

# THE TRAFFIC ROUTES OF TYROSINASES ON THEIR WAY TOWARDS MELANOSOMES

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In the past few years, many studies have added their contribution to the knowledge on tyrosinase, TRP-1 and TRP-2 traffic to melanosomes. Using cell lines having the hypopigmentation syndromes phenotypes, important progress has been made in understanding each step of melanosomal proteins transport to their target organelles, the melanosomes. In the present review we follow each of the three proteins in their journey from nascent polypeptide chains to maturation and further on the routes towards their site of activity. We also discuss the role of tyrosinases in pigmentation and the factors that contribute to the regulation of their melanocyte-specific expression. The sorting signals involved in their intracellular traffic and the molecules they interact with are next reviewed. Finally, we focus on the latest studies regarding the regulation of tyrosinase activity mediated by the ATP7A copper transporter.

**Key words:** tyrosinase, TRPs, pigmentation, biosynthesis, traffic.

## INTRODUCTION

Pigmentation in mammals is the result of synthesis and distribution of melanin in the skin, hair bulbs and eyes. Melanins are produced by pigmented cells called *melanocytes* in specialized cytoplasmic organelles known as *melanosomes*. Two types of melanins are being synthesized: red/yellow pheomelanin and brown/black eumelanin. Melanins are present in bird feathers, in hair, eyes and skin of mammals, in skin and scales of many fishes, amphibians and reptiles, in the ink of cephalopods (octopus, calamari) and in numerous invertebrate tissues. They are polymers which vary in size and complexity. Feathers and hair have different colours such as pale yellow, orange, yellow-brown, red, brown and black, which may result from melanins in different stages of formation or subdivision in granules. The melanization degree depends on the relative concentrations of copper and tyrosinase. Dark hair contains increased traces of copper in comparison with fair hair. If the copper intake decreases substantially to less than one milligram per day, animal fur becomes progressively lighter. This effect is reversed by a copper-rich diet.

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Melanogenesis is a complex metabolic pathway that processes L-tyrosine to melanin by a series of oxido-reducing and isomerisation reactions catalysed by an enzyme family localized in the melanosomal membrane (1). *Tyrosinase* is a key enzyme in this synthesis and catalyses three different reactions in melanogenesis. Two related proteins were described: *TRP-1* (*tyrosinase-related protein-1*) and *TRP-2* (*tyrosinase-related protein-2*), which also catalyse reactions in melanogenesis. These proteins are approximately 40% homologous and there are results indicating their provenience from a common ancestor. Tyrosinase initiates melanogenesis but there are different other factors which intervene in modulating the quantity and quality of produced pigment. DOPAquinone is the key intermediate in the formation of pheomelanin in the detriment of eumelanin synthesis. The type of melanin synthesised is influenced by the action of several factors: sulphhydryl compounds as cystein precursors, MSH (*melanocyte-stimulating hormone*) and TRP-1.

Cystein interacts non-enzymatically with DOPAquinone to form cysteinilDOPA, a direct precursor of pheomelanin. Reaction kinetics between sulphhydryl and DOPAquinone is far more rapid than DOPAquinone cyclisation to LeucoDOPAchrom and therefore cystein concentration is critical for the synthesis of a certain type of melanin.

Eumelanogenesis is controlled by MSH and all three tyrosinase family proteins are trafficked from TGN to ellipsoidal stage II melanosomes containing the structural matrix composed of filaments/lamellae (Fig. 1, upper panel). In contrast, pheomelanogenesis is regulated by ASP (*agouti signal protein*) and only tyrosinase is transported from stage I melanosomes to stage II, which are in this case spherical organelles similar to lysosomes (Fig. 1, lower panel) (2).

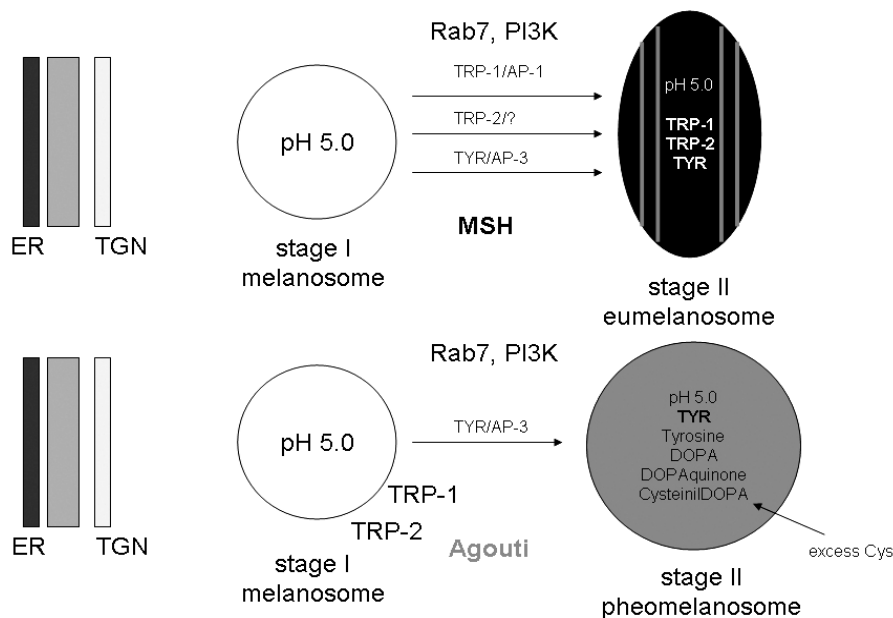


Fig. 1. – Structural difference and composition in tyrosinases of eumelanosomes (upper panel) and pheomelanosomes (lower panel).

In mice, mutations at *e/extension/Mc1r* locus coding for MSH (melanocyte stimulator hormone) receptor and at *a/agouti* locus coding for a MSH antagonist modulate melanogenesis affecting fur colour. It has been shown that variations in human *Mc1r* gene are more frequent in red hair than in brown or black hair people. Intracellularly, eumelanogenesis can be favoured by factors that modulate tyrosinase activity.

The presence and function of TRP-1 is not essential for pigmentation in mice but brown rather than black eumelanin synthesis in mutant mice indicates its role in eumelanogenesis synthesis and regulation. Moreover, in human cell lines, TRP-1 expression was detected only in cells containing eumelanin. TRP-1 DHICAoxidase activity contributes to eumelanin synthesis. It has also been shown that *b locus* is important in influencing melanosome structure (3).

More recent studies have explained the difference in mouse *versus* human melanogenesis. In mouse, TRP-1 functions as a DHICAoxidase which leads to melanin formation from DHICA precursor (4, 5). Tyrosinase is able to use tyrosine, DOPA and DHI (dihydroxyindole) as substrates but not DHICA; therefore, the activity of TRP-1 is needed for black melanin synthesis. Mutations in TRP-1 reduce this function, decrease eumelanin polymerisation and produce brown melanin (6). In human, TRP-1 does not function as a DHICAoxidase and tyrosinase can use DHICA in addition to tyrosine, DOPA and DHI as substrate (7). In mouse, TRP-1 has also a role in stabilising tyrosinase activity. Mutations in TRP-1 affect this function which interferes with melanin production explaining OCA3 phenotype. Following DOPAchrome synthesis, TRP-2 or metallic ions (copper, zinc) favour DHICA and not DHI formation. Melanins obtained from DHICA differ from those obtained from DHI in their solubility, flocculence and colour. Melanins generated from DHICA are brown, relatively soluble and have a low molecular weight while those generated from DHI are black, insoluble and with large molecular weight (8).

Regarding TRP-2 physiological role, there are no clear evidences, but mutations in *slaty/Tyrp2* locus can occur and lead to abolition of TRP-2 enzymatic activity, as in the case of *slaty-light*. In human there are studies on chromosomal deletions involving 13q31-q32, but they are not related with pigmentary disorders. However, it is possible that TRP-2 protects the melanocyte from the cytotoxicity of indol decarboxylated intermediates through limitation of their formation (9).

Despite multiple studies which helped characterizing all three genes there are still questions without answer regarding the occurrence of other eumelanogenic proteins, other functions associated to TRP-1 activity, characterisation of the pheomelanogenesis process or the structure of the melanin polymer.

#### REGULATION OF PIGMENTATION GENES TRANSCRIPTION

Skin and hair colour is determined by the relative proportions of pheo- and eumelanin. The ratio eumelanin/pheomelanin is regulated in mouse by *agouti*, *POMC*

and *extension* genes. These loci code for the agouti signalling protein (ASP), proopiomelanocortin (precursor of MSH) and MSH receptor (melanocortin 1 receptor (MC1R)). ASP is produced in the hair follicles of animals and acts on melanocytes to determine their switch from eu- to pheomelanin production contrary to the effect of MSH. Eumelanin and pheomelanin are different not only in colour, but also in the chemical composition. They even determine the structure of the melanosome in which they are synthesized and deposited (10).

Melanin synthesis is directly regulated by the enzymatic function of melanogenic proteins and hence by the transcription of their coding genes. Microphthalmia-associated transcription factor (MITF) which belongs to the basic-helix-loop-helix-zipper (bHLH-zip) is the main regulator of melanogenesis genes expression (11) and is essential for melanoblast development.

Promoter region sequences were compared with the regulatory elements involved in pigment producing cells gene expression. Thus, consensus sequences in mouse and human tyrosinase and TRP-1 were identified, which are present also in TRP-2; they were named *M box* and *E box* (Fig. 2). Microphthalmia gene product can bind to these sequences and activate transcription of tyrosinase genes (12).

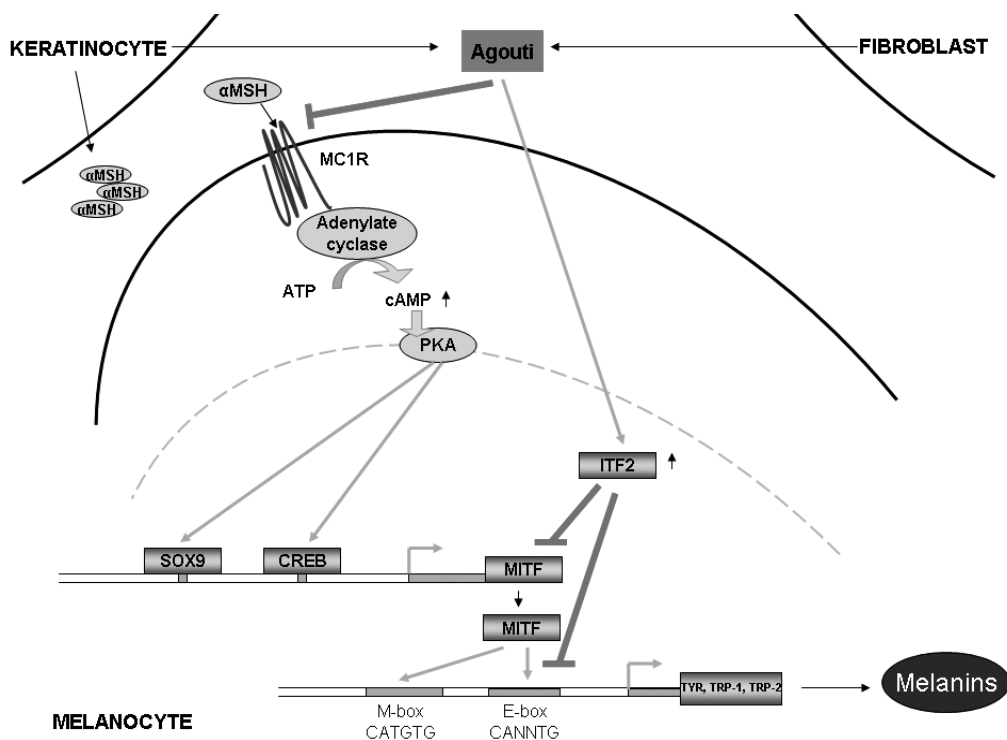


Fig. 2. – Tyrosinases transcription regulation.

MITF activates tyrosinases expression *via* PKA signalling pathway. MITF gene is highly conserved from human to mouse, birds and even fish. MITF expression is regulated by a very well described pathway. UV radiation triggers an increased expression of p53 protein in keratinocytes and p53 determines cells to produce MSH, which binds to MC1R found on melanocytes surface. Ligand binding to MC1R activates adenylate cyclases, which produce cAMP. Cyclic AMP activates CREB (13) that in turn promotes MITF expression (14). In the presence of MSH, tyrosinase expression increases, which leads to eumelanin production.

Recent data demonstrate direct implication of tyrosinase in melanogenesis (15). Using TYR-small interfering RNA (siRNA) the authors obtained an inhibitory effect on melanogenesis. They showed a specific down-regulation of tyrosinase at both mRNA and protein levels with no interference with TRP-1 and TRP-2 expression. Moreover, the treatment decreased viability of human epithelial melanocytes (HEMs) exposed to UV radiation.

In the presence of ASP (Agouti in Fig. 2) which competes with MSH for MC1R, a decrease in expression of the genes involved in the production of eumelanin including tyrosinase, TRP-1 and TRP-2 was observed. This competes with eumelanogenesis in favour of constitutive pheomelanin production (16). During pheomelanogenesis the expression of other genes is augmented. One of the most important is ITF2 (SEF2), member of class A of bHLH transcription factors called E proteins. ITF2 may act as inhibitor of melanosomal genes transcription but also through inhibition of *Mitf* transcription (10). Recent microarray experiments have revealed that ASP decreases Rab27A and dynein expression, key elements in melanosome transport, which inhibits melanogenesis (17).

Unlike mouse ASP, human ASIP does not significantly affect melanosomal genes transcription but only TRP-1 protein translation. Its expression generates a decrease in mRNA synthesis for PPAR $\beta$ , eIF-4B, RRM2, MINOR and EV12B (18). In principle, all intracellular signalling pathways can affect one or several parameters of pigmentation and it is clear that in this way they influence melanocyte specific processes but also basic processes that modulate cell functioning (16).

#### INTRACELLULAR TRAFFIC OF TYROSINASES

Similar to other membrane proteins, tyrosinase is synthesised in the endoplasmic reticulum (ER), where it acquires the proper folded conformation then is trafficked through the Golgi apparatus towards melanosomes. Proteins that do not succeed in acquiring the correct 3D structure are retained in the ER by prolonged chaperone molecules interaction and sent to degradation following retrotranslocation in the cytosol. Details regarding cell mechanisms of melanosome biogenesis and assembly started to be discovered between 1995 and 2000. During

the same timeframe, electronic microscopy studies have revealed melanin distribution and tyrosinase activity (19). Tyrosinase and TRPs genes have been cloned and their sorting signals identified. Correlations between defects in their transport and human pigmentation disorders or different animal fur colours phenotypes were unravelled.

In 2001, Raposo *et al.* (20) have described tyrosinase transport from TGN to melanosomes in clathrin coated vesicles. Sprong *et al.* (21) described in the same year tyrosinase and TRP-1 glycosphingolipid-dependent post-Golgi traffic. In the absence of these molecules, melanosomal proteins accumulate in the pericentriolar TGN and cells are hypopigmented.

In 2004, Maxfield *et al.* (22) described the pericentriolar localization of early endosomes. During the latest years the role of proteins involved in tyrosinases intracellular traffic has been studied on cell lines having the hypopigmentation syndromes phenotypes. Hermansky-Pudlak syndromes are genetic deficiencies associated with mutations in genes involved in melanosomal protein sorting which are 15 in mouse and 8 (HPS-1-8) in human. Most mutations affect genes which encode AP or BLOC subunits. Mutation of *lyst* protein coded by a gene involved in Chediak-Higashi syndrome and in *beige* phenotype in mouse functioning downstream of AP-3 prevents melanosomal and lysosomal protein segregation from endosomes. Griscelli syndrome manifests as a deregulation of the MyosinVa-RAB27a-melanofilin/SLAC2a complex which connects stage IV melanosomes to actin filaments in melanocyte dendrites (23).

For the optimal transport of proteins in the secretory pathway the maintenance of an intraluminal acidic pH is essential. Abnormal processing and traffic of tyrosinase in OCA2 (24), the most common form of albinism, or in amelanotic melanoma (25, 26) is due to defects in the mechanism of organellar pH regulation.

#### FIRST STEP: ENDOPLASMIC RETICULUM

Melanosomal membrane proteins are transported co-translationally in the ER by an N-terminal signal peptide as all proteins destined to enter the secretory pathway. While they are translocated in the ER, polypeptide chains undergo post-translational modifications. N-linked glycans trimming by resident glycosidases and the interaction with chaperone molecules results in correct folding of melanosomal proteins, which are then exported from ER. It is well known that protein misfolding or aggregation leads to their ER retention and further degradation. ER quality control function ensures the sorting of correctly processed proteins for their transport to Golgi apparatus.

Studies performed on B16F1 mouse melanoma cells have shown that the ER retention time is different for each tyrosinase protein: while TRP-2 is retained in the ER for 30 minutes (27), TRP-1 is processed in only 20 minutes (28) and

tyrosinase is retained in the organelle lumen approximately 3 hours (29). Tyrosinase and TRP-1 which are highly homologous in their amino-acids sequence presents different biosynthesis and maturation kinetics. While TRP-1 molecules are maturing rapidly and leave ER, a large proportion of tyrosinase polypeptides is retained in the ER for a longer period and sent to degradation. This degradation involves protein ubiquitination and activation of cytosol proteasomes.

Dynamic association with calnexin and calreticulin, which bind to tyrosinase and TRP-1 oligosaccharides facilitates their proper folding. The difference in their N-linked glycans can explain differences in their ER maturation kinetics. Another difference is that blocking TRP-1 interaction with calnexin does not impair protein maturation, since following the action of Golgi endomannosidases TRP-1 molecules bearing complex glycans were observed. In NB-DNJ treated cells the polypeptide binds BiP and acquires a stable conformation through formation of S-S bonds. It has been demonstrated that an important role in folding and stability of TRP-1 is played by the post-translational formation of disulphide bonds which is mediated by CNX interaction in the ER (28).

TRP-2 is folded in the ER in the presence of calnexin until a DTT-resistant conformation is obtained, which allows export to Golgi. In the presence of N-glycosylation inhibitors which prevent CNX association, TRP-2 is rapidly sent to proteasomal degradation. Studies demonstrate that TRP-2 follows a maturation pathway different from tyrosinase and TRP-1 (27).

In the early stages of ER processing N-linked glycans of the immature tyrosinase (Glc3Man9GlcNAc2) are sequentially cleaved by the  $\alpha$ -glucosidases I and II. Tyrosinase monoglycosylated glycans (Glc1Man9GlcNAc2) interact with calnexin and calreticulin which improve folding efficiency of the nascent chain (30). The role of glycosylation in the correct folding of tyrosinase was studied by Branza-Nichita *et al.* (29). Experiments showed that mouse tyrosinase mutants with less than two of the six sites occupied do not interact with calnexin and have no enzymatic activity. Any pair of combinations between the four occupied sites (1, 4, 5 and 6) in the normal protein ensures CNX interaction and partially restores the protein enzymatic activity. Folding kinetics and optimal activity is provided by the occupation of sites 1 and 6. It has been demonstrated that partial activity is obtained following incomplete copper ions loading.

Properly folded tyrosinase is released from the complex with chaperones during the glucose cleaving reaction catalysed by  $\alpha$ -glucosidase II. The export rate from the ER after incorporation into transport vesicles seems to be determined by both folding dynamics and cytosolic tale sorting signals. The CuB copper binding site is essential not only for tyrosinase ER export, but also for its proper Golgi maturation, followed by loading of two copper ions. There are studies showing that in fibroblasts deficient in Menkes copper transporter (mostly located in TGN) tyrosinase is inactive although it is processed to its mature form (31).

One of our recent studies showed that the transmembrane (TM) domain of tyrosinase plays an important role in tyrosinase folding indirectly favouring CNX interaction (32). This conclusion was drawn after studying the intracellular behaviour of tyrosinase chimera having the TM domain replaced by TMs from other proteins or by a GPI anchor, in parallel with the soluble form of the protein. We saw that ER membrane tethering restores CNX interaction absent in the case of soluble tyrosinase which is retained in the ER by CRT and BiP and sent to degradation.

#### SECOND STEP: TRANSPORT THROUGH GOLGI APPARATUS

Properly ER folded tyrosinases continue their journey in the secretory pathway by crossing the Golgi stacks. Vesicles containing melanosomal proteins bud from the ER and are trafficked forward by *dinein/dinactin* all along the microtubules towards *cis*-Golgi. In the Golgi, the *spectrin* network stabilises vesicles and continues the anterograde transport (17). During the transport from *cis*- to *trans*-Golgi the oligomannosidic N-linked glycans are processed to complex glycans resistant to EndoH digestion. Tyrosinase and TRP-1 are different in the nature and processing of glycans. Both have hybrid and complex N-linked glycans but only tyrosinase has O-linked glycans (33).

In the case of TRP-2, studies on B16F1 mouse melanoma cells (34) have shown that its post-ER traffic is distinct from that of TRP-1. This was demonstrated in the presence of monensin, a monovalent ionophore which participates in Na<sup>+</sup> ion exchange with H<sup>+</sup>. Monensin treatment does not affect TRP-1 processing to its EndoH resistant form. However, TRP-2 is retained in the EndoH-sensitive form with oligomannosidic glycans. The majority of studies describe a blockage between medial and *trans*-Golgi produced by monensin. Soluble proteins are retained in their EndoH-sensitive form, while transmembrane proteins are processed to their EndoH-resistant mature form. TRP-2 follows the soluble proteins pathway, sensitive to monensin treatment which affects cholesterol-rich membranes, where TRP-2 but not TRP-1 is probably localized. Between the two proteins there is also a difference in the speed of Golgi traffic, indicating once more that they take different routes. While TRP-2 passes Golgi in 2h30, TRP-1 is matured in only 40 minutes.

#### THIRD STEP: TGN AND POST-GOLGI TRAFFIC

*Trans*-Golgi compartment is a key sorting station from where the mature proteins are directed towards intra- and extracellular destinations *via* transport vesicles. At the TGN, sorting vesicles which present spectrin mosaics are transported to next compartments in conjunction with other motor proteins (17).

Membrane protein sorting at TGN is controlled by transport signals in the cytoplasmic domain which contain tyrosine and/or di-leucine residues which are

often decoded by heterotetrameric complex adaptor proteins (AP). These are composed of two large subunits ( $\alpha$ ,  $\gamma$ ,  $\delta$  or  $\epsilon$  and  $\beta$ ), a medium subunit ( $\mu$ ) and a small subunit ( $\sigma$ ) (35). Tyrosine-based signals are mainly recognized by AP  $\mu$  subunits while di-leucinic [DE]xxxL[LI] signals can interact with  $\mu$  subunits of AP-1 or AP-2, a combination of  $\gamma$  and  $\sigma 1$  of AP-1 or  $\delta$  and  $\sigma 3$  subunits of AP-3 (36). Some melanosomal proteins such as tyrosinase, TRP-1 and Pmel17 have one or both sorting signals in their amino-acids sequence (35).

Up to now, four adaptor proteins complexes have been identified: AP-1, AP-2, AP-3 and AP-4 (37). AP-1, AP-3 and AP-4 function at the TGN or endosomal site while AP-2 functions at the plasma membrane (38).

The role of APs in melanosomal protein transport has been first unravelled on melanocytes isolated from patients with Hermansky-Pudlak type 2 syndrome (HPS-2) with mutations in the  $\beta 3A$  subunit of AP-3. In these cells tyrosinase is misrouted to multivesicular bodies (MVB) (39). Moreover, the lack of a functional AP-3 leads to a hypopigmented melanosomes phenotype.

Both AP-1 and AP-3 have been proposed initially to be a part of the TGN sorting machinery, but recent data suggest that they mediate protein sorting at the endosomal level in melanocytes in partially redundant pathways (40).

Tyrosinase and LAMP-2 have been the first cargo proteins identified to bind AP-3 (41). Both contain the di-leucinic signal which binds  $\delta$ -adaplin/  $\sigma 3$ -adaplin AP-3 hemicomplex. Based on electronic microscopy studies it has been initially hypothesised that tyrosinase is transported in coated vesicles from Golgi to premelanosomal vesicles (42, 43).

By surface plasmon resonance (SPR) studies the interaction between the di-leucinic signal of tyrosinase and AP-3 was demonstrated (41). Also, it has been shown that AP-3 associates with clathrin (44) and binds the cytosolic domains of membrane proteins (45). AP-3 is present in the endocytic compartment and can play a role in the transport from early to late compartments (46) on the way to lysosomes, melanosomes or dense granules in the platelets (lysosome-related organelles, LRO). Later, the presence of two sorting determinants in the cytosolic tail has been demonstrated: i) a di-leucinic signal sufficient for tyrosinase transport to melanosomes/lysosomes and ii) a tyrosine-based signal (Y-X-X- $\emptyset$ ), where  $\emptyset$  is a bulky hydrophobic amino-acid, necessary for tyrosinase traffic to lysosomes by an indirect pathway from the plasma membrane. Both sorting signals are conserved and are present in the cytosolic domains of tyrosinase and TRPs in vertebrates (47). However, we found that tyrosinase mutants lacking the cytoplasmic tail were able to pass through the Golgi and reach the plasma membrane in the absence of these sorting signals, indicating that alternative transport pathways should be also considered (32).

Regarding TRP-1, transfection experiments performed in fibroblasts and B16 mouse melanoma cells have shown that a C-terminal di-leucine signal is required

for correct sorting to lysosomes/melanosomes (48). In the absence of this signal the protein is directed through the constitutive pathway to plasma membrane (49).

Recent *in vitro* studies on transport vesicles derived from TGN have shown that AP-1 and AP-3 mediate melanosomal and lysosomal protein sorting on distinct post-Golgi traffic pathways (50). The same study demonstrates that transport vesicles budding depends on temperature, nucleotides, cytosol, Arf1 and adaptor. Experiments confirmed that TRP-1 (Fig. 3 – dashed line) sorting requires AP-1 and tyrosinase (Fig. 3 – round dotted line) sorting is mediated by AP-3. Clathrin depletion abrogates both traffic pathways demonstrating that AP-1 and AP-3 generate clathrin coated vesicles.

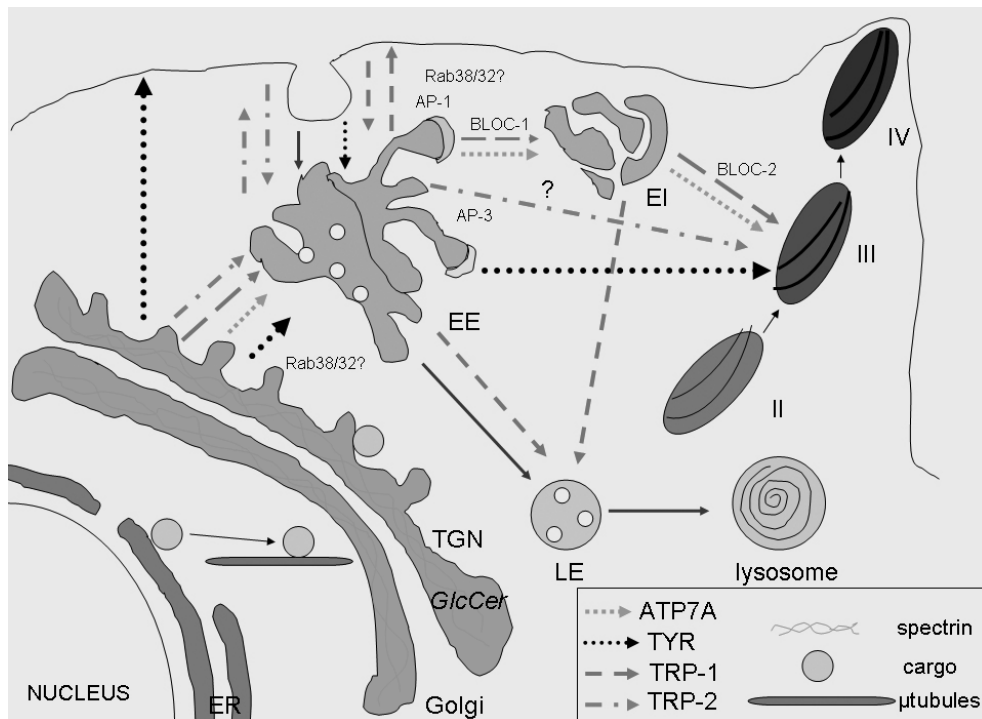


Fig. 3. – Traffic of tyrosinase (round dotted line), TRP-1 (long and small dashed line) and TRP-2 (dashed dotted line) from TGN to melanosomes.

In case of TRP-2 (Fig. 3 – dashed line), studies performed on B16 cells (34) have shown that its post-Golgi traffic follows a common pathway with the cargo destined for plasma membrane *via* early endosomes. TRP-2 is rapidly recycled from plasma membrane, similarly to TGN38 and furin. In the presence of chloroquine (CQ), a quaternary amine which accumulates in acidic compartments and interferes with endocytic pathways, TRP-2 no longer colocalizes with TGN,

but accumulates in vacuolae positive for early and late endosome markers where it degrades. Post-Golgi pathway followed by TRP-2 is distinct from that of TRP-1, which is not affected by CQ treatment and is transported to melanosomes. Unlike tyrosinase and TRP-1, TRP-2 (and OA1) has no di-leucinic signal in its cytosolic tail but has a tyrosine-based signal. Usually this is recognized by AP-1 in the TGN and/or endosomes and by AP-2 at the plasma membrane.

Melanosomal proteins transport to melanosomes is mediated also by products of other genes associated with HPS than AP. These genes code for subunits of protein complexes called BLOC-1, -2 and -3 (*biogenesis of lysosome-related organelles complex*). All BLOC complexes are expressed in a variety of cell types and exist as both soluble and transmembrane forms (51).

Functional connexions between BLOC and AP-3 complexes were recently studied by Di Pietro *et al.* (52) through biochemical, genetics and cell biology methods. This study reveals physical and functional interactions between BLOC-1, BLOC-2 and AP-3 involved in cargo traffic from endosomes. BLOC-1 interacts with BLOC-2 or AP-3 in the endosomal membranes and regulates their association/dissociation. Melanocytes deficient in AP-3 have a rather abnormal TRP-1 traffic, which is severely affected in BLOC-1 deficient cells. The conclusion that TRP-1 does not have a totally AP-3-independent traffic is in line with a previous study (39). TRP-1 traffic is partially dependent on BLOC-2. Defective function of either of the three complexes results in missorting of TRP-1 from early endosomes to plasma membrane (Fig. 3 – small dashed lines). A fraction seems to be sent to degradation in lysosomes (Fig. 3 – small dashed lines) (52). Tyrosinase instead (Fig. 3 – round dotted line) is transported to melanosomes by a mechanism dependent on AP-3, but not BLOC-1 (39). Di Pietro hypothesis is that BLOC-1 can be recruited by AP-3 positive vesicles first after their budding from the endosomal membrane.

Research performed more recently sustains the idea that melanosomes maturation needs two transport pathways from late endosomes to melanosomes: one mediated by AP-3 and the other mediated by BLOC-1 and BLOC-2, which are deficient in various forms of HPS (53). Data suggest that TRP-1 follow by default a BLOC-1 mediated pathway and AP-3 participates in other cargo transport. It has been shown that loss of BLOC-2 function interferes with TRP-1 transport from an intermediary endosomal compartment partially located downstream of BLOC-1 to melanosomes redirecting the protein to late endosomes/lysosomes for degradation.

Traffic through early endosomes requires also the function of other protein complexes such as HOPS-*homotypic* fusion and vacuole *protein sorting* (subunit Vps33a) and Rab GGT II (subunit alpha) and Rab38 regulates transport through endosomal intermediates. HOPS could regulate crucial SNARE interactions needed for endosomes-melanosomes traffic (23). Melanocytes are among the few cell types expressing both Rab32 and Rab38 which have possibly a redundant function in transport of proteins to melanosomes (54). It has been shown that Rab7 is

involved in TRP-1 traffic which is redistributed into the cell in the absence of the endosomal protein.

The model proposed by Setty for BLOC-1 function is the following: BLOC-1 complex promotes protein export from early endosome membranes to melanosomes. Enhanced traffic towards plasma membrane and endosomal content of TRP-1 would reflect inefficient cycles of endocytosis and recycling which result from incapacity in *emptying* the endosomes in melanosomes. This model explains the complete absence of TRP-1 from melanosomes, vacuolar endosomes accumulation and inefficient recycling of TRP-1 in BLOC-1 deficient cells. TRP-1 accumulation in a partially distinct compartment downstream of BLOC-1 in BLOC-2 deficient cells is in favour of the model by which endosomes-derived vesicles in BLOC-1 pathway are trafficked through an intermediary compartment from where BLOC-2 facilitates fusion with melanosomes. This intermediary compartment can be the one from which Rab 38 regulates tyrosinase and TRP-1 transport to melanosomes (54).

These discoveries put in evidence the importance of endosomes as intermediates in the post-Golgi traffic to lysosomes/melanosomes and the adaptability of endosomal system in specialized cell types such as melanocytes.

In melanocytes, tyrosinase and TRP-1 are transported to melanosomes by an intracellular pathway dependent on glycosphingolipids (21). Recent studies have shown that luminal domain of melanosomal proteins contains informations both necessary and sufficient for their sorting to melanosomes. Hence, lysosomal and melanosomal proteins contain different informations which are most probably transduced to cytosol where adaptor complexes differentiate between transport signals and send the proteins to target organelles. In this process an essential role is played by glucosylceramide (GlcCer) (55). This can be important for correct sorting of melanosomal proteins by several mechanisms: i) participation to oligomerisation of proteins which have in this form an increased affinity for adaptor protein binding; in the absence of GlcCer the APs interact with lysosomal proteins which compete with tyrosinases for their binding; ii) GlcCer can induce melanosome protein aggregation by forming lipid domains of glycosphingolipids/cholesterol; iii) GlcCer modifies the pH of TGN lumen to favour tyrosinase and TRP-1 oligomerisation.

In GM95 cells deficient in glucosylceramide synthase (GLC negative) tyrosinase accumulates in the Golgi apparatus and in the multivesicular bodies while TRP-1 is trafficked in structures similar to melanosomes where it colocalizes with LAMP-1, but is also recycled to plasma membrane like in AP-3 deficient cells. Hence, a hypopigmentation due to transport defects is produced and stage II–IV melanosomes are lacking. Pigmentation can be restored by transfection with a tyrosinase chimera having a longer transmembrane domain which is not retained in the TGN or by NH<sub>4</sub>Cl/bafilomycin A treatment, but TRP-1 sorting and melanosome assembly remains affected.

## FINAL STEP: ACQUIRING ACTIVITY

Tyrosinase activity is dependent on binding and function of two copper ions in its catalytic site (56), which is facilitated by the ATP7A copper transporter, mutated in the Menkes disease (31). Recently performed experiments (53) have shown that tyrosinase binds copper only transiently in the TGN of murine melanocytes. To catalyze melanogenesis initiating reactions, tyrosinase is reloaded with copper in the melanosomes. Copper is transported in melanosomes by ATP7A, which does not only localize to Golgi and TGN, but also in pigmented melanosome membranes. As in the case of TRP-1 (but not tyrosinase), ATP7A is transported to melanosomes by BLOC-1 (Fig. 3 – square dotted line). This is how the activity of the metalloenzyme is spatially controlled. These recent discoveries explain the phenotype of melanocytes having BLOC-1 mutations in the Hermansky-Pudlak syndrome. Defects in ATP7A localization contribute to hypopigmentation and probably other systemic defects present in patients manifesting this disease. In these cells the copper transporter is localized predominantly in the perinuclear area and peripheral early endosomes where it colocalizes with TRP-1 or EEA1 and syntaxin 13, respectively. This observation demonstrates that ATP7A remains at the Golgi apparatus membranes, vacuolar and tubulovesicular endosomes and multivesicular late endosomes.

It has also been shown that ATP7A and TRP-1 are in close proximity in an intermediary transport compartment before BLOC-1 interaction, although it does not need TRP-1 for traffic to melanosomes.

In conclusion, it was demonstrated that tyrosinase is inactive in BLOC-1 mutants; exposure to L-DOPA has raised tyrosinase activity only in TGN and adding copper sulphate has led to tyrosinase activation in melanosomes. Tyrosinase is initially activated in TGN, but it is inactive in unpigmented melanosomes because of the lack in copper. Studies show that tyrosinase inefficiently binds copper ions in TGN and loses them in the endosomes on its way to melanosomes. This is a protective mechanism of the cell against the toxic effect of reaction intermediates of tyrosinase which is active only in melanosomes.

Tyrosinase is inactive at acidic pH probably due to protonation of histidines which coordinate copper ions. Melanosomes become progressively more alkaline during maturation which creates proper conditions for copper rebinding and tyrosinase reactivation (57).

Tyrosinase activation is associated also with phosphorylation of serine residues in its cytosolic domain by PKC-  $\beta$ I (58) and formation of a complex between phosphorylated tyrosinase and TRP-1 (59).

It has been recently shown that tyrosinase activity can be also inhibited by factors affecting cell-cell signaling between keratinocytes and melanocytes (60).

## CONCLUSIONS

Correlations between skin and eye pigmentation in mammals and tyrosinase/TRPs expression were first observed in the '90s after cloning their coding genes. Multiple studies were performed since then including gene expression, post-translational modifications, intracellular localization, traffic pathways and essential protein interactions. Nevertheless, despite the progress made in the recent years regarding detailed knowledge on transport pathways of tyrosinase and TRP-1 highlighting the role of endosomes in the TGN to melanosomes transport there are still many questions remaining. The traffic pathway of TRP-2 from Golgi to melanosomes has not been discovered yet, although it has been proposed that it trafficks through early endosomes by recycling from the plasma membrane. Another unresolved issue is the involvement of ubiquitinary protein complexes AP-3, BLOC and HOPS in forming endosomal domains that function in tissue-specific sorting events. There is a need of investigations regarding SNARE distribution between endosomal and melanosomal membranes and protein-protein interaction mediating subcompartmentalization of the endosomal system.

Finally, we need to know more in depth the melanosomal intraluminal milieu to understand the conditions necessary for the proper interaction of proteins participating in melanogenesis.

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