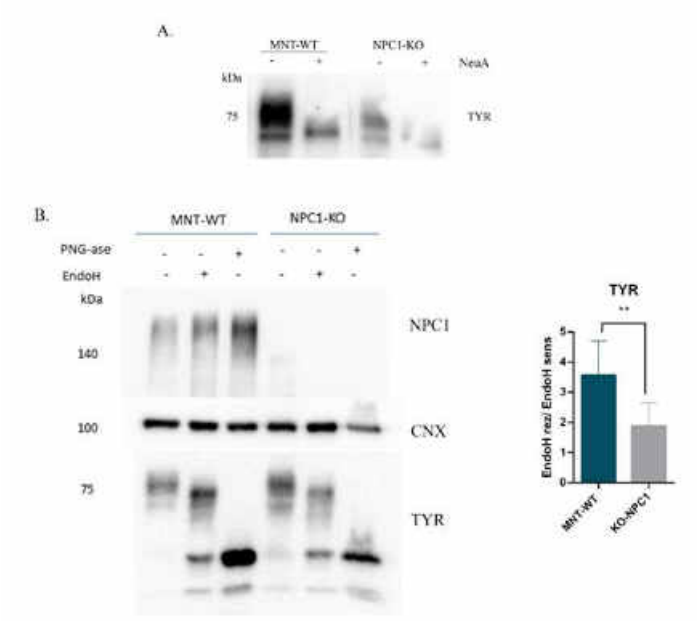

Figure 6. Evaluation of TYR processing.

(A) TYR expression following NeuA digestion was determined by Western blot. (B) Cell lysates were incubated with PNGase and EndoH respectively, and proteins of interest were determined by Western blotting. Near is the graphical representation of the ratio of EndoH rez/ EndoH sense bands in NPC1-KO vs MNT-WT cells.

To unravel the TYR pathway in the absence of NPC1 protein, we evaluated the extracellular secretion process of TYR in melanoma cells. NPC1-KO and MNT-WT cells were cultured for 72 h and the expressions of several secreted proteins were determined by Western blot from the collected culture medium. As presented in Figure 7, although LAMP-2 protein level is increased in the medium of NPC1-KO cells, TYR expression is decreased in extracellularly secreted vesicles. While TYR secretion is not accelerated or enhanced, the decrease in TYR levels could be determined by other intracellular sequestration and accelerated degradation processes. Foltilin-1, which is an exosome-resident protein (Phuyal et al. 2014), was identified in both cell lines, suggesting that the extracellular secretion process is not affected in the absence of NPC1 protein.

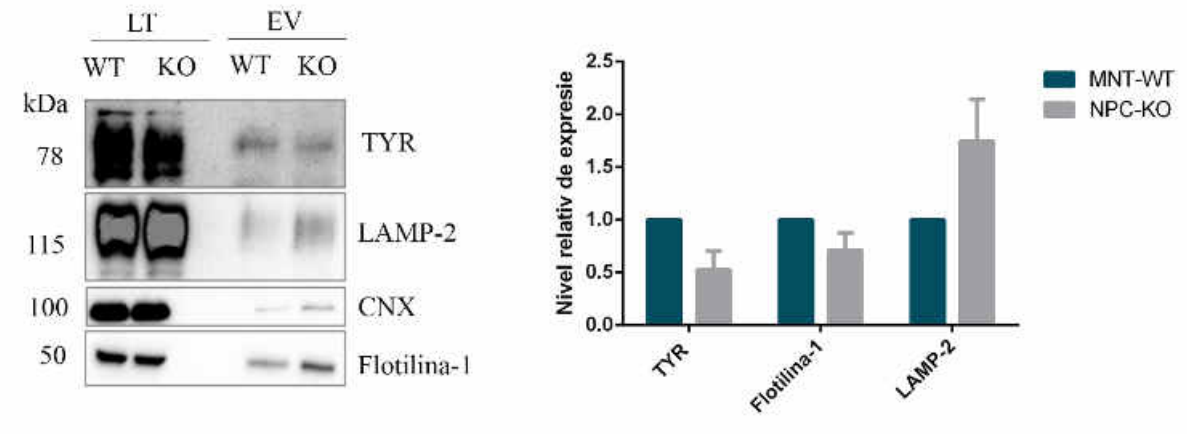

Figure 7. Influence of NPC1 protein on extracellular secretion.

TYR, LAMP-2, CNX and flotillin-1 proteins were analyzed by Western blot and the bands were quantified.

The degradation rate of TYR in the absence of NPC1 protein was studied using a protein synthesis inhibitor, cycloheximide (CHX). MNT-WT and NPC1-KO cells were incubated with

CHX and harvested at different time points (0 h, 30 min, 1 h, 2 h, 3 h, and 4 h). In the parental cell line the half-life of TYR is approximately 3.5 h, compared to the NPC1-KO cell line where the half-life of TYR is 1.5 h (Figure 8). Thus, in the absence of NPC1 protein, TYR degradation is accelerated. Therefore, in NPC1-KO cells it can be assumed that reduced levels of TYR are processed to mature proteins, but exhibit poor quality control in the secretory pathway, leading to or accelerated degradation of TYR.

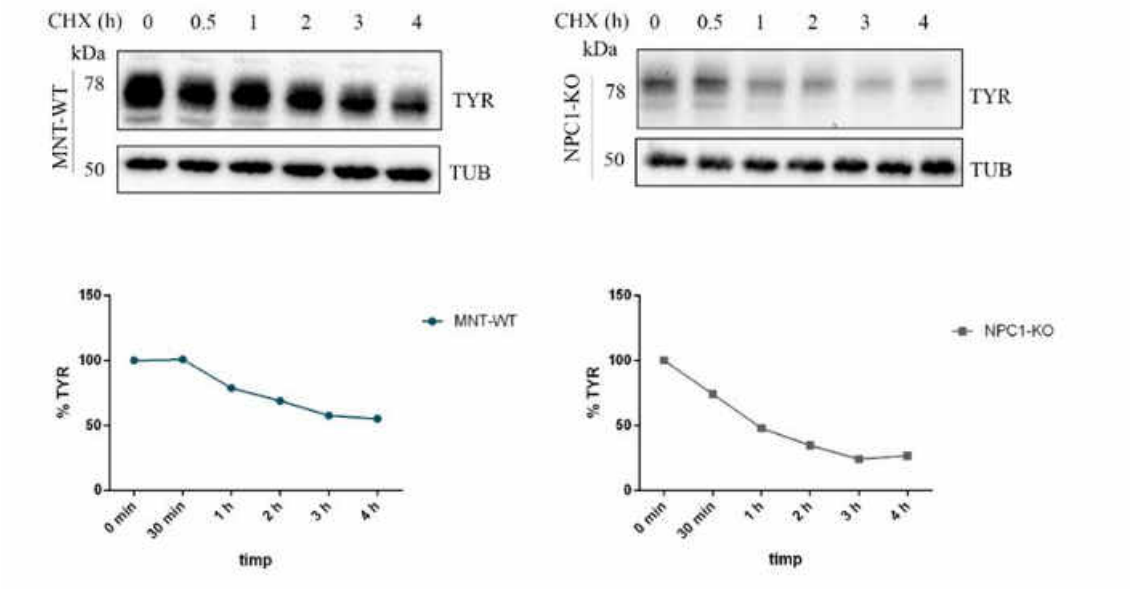

Figure 8. TYR degradation

The two cell lines were treated with CHX and harvested at different time points. TYR expression was determined by Western blot. TUB was used as a loading control.

Various proteins are involved in the maturation process of melanosomes, of which PMEL17 and TYR are essential. We investigated the pathway of melanosome biogenesis in cells depleted of NPC1 protein by performing subcellular localization studies of different proteins associated with the endosomal or melanosomal pathway. Fluorescence microscopy and subcellular separation of organelles on sucrose gradients were used to characterize the influence of NPC1 protein in LRO, in melanosomes, and implicitly in melanogenesis.

We analyzed the localization of TYR by confocal immunofluorescence microscopy in the two compared cell lines. In MNT-WT cells, TYR was distributed as punctate structures scattered

throughout the cytoplasm, with some concentrated in the perinuclear region (Figure 9). Images in BF showed TYR coincident with pigment granules accumulated in the juxtanuclear region, at the cell periphery and cell tips, thus indicating the localization of TYR in pigmented, mature melanosomes. On the other side, in NPC1-KO cells, the intensity of TYR staining was lower, but also its intracellular localisation was very different. TYR was concentrated along extended regions at the cell periphery, close to the plasma membrane. Moreover, NPC1-KO cells lacked mature, highly pigmented melanosomes.

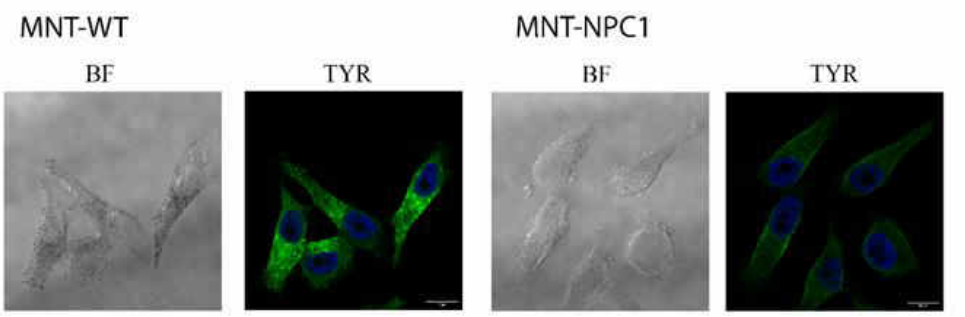

Figure 9. Confocal microscopy visualization of TYR.

TYR was labeled with anti-PEP7 antibody (1:300, ON), nuclei with Hoechst, and melanin vesicles were visualized in BF.

We found that TYR detected in MNT-WT cells co-localized extensively with RAB38, a RabGTPase involved in the cargo trafficking to and from mature melanosomes (Figure 10). In contrast, in NPC1 deficient cells, their co-localization was restricted to peripheral compartments. A similar distribution was observed for TYRP-1, a protein involved in melanin biosynthetic process. Furthermore, there was a detected also a decrease in fluorescence intensity for TYRP-1 (Figure 10). Decreased pigmentation and changes in the expression and subcellular localization of proteins normally associated with mature melanosomes indicates that NPC1 protein is important for melanosome biogenesis and/or maturation. Since the sorting of melanogenic enzymes to maturing melanosomes originates from early endosomes, we investigated the localization of EEA1 protein, a protein associated with early endosomal membrane domains. We found out that the distribution of EEA1 protein was not altered in NPC-1 KO cells compared to

WT MNT-1 cells (Figure 10), but TYR and EEA1 showed different localization in both cell lines. On the other hand, in both cell lines there was a partial co-localization of TYR with CD63, a tetraspanin involved in PMEL17 regulation (Figure 10). PMEL17 (HMB-45 positive), another protein involved in melanogenesis showed in MNT-WT cells partial co-localization with TYR in punctate structures located at the cell periphery, but also intracellularly. In contrast, in NPC1-KO cells, PMEL17 was also shifted peripherally where it partially co-localize with TYR.

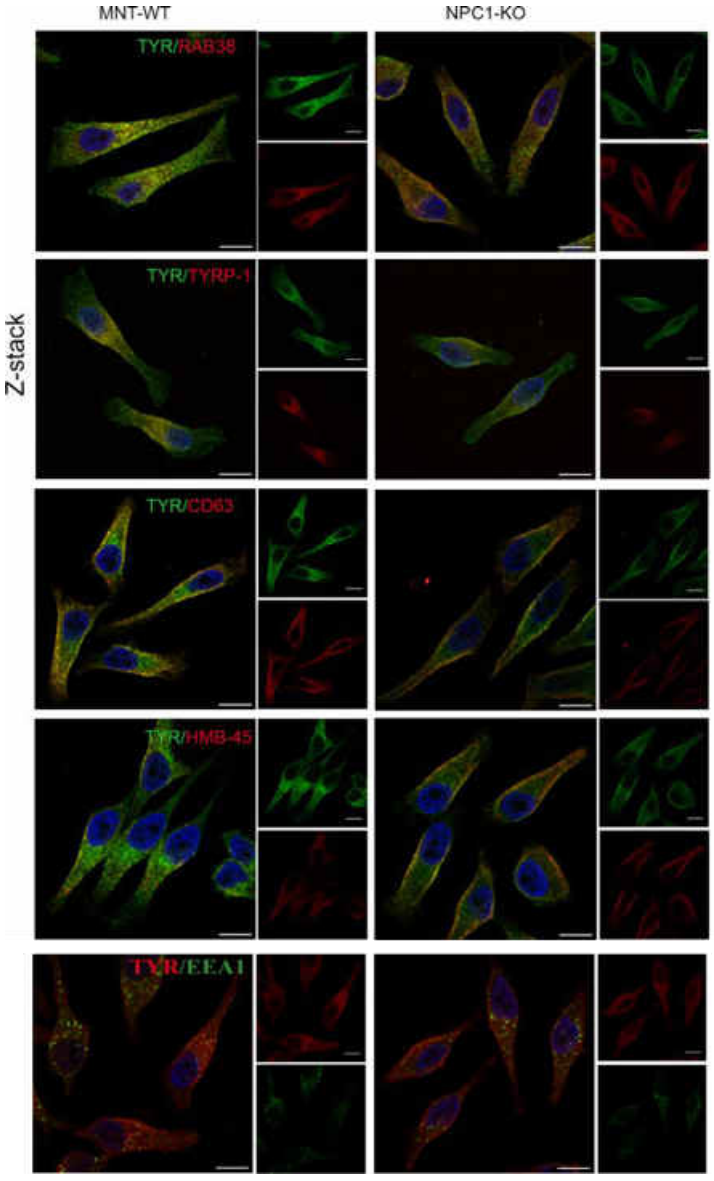

Figure 10. Co-localization of TYR with TYRP-1 protein, Rab38, CD63 and HMB-45

MNT-WT and NPC1-KO cells were incubated with the TYR antibody (anti-Pep7) and an antibody against one of the melanosome-associated proteins: Rab38, CD63, or HMB-45-reactive mature PMEL, or against EEA1. Nuclei were counterstained with Hoechst and pictures were acquired with a 63x objective.

Furthermore, we investigated whether the NPC1 protein influences the maturation process of PMEL17 protein and the biogenesis of melanosomes. The level of the immature (P1/P2) and M-beta forms of PMEL17, recognized by the PEP-13 and PMEL17 antibodies, were not affected in NPC1 deficient cells (Figure 11A and B). Interestingly, a fibril-associated fragment containing the repeat domain (RPT) was overexpressed in the absence of NPC1 (Figure 11C). Western blot analysis revealed that there was a statistically significant increase in soluble and insoluble forms of PMEL17 in the NPC1 deficient cells. During the fibril formation process, PMEL17 traffics to the plasma membrane before transfer to the early endosome/premelanosomes and the accelerated fibril formation could occur at these levels.

The PMEL17 fibrils at the cell membrane were determined by flow cytometry. Similar expression of surface PMEL17 was observed in both cell lines.Therefore, in the NPC1-KO cells there an increase in the mature PMEL17 protein (RPT domanin), which is accumulated in immature melanosomes or MVB.

Thus, in NPC1-deficient cells, the trafficking of TYR and TYR-related proteins is diverted from melanosomes to lysosomes and TYR is degraded more rapidly. Consequently, melanin biosynthesis is reduced, the formation of the PMEL17-melanin matrix in early melanosomes is affected and these organelles remain immature.

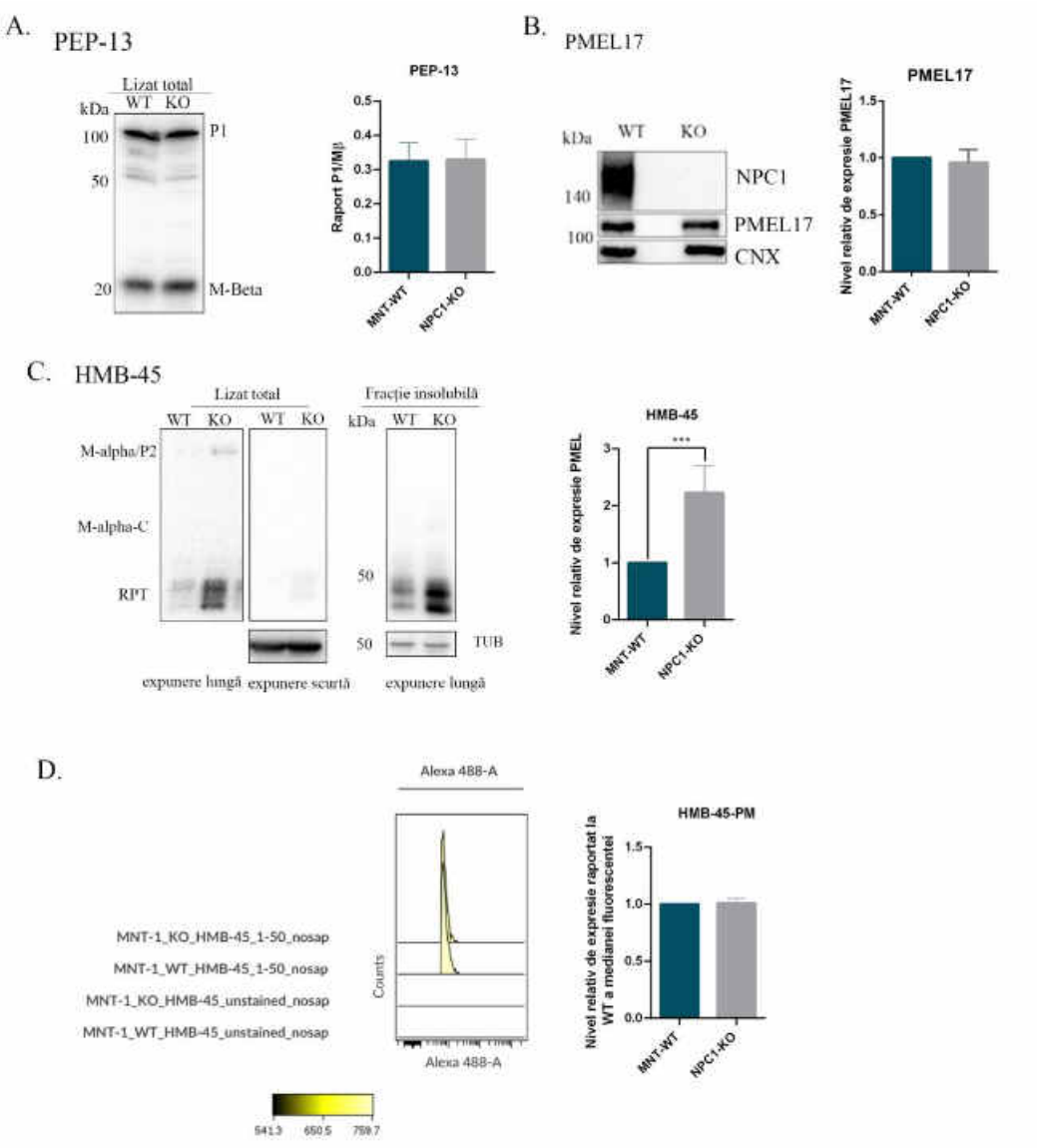

Figure 11. PMEL17 protein processing in MNT-WT vs NPC1-KO.

(A, B, C) The expression level of different processing forms of PMEL17 protein was detected by Western blot and the quantification of PMEL protein in the two compared cell lines was plotted. Detection of PMEL17 protein was performed with anti-PEP13 (A), anti-PMEL17 (B) and HMB-45 (C) antibodies. (D) Quantification of mature PMEL17 protein expression from the PM by flow cytometry. Non-permeabilized cells were labeled with HMB-45 antibody, incubated with anti-mouse-AlexaFluor488 secondary antibody, and intensity was detected by FACS.

4. Conclusions

Rare lysosomal diseases such as Niemann-Pick have been of increasing interest to researchers in recent years. Although there are many studies on the function of NPC1 protein, the whole mechanism has not yet been fully elucidated. The NPC1 protein has an essential role in lipid metabolism, being involved in the transport of glycosphingolipids and cholesterol at the endo-lysosomal level, but it also has major implications in the optimal functioning of the endo-lysosomal system.

In the present study I chose to investigate whether the NPC1 protein also has a role in the maturation of the endo-lysosomal pathway. Thus, we chose as a study model the maturation pathway of melanosomes, which are organelles related to lysosomes and originate from early endosomes.

We generated a novel cell line depleted of the NPC1 protein by the CRISPR/CAS9 method and observed that the NPC cell phenotype is associated with major pigmentation defects. The NPC1-KO cell line showed the cellular characteristics specific to the disease, such as increased lysosomal volume, increased LAMP-2 protein expression, cholesterol accumulation, and glycosphingolipid accumulation.

In order to identify the cause of pigmentation defects, we investigated the fate of TYR and the PMEL17 protein, which are two essential proteins in the process of melanosome biogenesis and melanin biosynthesis.

Following the study carried out, we can reproduce the main observations and conclusions regarding the influence of the NPC1 protein on melanogenesis:

-Proteomics data as well as Western blot results indicated a drastic reduction in TYR expression in the absence of NPC1 protein. In addition, the decrease in the expression level of TYR-related proteins, namely TYRP-1 and DCT, was also noted by immunoblotting.

-Through qRT-PCR experiments we demonstrated that TYR is not affected at the transcriptional level, but at the post-translational level. The comparable expression level of the transcription factor MITF in the two cell lines (MNT-WT and NPC1-KO) supports these observations.

-In the NPC1-KO cell line the degradation rate of TYR is increased, explaining the low level of TYR and the reduction of melanin synthesis in KO cells.

-The study of the N-glycosylation process showed that the ratio between the mature vs. immature fraction is lower in the absence of NPC1 protein, suggesting that the NPC1 protein could be involved in its maturation.

-In the presence of BafA we observed that in NPC1-KO cells the pigmentation is partially recovered and the level of TYR increases significantly. BafA inhibits lysosomal degradation, but may also inhibit some pathways involved in retrograde transport from the Golgi to the ER or LRO to the TGN. Thus, the different forms of N-glycosylation (resistant EndoH) identified in the presence of BafA may represent glycoforms redirected from the LRO to the Golgi, and then from the TGN in melanosomes, avoiding lysosomal degradation.

-In the absence of the NPC1 protein, the distribution of the remaining TYR is altered. While in MNT-WT cells TYR presents a specific localization of mature melanosomes, at the perinuclear level and in dendritic ends, in NPC1-KO cells TYR is redistributed towards the cell periphery, in Rab38, HMB-45 and CD63 positive vesicles. TYR remains in some vesicles that belong to the LRO, in MVBs or immature melanosomes that contain amyloid fibrils but are not able to mature.

-Unlike TYR, in NPC1-KO cells the PMEL17 protein is not affected at the level of synthesis or degradation. Even though the expression of the PMEL17 polypeptide is the same in both cell lines (MNT-WT and NPC1-KO), the expression level of the fibril-specific RPT domain recognized by the HMB-45 antibody is significantly increased in the line depleted of the NPC1 protein. This fact may be due to a more efficient fibril generation process. Thus, in NPC1-KO cells PMEL17 traffic is not affected, it reaches the MVB, where it is processed and fibril formation occurs.

-In conclusion, in the absence of the NPC1 protein, TYR trafficking to melanosomes is impaired and although PMEL17 fibrils are generated, melanosome biogenesis is impaired.

-This thesis describes for the first time a new role of the NPC1 protein in the biogenesis of melanosomes.

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