Traffic 2008; **9:** 951–963
Blackwell Munksgaard

doi: 10.1111/j.1600-0854.2008.00740.x

Glycolipid-Dependent Sorting of Melanosomal from Lysosomal Membrane Proteins by Lumenal Determinants

Sophie Groux-Degroote[1,2], Suzanne M. van Dijk[1,3], Jasja Wolthoorn[1,4], Sylvia Neumann[1], Alexander C. Theos[5], Ann M. De Mazière[3], Judith Klumperman[3], Gerrit van Meer[1,*] and Hein Sprong[1,6]

[1]Membrane Enzymology, Bijvoet Center and Institute of Biomembranes, Utrecht University, 3584CH Utrecht, The Netherlands
[2]Current address: Unité de Glycobiologie Structurale et Fonctionnelle, UMR CNRS 8576, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq, France
[3]Cell Microscopy Centre, Department of Cell Biology, Institute of Biomembranes, University Medical Centre Utrecht, 3584CX Utrecht, The Netherlands
[4]Current address: Analytical Research Department, TNO Quality of Life, 3700AJ Zeist, The Netherlands
[5]Department of Human Science, Georgetown University, Washington, DC 20057, USA
[6]Current address: Laboratory for Zoonoses and Environmental Microbiology, National Institute of Public Health and Environment (RIVM), 3720BA Bilthoven, The Netherlands
**Corresponding author: Gerrit van Meer, g.vanmeer@uu.nl*

Melanosomes are lysosome-related organelles that coexist with lysosomes in mammalian pigment cells. Melanosomal and lysosomal membrane proteins share similar sorting signals in their cytoplasmic tail, raising the question how they are segregated. We show that in control melanocytes, the melanosomal enzymes tyrosinase-related protein 1 (Tyrp1) and tyrosinase follow an intracellular Golgi to melanosome pathway, whereas in the absence of glycosphingolipids, they are observed to pass over the cell surface. Unexpectedly, the lysosome-associated membrane protein 1 (LAMP-1) and 2 behaved exactly opposite: they were found to travel through the cell surface in control melanocytes but followed an intracellular pathway in the absence of glycosphingolipids. Chimeric proteins having the cytoplasmic tail of Tyrp1 or tyrosinase were transported like lysosomal proteins, whereas a LAMP-1 construct containing the lumenal domain of Tyrp1 localized to melanosomes. In conclusion, the lumenal domain contains sorting information that guides Tyrp1 and probably tyrosinase to melanosomes by an intracellular route that excludes lysosomal proteins and requires glucosylceramide.

Key words: adaptor complex, glycosphingolipids, lysosomal protein, melanosome assembly, melanosomal enzyme, protein sorting, secretory lysosome

Received 21 December 2007, revised and accepted for publication 25 March 2008, uncorrected manuscript published online 29 March 2008, published online 15 April 2008

Melanin pigment is synthesized in melanosomes, specialized organelles in melanocytes that are situated along the secretory pathway but originate from a coated endosome (1). Pigmentation-related disorders are caused by defects in the formation of proper melanosomes. The main form of oculocutaneous albinism is OCA2, which has been traced to mutations in the P-protein (2), a melanosomal membrane protein of unknown function that has homology to anion channels. Other forms are caused by defects in melanosomal enzymes, notably of the first two enzymes in the pathway, tyrosinase and tyrosinase-related protein 1 (Tyrp1). Yet, other forms display no appreciable defect in the enzymes themselves, but in their routing to the melanosomes. For example, Hermansky–Pudlak syndrome type II patients bear mutations in the β3A subunit of the heterotetrameric adaptor protein (AP)-3 complex (3). This adaptor complex has been shown to bind to a dileucine signal in the cytosolic tail of tyrosinase (4), but probably not Tyrp1 (5), and is involved in tyrosinase transport to the melanosomes (5,6). Notably, AP-3 also binds to the cytoplasmic tails of lysosomal membrane proteins (4), the bulk of which in melanocytes is transported to the lysosomes and not to the melanosomes (1). Moreover, an increase in cell surface appearance of lysosome-associated membrane protein 1 (LAMP-1) has been well characterized in AP-3-deficient cells (3,7–9). In this study, we address how melanocytes differentially sort their lysosomal and melanosomal membrane proteins to better understand the potentially conserved, but as yet poorly understood, mechanisms of sorting between endocytic organelles that share common progenitor compartments.

We have previously reported that the glycosphingolipid-deficient melanocyte line GM95 lacks pigmentation because of defects in transport of tyrosinase (10). Tyrosinase accumulates in the Golgi area of GM95 cells, whereas Tyrp1 still reaches melanosome-like structures. However, also Tyrp1 transport was changed because an increased amount reached the cell surface, and its endocytosis as measured by the uptake of anti-Tyrp1 antibodies is increased. GM95 cells do not synthesize glycosphingolipids because they lack a functional glucosylceramide synthase (GCS). Protein sorting and melanin synthesis could be restored by transfection with functional GCS,

showing that glycosphingolipids are required for intracellular transport of melanosomal proteins. Conversely, knocking down GCS by RNA interference (RNAi) inhibited pigmentation (11). Exactly which glycosphingolipids are involved in melanosomal protein sorting and at which step of the transport route has remained unclear.

The glycosphingolipid glucosylceramide (GlcCer) is synthesized by GCS in all mammalian cells and serves as the basis for a highly polymorphic class of complex glycosphingolipids (12). Glycosphingolipids are of vital importance for mammals, and defects in GCS expression are embryonically lethal in mice (13). There is ample evidence to suggest that glycosphingolipids are not distributed randomly over membrane surfaces but that they concentrate with cholesterol at specific locations into lipid rafts and that this forms the basis for their selective transport in the vacuolar membrane system of mammalian cells (14). Originally, this mechanism was proposed to drive the sorting of apical from basolateral membrane proteins in the secretory pathway of epithelial cells (15). In neuroendocrine cells, lipid rafts were reported to sort proteins at the Golgi into the secretory granules (16). Independently, evidence has been provided for a role for lectins in sorting membrane proteins into the apical transport pathway (17). The missorting of melanosomal proteins in the absence of glycosphingolipids (10) suggests that protein sorting toward lysosome-related organelles also may involve glycosphingolipid rafts or lectins.

In this study, we addressed the role of glycosphingolipids in the biogenesis of functional organelles. We demonstrate that the glycosphingolipid GlcCer, and not a higher glycosphingolipid, is required for the intracellular sorting of melanosomal proteins and melanosome biogenesis. In contrast, the sorting of lysosomal membrane proteins by an intracellular pathway only occurred in the absence of glycosphingolipids. From experiments in which domains were swapped between melanosomal, lysosomal and plasma membrane proteins, we conclude that the discrimination between melanosomal and lysosomal proteins depends on lumenal sorting information in the cargo proteins. We discuss how this may be linked to the glycosphingolipids.

Results

Melanosome biogenesis requires GlcCer, not higher glycolipids

The wild-type mouse melanocyte cell line MEB4 expresses three major glycolipids: GlcCer, lactosylceramide (LacCer) and the ganglioside GM3 (18). To address which glycosphingolipid is required for pigmentation, we reduced their levels by enzyme knockdown. MEB4 cells were stably transfected with vectors that direct the synthesis of small interfering RNAs against ceramide transport protein (CERT) (19), GCS, LacCer synthase (LacCerS) and lamin as a control. Knocking down CERT or GCS decreased the GlcCer and GM3 levels to $<30\%$ of control values. This reduction was sufficient for creating a pigmentation defect (Figure 1A). Knocking down LacCerS did not significantly change the level of newly synthesized GlcCer but reduced GM3 (through a reduction in LacCer) by a similar 70% as in the CERT and GCS knockdown. This had no effect on cell pellet color, showing that the level of newly synthesized GlcCer, not LacCer or GM3, determined pigmentation.

The pigmentation defect in the absence of glycosphingolipids is accompanied by a change in the transport route of Tyrp1 (10). Like tyrosinase, Tyrp1 is normally transported from the Golgi to the melanosomes without passing over the cell surface. However, in GM95 cells, the presence of Tyrp1 on the cell surface and its endocytosis as measured by the uptake of anti-Tyrp1 antibodies are increased (10). The level of antibody uptake reflects Tyrp1 'missorting'. It inversely reflects how efficiently Tyrp1 is transported to the melanosomes through the intracellular route. Tyrp1 missorting in GM95 cells was corrected by transfection of GCS and by exogenous glucosylsphingosine (GlcSph; Figure S1), which yields GlcCer independent of GCS (10). Knocking down CERT or GCS in MEB4 cells induced anti-Tyrp1 antibody uptake. Addition of GlcSph to CERT or GCS knockdown cells restored the intracellular transport of Tyrp1 (Figure 1B) and pigmentation (not shown). In contrast, knocking down LacCerS in MEB4 cells did not induce antibody uptake. We conclude that the intracellular transport route of Tyrp1 requires GlcCer and that LacCer or more complex glycosphingolipids are not needed.

The distribution of tyrosinase and Tyrp1 in cells with and without glycosphingolipids

To elucidate the role of glycosphingolipids in the transport of melanosomal enzymes, we first wanted to compare at high resolution the intracellular localization of tyrosinase and Tyrp1 in the presence and absence of glycosphingolipids in MEB4 and GM95 cells (10). In wild-type MEB4 cells, tyrosinase and Tyrp1 are largely separated from the lysosomal marker LAMP-1 by immunofluorescence microscopy (Figure 2), corroborating that melanosomes and lysosomes are distinct organelles with different protein composition (1). In GM95 cells, tyrosinase transport is blocked in the Golgi area (10) (Figure 2). To define this block in more detail, we treated GM95 cells with microtubule-depolymerizing nocodazole, leading to dispersion of the Golgi into numerous 'ministacks' (20). Within ministacks, tyrosinase still largely colocalized with the *trans* Golgi marker α2,6-sialyltransferase. Immunogold labeling of ultrathin cryosections of GM95 cells localized tyrosinase not only in the *trans* Golgi network (TGN) but also in nearby multivesicular bodies (MVBs). In contrast, Tyrp1 displayed a typical melanosomal pattern also in GM95 cells, but a significant fraction of Tyrp1 was found

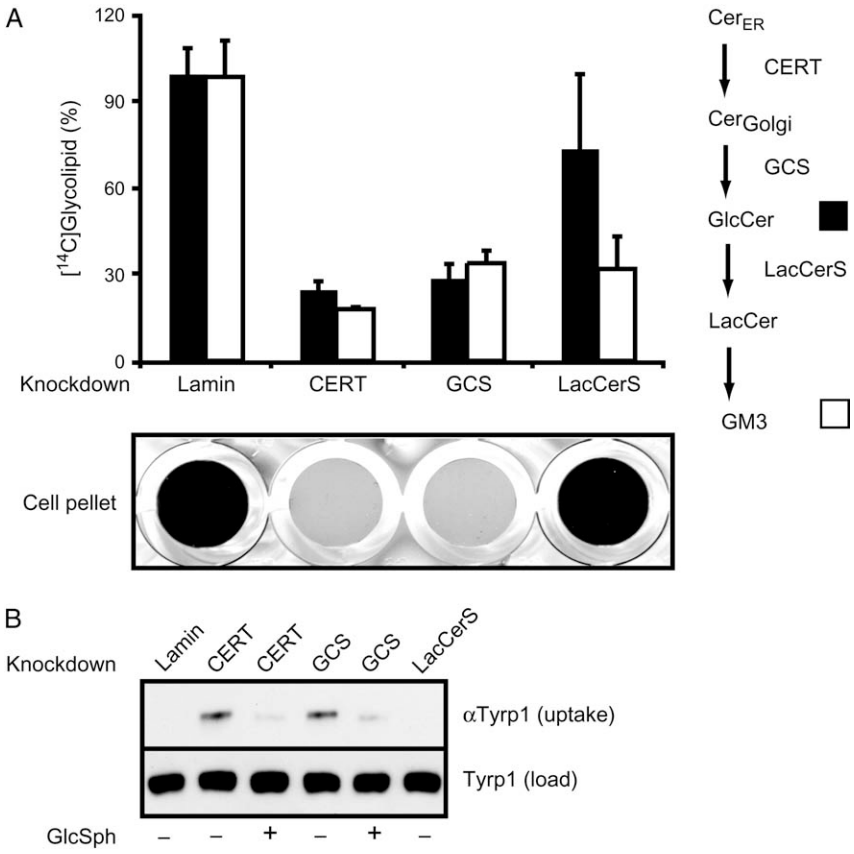

Figure 1: Role for GlcCer but not for LacCer in pigmentation. A) MEB4 cells stably expressing RNAi plasmids against CERT, GCS, LacCerS or lamin as a control were incubated overnight with [^{14}C]serine. Lipids were extracted, separated by two-dimensional-thin-layer chromatography and radiolabeled lipids were quantified as described in *Materials and Methods*. Values are expressed as a percentage of GlcCer (black bars) or GM3 (open bars) in control cells (RNAi against lamin) and are the mean of two independent stable clones ± range. Parallel cell monolayers were scraped, pelleted and photographed. B) Independently, 3-cm dishes having the same number of MEB4 cells expressing RNAi against the indicated proteins (knockdown) were incubated with αTyrp1 antibody (TA99) for 3 h at 37°C. After washing, the internalized antibody was visualized by blotting using a horseradish peroxidase-conjugated secondary antibody. Total Tyrp1 in the samples was detected by Western blotting using anti-pep1 (load). Tyrp1 missorting induced by CERT or GCS knockdown was corrected by the continuous presence of GlcSph.

to colocalize with LAMP-1 in endosomes (Figure 2 and Table S1).

Melanosome assembly strictly depends on GlcCer, pigmentation does not

Next, we assessed by electron microscopy whether GlcCer was required for both the pigmentation and the presence of mature melanosomes. MEB4 melanoma cells display all stages of melanosome biogenesis (21,22), that is, stage I, equivalent to an endosome with a bilayered coat; stage II, with a fibrillar content; stage III, with increasing melanin deposition and the mature stage IV (Figure 3). Immunogold labeling revealed that the melanosomal enzymes tyrosinase, Tyrp1 and Pmel17 occur in the melanosomal stages I–IV and less extensively in early endosomes and MVBs (Table S1), in line with previous observations (1,5). In contrast, electron microscopical analysis of glycosphingolipid-deficient GM95 cells revealed that they were completely devoid of the characteristic stages II, III and IV melanosomes, although a subpopulation of MVBs and lysosomes contained a core with regular transverse and longitudinal striations, which were rare in MEB4 cells (Figure 3B). These striations were different in appearance from the flexible fibrils in stage II melanosomes. The MVBs containing striated bodies labeled positive for the lysosomal markers LAMP-1 and cathepsin D as well as for the melanosomal enzymes tyrosinase and Tyrp1 (Table S1). GM95 cells could not be labeled with the anti-Pmel17 antibody HMB45 because of a defect in GM95 cells that is independent of the lack of GCS activity (Figure S2). Unfortunately, HMB45 was the only available antibody to detect mature Pmel17 in mouse melanosomes and thus potential missorting of Pmel17 in GM95 cells could not be evaluated. The knockdown of GCS but not of LacCerS abolished pigmentation of MEB4 cells (Figure 1), while transfection of GM95 cells with a GCS producing GlcCer in the endoplasmic reticulum (ER) restored pigmentation (10). This correlated with the disappearance of pigmented melanosomes from MEB4 cells after knocking down GCS (MEB4-aGCS) but not LacCerS (MEB4-aLacCerS) and conversely with the recovery of tyrosinase-positive organelles morphologically identical to stage II and melanized stages III and IV melanosomes in the GM95–GCS cells (Figure 3).

Two other treatments restored pigmentation of GM95 cells: transfection with a tyrosinase construct with an elongated transmembrane domain that no longer accumulated in the Golgi area (10) and an incubation with ammonium chloride (NH_4Cl) or bafilomycin A (Figure S3). However, these treatments that did not induce GlcCer synthesis (not shown) did not induce proper melanosome assembly nor did they correct the missorting of Tyrp1

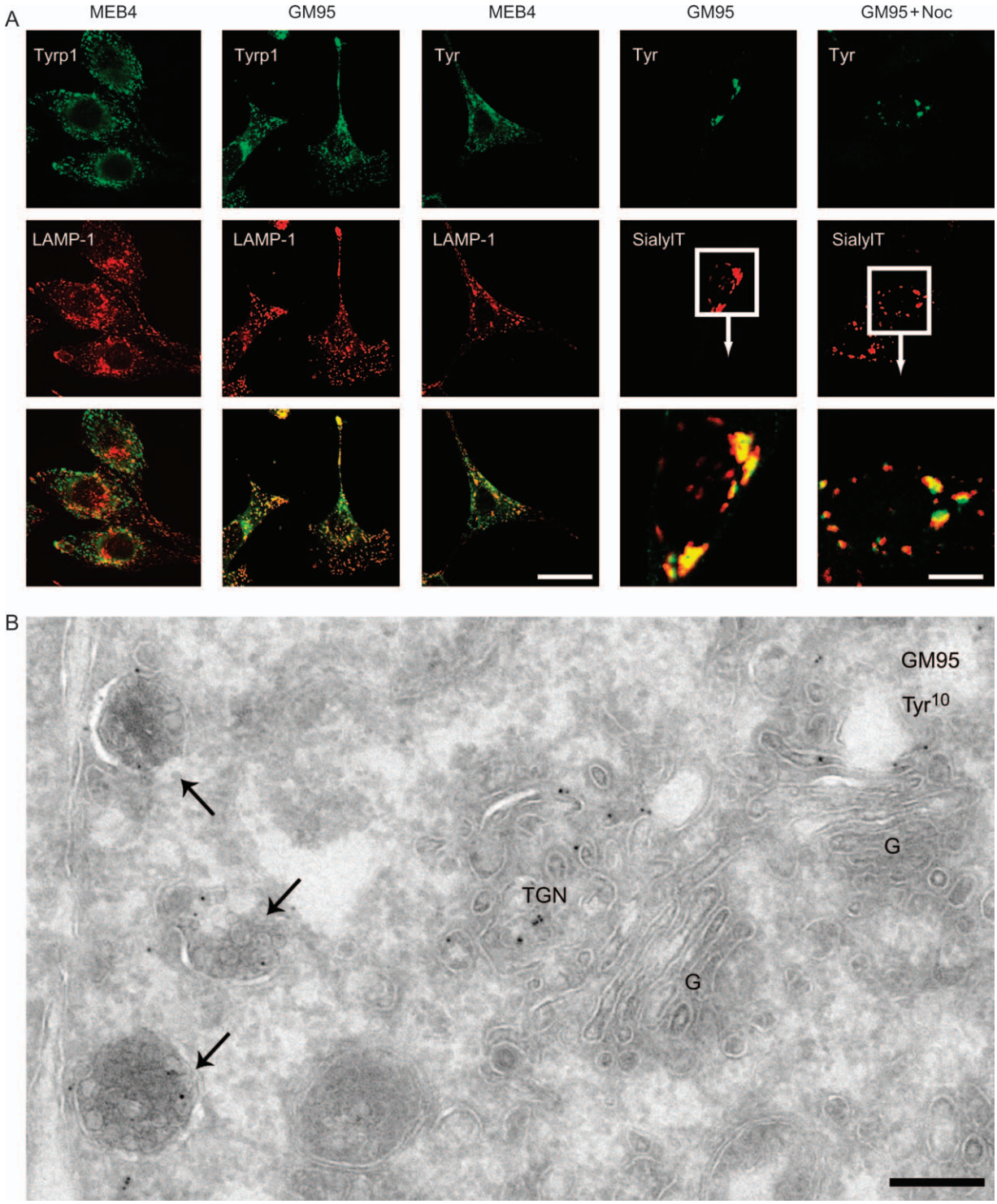

Figure 2: Localization of tyrosinase and Tyrp1 in MEB4 and GM95 cells. A) MEB4 cells, GM95 cells and GM95 cells transfected with the myc-tagged *trans*-Golgi marker α2,6-sialyltransferase and (mock) treated with 10 M nocodazole for 3 h, were labeled for Tyrp1 (anti-pep1), LAMP-1 (1D4B), tyrosinase (Tyr; anti-pep7) and sialyltransferase (SialylT; 9E10). The bottom row shows the merged images (bar, 20 μm) and the magnified merges of the boxed areas (bar, 7 μm). The antibodies to the cytoplasmic tail (anti-pep1) and the lumenal domain of Tyrp1 (TA99) showed perfect colocalization in MEB4 cells (not shown). B) An ultrathin cryosection of GM95 cells was labeled with anti-tyrosinase antibody and 10-nm gold particles. Tyrosinase is accumulated in the TGN and present in nearby MVBs (indicated by arrows). G, Golgi apparatus. Bar, 200 nm.

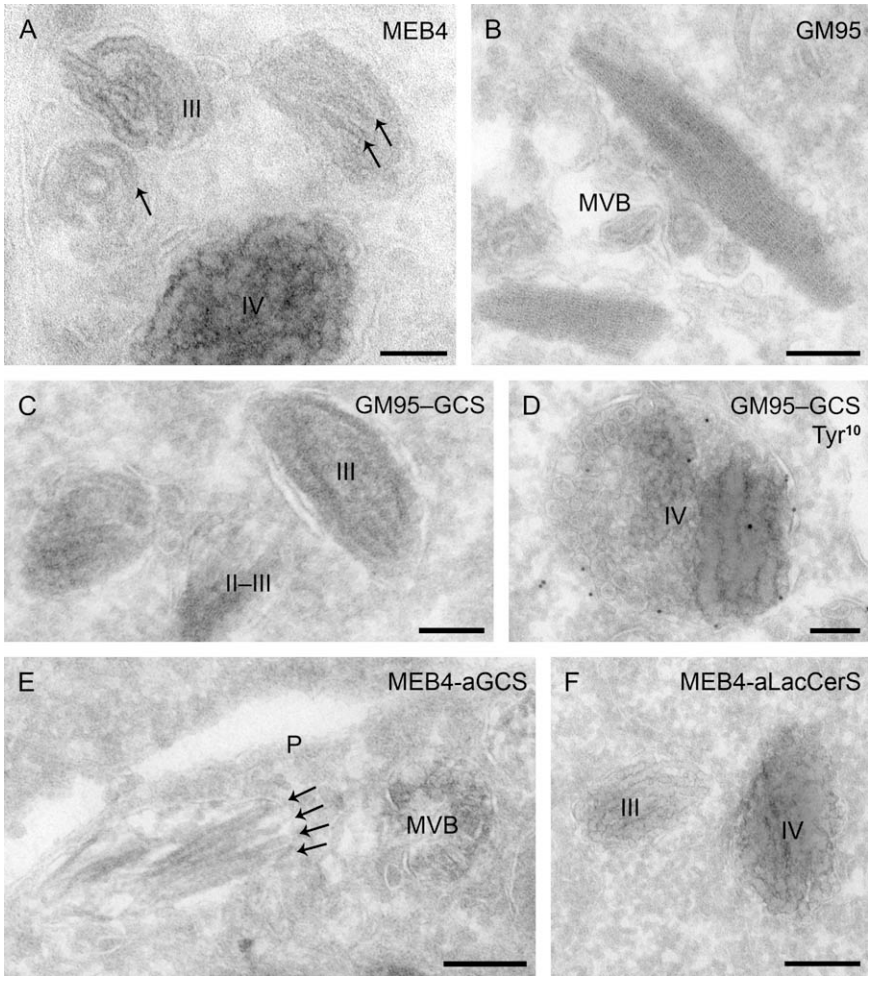

Figure 3: Melanosome formation in MEB4 but not in glycolipid-deficient GM95 cells. A–F) Ultrathin cryosections. A) MEB4 cells contain typical stage II melanosomes traversed by weakly banded fibrils of characteristic width (indicated by arrows) and stages III and IV melanosomes with increasing melanin deposition visible as electron density increases. B) GM95 cells, as well as GM95 cells transfected with tyrosinase–myc (shown here), have no characteristic stages II, III or IV melanosomes. Part of their MVBs contains rigid, longitudinally and transversely striated bodies. C and D) GM95 cells transfected with GCS display true melanosomes with fibrils undergoing melanization. These melanosomes can be labeled with anti-tyrosinase antibodies and 10-nm gold particles (D). E) MEB4 cells stably transfected with RNAi plasmids against GCS (MEB4-aGCS) have no characteristic melanosomes. Sometimes, longitudinal striations are found (indicated by arrows) that differ in width and structural organization from the fibrils and striations observed in MEB4 (A) or GM95 (B) cells, respectively. F). MEB4 cells stably transfected with RNAi plasmids against LacCerS (MEB4-aLacCerS) display all types of melanosomes. P, plasma membrane. Scale bars, 100 nm.

through the cell surface. Together, these results reinforce the notion that pigmentation can be induced in the absence of GlcCer but that GlcCer is required for correct protein sorting and melanosome assembly.

Intracellular transport of melanosomal proteins and routing through the surface of lysosomal proteins in melanoma cells, depending on their lumenal domain

Because the biogenesis of melanosomes requires segregation of melanosomal from lysosomal proteins, we compared their traffic in MEB4, GM95 and GM95–GCS melanocytes using the uptake of exogenous antibodies as a measure for routing through the cell surface. In wild-type melanocytes and MEB4 melanocytes, Tyrp1 reaches melanosomes by a strictly intracellular pathway (10,23,24). This was also observed for GM95–GCS cells. In these cells, part of the lysosomal LAMP-1 and -2 traveled over the cell surface (Figure 4A). In contrast, in GM95 cells, Tyrp1 was missorted, while now, LAMP-1 and -2 followed an intracellular pathway. Thus, the intracellular routing of Tyrp1 to the melanosomes depended on glycosphingolipids, and LAMPs were routed by an intracellular pathway only under conditions of Tyrp1 missorting.

The opposite transport behavior of the melanosomal and lysosomal proteins in MEB4 and GM95 cells implied that next to similar cytosolic sorting motifs, they must contain additional sorting information. To test whether sorting information resides in their lumenal domains, we tested three chimeras that contained the cytoplasmic tail of melanosomal proteins but the lumenal domain of plasma membrane proteins: CTT, lumenal domain of CD8 and transmembrane and cytoplasmic tail of Tyrp1; CCT, lumenal and transmembrane domains of CD8 and cytoplasmic tail of Tyrp1; and CCY, lumenal and transmembrane domains of CD25 and cytoplasmic tail of tyrosinase. First, we tested the effect of the lumenal domain on the steady-state accumulation of tyrosinase and Tyrp1 in the melanosomes. All chimeras fully colocalized with LAMP-1 both in MEB4 and in GM95 cells (Figure 5). This implies that the lumenal and/or transmembrane domains of tyrosinase and the lumenal domain of Tyrp1 were necessary for directing them to melanosomes; the cytosolic domains by themselves were sufficient for lysosomal targeting.

Next, we studied whether the altered destination of the tyrosinase and Tyrp1 chimeras correlated with a change in

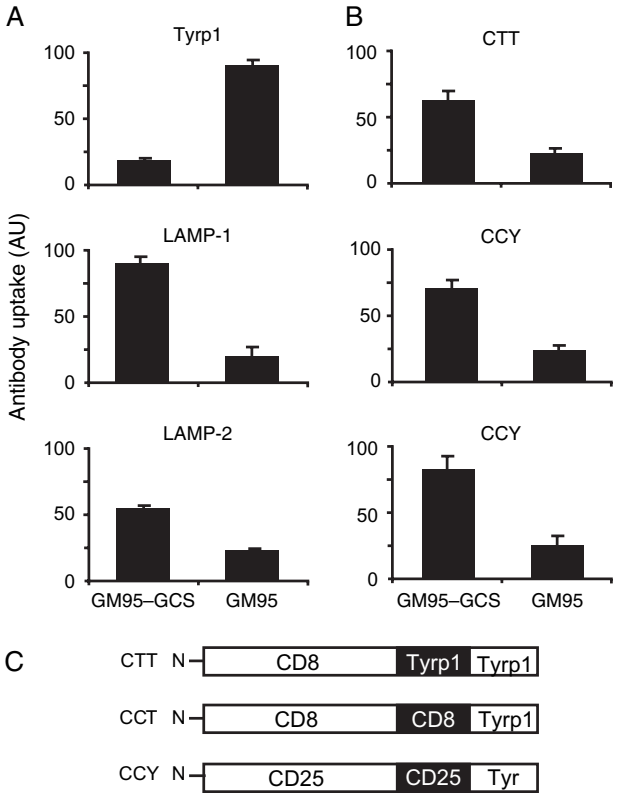

Figure 4: Transport of Tyrp1 and LAMPs in glycosphingolipid-deficient cells. A) GM95 and GM95–GCS cells were incubated with antibodies against the lumenal domain of Tyrp1, LAMP-1 or LAMP-2 for 3 h at 37°C. Internalized antibodies were visualized by Western blotting and quantified. Data are means of at least two independent experiments ($n \geq 2$). To allow a quantitative comparison, different amounts of each sample were loaded on gel and the total amount of LAMP-1 or Tyrp1 in the samples was measured as an internal control. Signals were quantified using the IMAGE QUANT software (*Materials and Methods*). B) GM95 and GM95–GCS cells were transiently transfected with plasmids containing the CTT, CCT and CCY constructs. After 1 day, the cells were incubated with antibodies against the lumenal domain of CD8 or CD25, and antibody uptake was determined as under (A). C) Chimera constructs: CTT, lumenal domain CD8 and transmembrane and cytoplasmic tail Tyrp1; CCT, lumenal and transmembrane domains CD8 and cytoplasmic tail Tyrp1; and CCY, lumenal and transmembrane domains CD25 and cytoplasmic tail tyrosinase. All amino termini are situated in the lumen and the carboxy termini in the cytosol. Transmembrane domains are depicted as black boxes.

routing. In MEB4 and GM95–GCS cells, we observed antibody uptake for each chimera just like for the lysosomal proteins, indicating routing through the cell surface (Figure 4). In contrast, in glycosphingolipid-deficient GM95 cells, essentially, no antibody uptake was observed. In addition, CTT routing through the cell surface was increased threefold in GM95 incubated with GlcSph (Figure S1), which restores all features of melanogenesis. Thus, replacement of the lumenal domain of Tyrp1 and the lumenal and the transmembrane domains of tyrosinase by those of plasma membrane proteins changed their trafficking pattern to that of lysosomal proteins.

To test directly whether the lumenal domain of Tyrp1 was sufficient for directing the protein into the intracellular transport pathway to melanosomes, we constructed a chimera that contained the lumenal domain of Tyrp1 and the transmembrane domain and cytoplasmic tail of LAMP-1 (TLL) (Figure 6A). In MEB4 cells, TLL colocalized with Tyrp1 and not with LAMP-1, while TLL did colocalize with LAMP-1 in GM95. The transport route of TLL was established in a pulse–chase and cell surface biotinylation experiment. The fraction of newly synthesized TLL present on the cell surface at various time-points during 1 h of chase increased up to sevenfold in GM95 cells compared with GM95–GCS cells (Figure 6B), just like that for Tyrp1 (10). This implies missorting of both TLL and Tyrp1 in glycosphingolipid-deficient GM95 cells.

Intracellular transport of melanosomal proteins is affected by AP-3 knockdown

Sorting of melanosomal and lysosomal proteins involves both AP-1- and AP-3-dependent steps (1,5). AP-1-mediated transport of three lysosomal enzymes through the mannose-6-phosphate receptors and the level of missorting to the cell surface were identical between GM95 and MEB4 cells (10). Because a dramatic increase in Tyrp1 routing through the cell surface like in GM95 cells was also found in pearl primary melanocytes that lack active AP-3 (23), we investigated AP-3-mediated sorting in GM95 cells. The expression levels of the various AP-1 and AP-3 subunits as well as their recruitment to membranes were similar in MEB4 and GM95 cells (Figure 7A). Furthermore, the ultrastructural localization of the AP-3 complex by immunogold labeling of ultrathin cryosections showed that in both cell types, AP-3 associated with buds evolving from tubular endosomal compartments (Figure 7B,D), just as previously shown in melanocytes (5) and non-melanosomal cells (25). To test whether AP-3-mediated sorting was functional in the absence of glycosphingolipids, the δ subunit of AP-3 was knocked down in MEB4 and GM95 cells by RNAi. Western blot analysis (Figure 7C) showed that the expression of δAP-3 was reduced to 20 and 30% in MEB and GM95 cells, respectively. In MEB4 cells, the AP-3 knockdown increased transport of both Tyrp1 and LAMP-1 over the cell surface. In GM95 cells, δAP-3 knockdown induced transport of LAMP-1 over the plasma membrane (like in fibroblasts; Figure S4) but did not lead to a further increase in Tyrp1 transport over the cell surface. The data show that AP-3 was directly or indirectly required for the intracellular transport of Tyrp1 in MEB4 cells and LAMP-1 in GM95 cells. In addition, the further increase in LAMP-1 routing through the plasma membrane after AP-3 knockdown in MEB4 cells indicates that also in MEB4 cells, part of LAMP-1 is transported by an AP-3-dependent intracellular route.

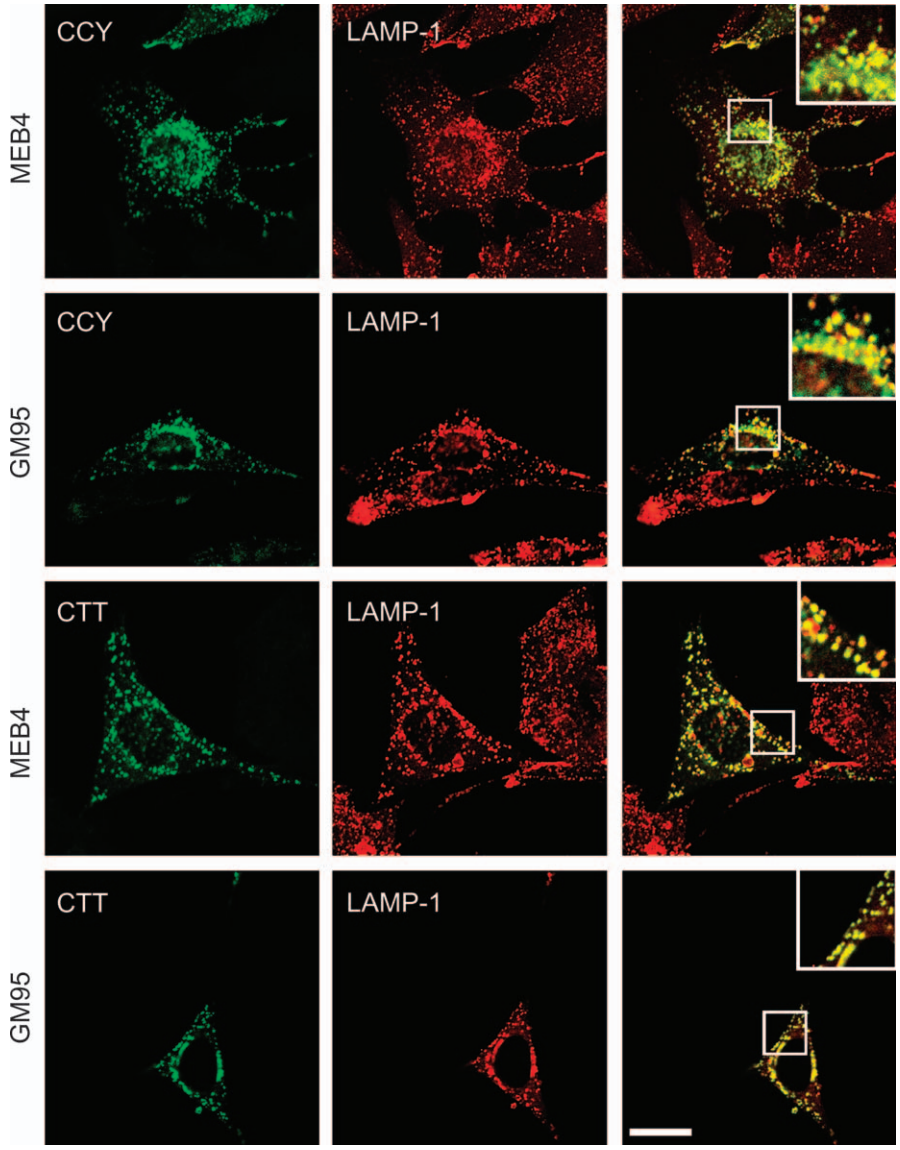

Figure 5: The lumenal domains of tyrosinase and Tyrp1 determine their melanosomal localization. MEB4 and GM95 cells were transfected with CCY (lumenal and transmembrane domains CD25 and cytoplasmic tail tyrosinase) or CTT (lumenal domain CD8 and transmembrane and cytoplasmic tail Tyrp1). Prior to fixation, cells were incubated with 20 μg/mL leupeptin for 3 h at 37°C to prevent lysosomal degradation of the lumenal CD25 and CD8 domains. Cells were double labeled with antibodies against LAMP-1 and CD25 (CCY) and CD8 (CTT) and analyzed. In contrast to tyrosinase (Figure 2), CCY is located to lysosomal structures in MEB4 as well as in GM95 cells. Also note the (strict) segregation between Tyrp1 and LAMP-1 in MEB4 cells (Figure 2) and the similar staining patterns of CTT and LAMP-1. The localization of CCT was similar to that of CTT (not shown). Bar, 10 μm.

Discussion

In melanocytes, the melanosomal proteins tyrosinase and Tyrp1 are transported to melanosomes by a strictly intracellular pathway that depends on glycosphingolipids. Unexpectedly, lysosomal membrane proteins, which in fibroblasts follow an intracellular pathway, were found to be present and endocytosed at the surface of melanocytes. In the absence of glycosphingolipids, this situation is reversed. Domain swapping showed that the lumenal domain of the melanosomal proteins contains sorting information for the intracellular pathway to the melanosomes that is both required and sufficient. This suggests that glycosphingolipids exert their critical effect on sorting at the lumenal surface of the sorting compartment.

GM95 cells are unable to synthesize glycosphingolipids, lack melanosomes and pigment, and missort melanosomal proteins (10). Furthermore, pigmentation, melanosomes and intracellular Tyrp1 transport were restored in GM95 by both GCS transfection and exogenous GlcSph. Knocking down CERT or GCS, but not LacCerS, in MEB4 cells mimicked the GM95 phenotype in loss of melanosomes and pigmentation and Tyrp1 missorting. This validates the GM95/GM95–GCS pair and the MEB4/MEB4-aGCS pair as systems to address the molecular role of GlcCer in pigmentation. A careful morphological analysis by electron microscopy demonstrated that GM95 cells and MEB4-aGCS did not contain stages II–IV melanosomes. Using this assay, we noticed that some treatments, notably transfection with tyrosinase with a longer transmembrane domain and treatment with NH_4Cl, restored pigmentation but not melanosome assembly. Also the intracellular transport of Tyrp1 to the melanosomes was not restored. In summary, GlcCer was not required for pigmentation but was essential for transport of melanosomal proteins to the

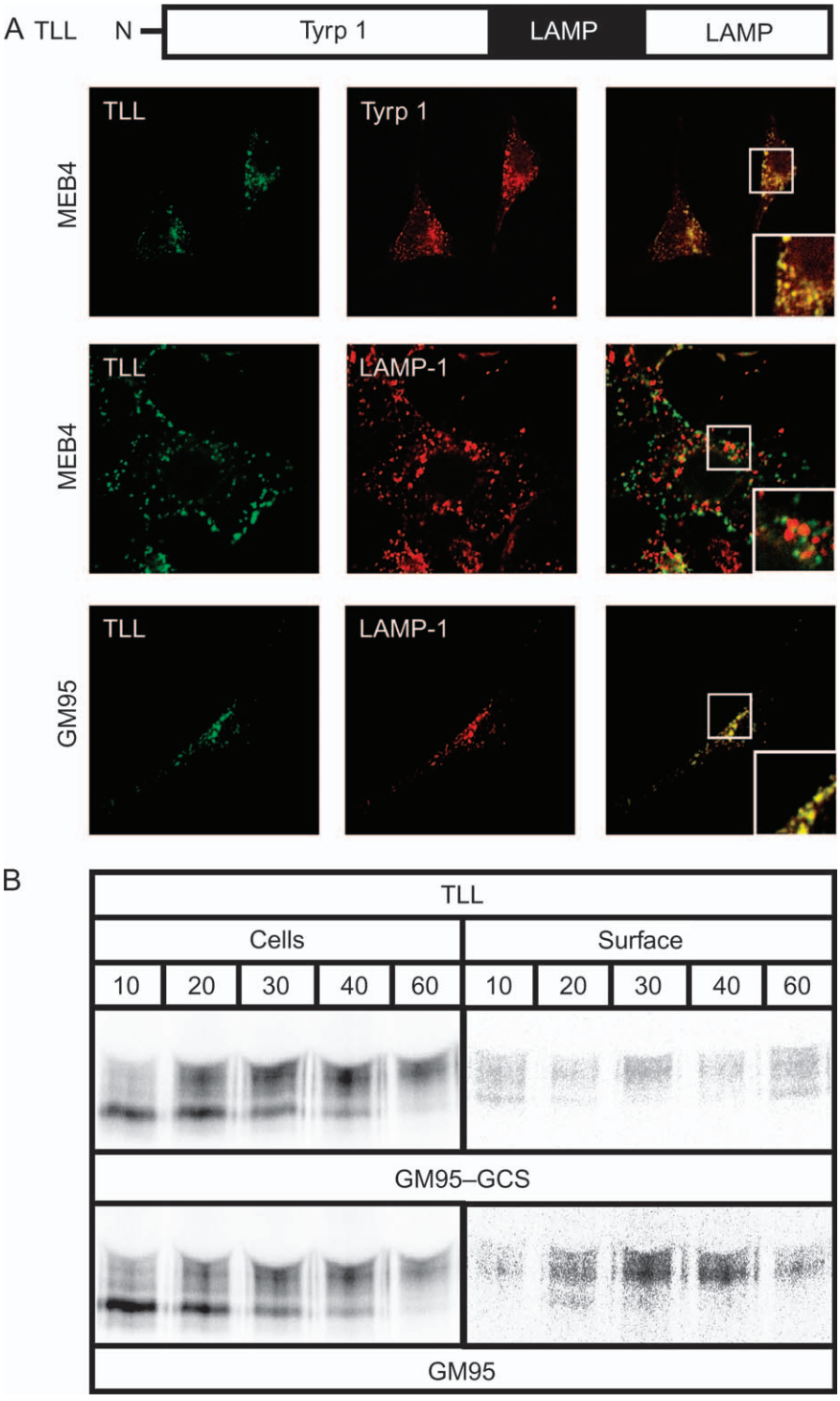

Figure 6: Localization and sorting of LAMP-1 having the lumenal domain of Tyrp1. A) The Tyrp1/LAMP-1 chimera (TLL): lumenal domain Tyrp1 and transmembrane (black box) and cytoplasmic tail of LAMP-1. MEB4 and GM95 cells were transiently transfected with TLL, incubated with 20 μg/mL leupeptin for 3 h at 37°C, fixed and labeled for Tyrp1 (anti-pep1), LAMP-1 (1D4B) and TLL (anti-HA/Y11) and viewed by fluorescence microscopy. B) GM95 and MEB4 cells transfected with TLL were pulse labeled for 15 min with Tran[^{35}S]label, chased for the indicated time (min) and biotinylated on ice. TLL was immunoprecipitated from detergent lysates with the anti-HA antibody. Immunoprecipitated protein was eluted from the beads, and part was analyzed by SDS–PAGE and autoradiography (Cells). Biotinylated TLL was immunoprecipitated from the remainder using streptavidin–agarose beads and analyzed by SDS–PAGE and phosphorimaging (Surface).

melanosomes by the intracellular pathway and melanosome maturation.

In wild-type melanocytes, MEB4 cells and GM95–GCS cells, Tyrp1 and tyrosinase [and Tyr-TM6; (10)] enter an intracellular pathway to the melanosomes. Surprisingly, in these cells, the lysosomal membrane proteins LAMP-1 and -2 did not enter the intracellular pathway to the lysosomes, which they do in fibroblasts (Figure S4), but reached the cell surface from where they were endocytosed. In GM95 cells and MEB4-aGCS cells, the opposite was observed. Now, Tyrp1 and Tyr-TM6 were missorted

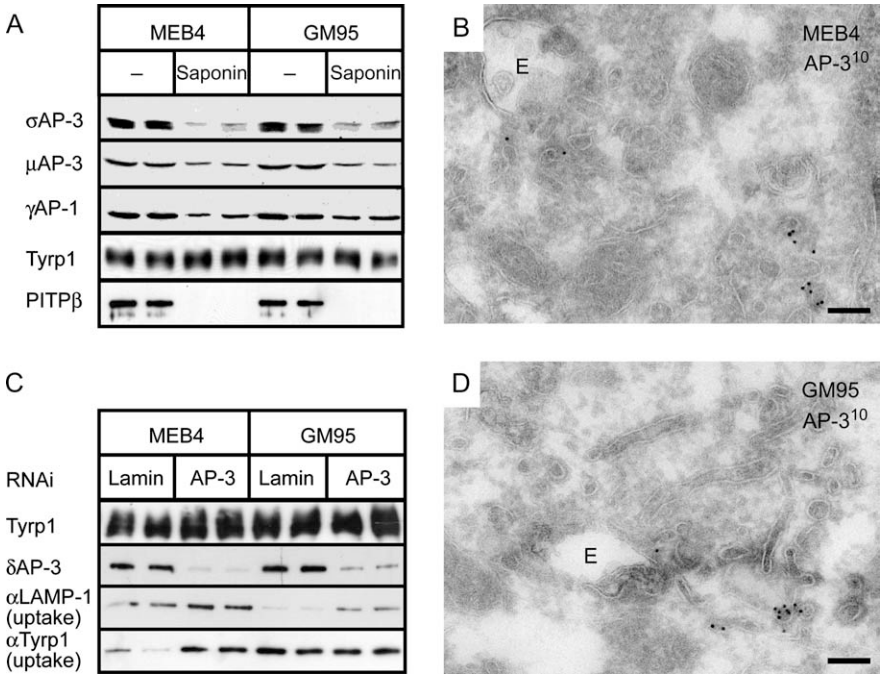

Figure 7: Knockdown of adaptor complex AP-3 affects transport of Tyrp1 and LAMP-1. A) The membrane binding of the γ subunit of AP-1 and the σ and μ subunits of AP-3 was investigated in saponin-treated MEB4 and GM95 cells as described in *Materials and Methods*. The membrane-bound melanosomal protein Tyrp1 and the soluble cytosolic protein PITPβ were used as controls for the permeabilization procedure. The experiment was performed three times with comparable results. B and D) AP-3 distribution in MEB4 and GM95 cells. Immunogold label (10-nm gold particles) for the δAP-3 subunit on ultrathin cryosections occurs in both MEB4 (B) and GM95 (D) cells on buds on endosome (E)-associated tubules that are often clathrin coated. Bar, 100 nm. C) MEB4 and GM95 cells were stably transfected with RNAi plasmids against lamin or δAP-3. The expression level of δAP-3 was determined by Western blotting using Tyrp1 as a loading control. In parallel dishes, an antibody uptake experiment was performed using αTyrp1 and αLAMP-1 antibodies, which bind to the lumenal domains of Tyrp1 (TA99) and LAMP-1 (1D4B), respectively. Internalized antibodies were quantified by Western blotting. Data are representative of three independent experiments.

to the cell surface (10), and LAMPs followed an intracellular pathway to the lysosomes. The opposite behavior of the melanosomal and lysosomal proteins fits the fact that lysosomal and melanosomal proteins must contain different targeting information. The domain-swapping experiments showed that the lumenal domain of the melanosomal proteins contains the specific targeting information. Replacement by the lumenal domain of non-specific plasma membrane proteins turned Tyrp1 into a typical lysosomal protein. Conversely, LAMP-1 possessing the lumenal domain of Tyrp1 was transported to melanosomes. These experiments showed that the lumenal domain of Tyrp1 was both necessary and sufficient for melanosomal targeting.

Because sorting of melanosomal and lysosomal proteins depends on cytosolic sorting machineries (26,27), the most likely scenario is that lumenal sorting information is transduced to the cytosol where adaptor complexes are likely responsible for the actual targeting of the proteins to their cognate organelles. Because mutations in AP-3 have been reported to result in distribution of tyrosinase and Tyrp1 and to Tyrp1 missorting very similar to the situation in GM95 cells (6,23), we tested the effect of knocking down AP-3 on the transport of Tyrp1 and LAMP-1. Knocking down AP-3 induced cell surface trafficking of Tyrp1 in MEB4 cells and increased the cell surface trafficking of LAMP-1 in both MEB4 and GM95 cells like in fibroblasts (Figure 7 and Figure S4). This shows that AP-3 was in some way involved in sorting melanosomal proteins into the intracellular pathway to the melanosomes in MEB4 but not in GM95 cells, whereas in contrast, it sorted lysosomal proteins into the intracellular pathway to the lysosomes in GM95 cells. Tyrp-1 still traveled to melanosomes in AP-3 (β3A)-deficient cells but not in BLOC-1-deficient cells (24). Because BLOC-1-deficient cells resemble GM95 cells, also BLOC-1 may be involved in the GlcCer-dependent pathway (23).

It has been reported that the interaction of the AP-2 adaptor complex with its cargo depends on multimerization of the AP-2-binding site (28). Possibly, the lumenal domains of melanosomal proteins cause them to oligomerize in the relevant sorting compartment, which then increases their affinity (or avidity) for adaptor complexes like AP-3 or BLOC-1 (24). Lysosomal proteins would lose the competition for adaptor binding from oligomerized melanosomal proteins and shift to a non-specific pathway

to the cell surface (7,25) from where they may be endocytosed through AP-2 (9). Indeed, by contrast to the enrichment of lysosomal cargo in AP-3-coated buds of HepG2 cells (25), no such enrichment of the late endosome/lysosome-resident LAMP-1 could be observed in AP-3-coated buds of melanocytic cells (5). Interestingly, purposeful overexpression of Tyrp1 by transfection in wild-type melanocytic cells increased the surface expression of LAMP-1 (24), supporting the idea of competition. This competition is probably indirect because in contrast to LAMP-1, Tyrp1 does not bind AP-3 directly (5). The role of the glycosphingolipids may be in the oligomerization of the melanosomal proteins. The lack of glycosphingolipids would result in the loss of clustering of the melanosomal proteins and reduce their affinity for the relevant adaptors. This would then result in binding of the adaptors to the lysosomal proteins by default, followed by the observed shifts in routing.

Our results show that glycosphingolipids are required for the proper functioning of the melanosomal targeting information in the lumenal domain of the melanosomal proteins tyrosinase and Tyrp1. Glycosphingolipids, more specifically GlcCer, may drive clustering of these melanosomal proteins by the formation of glycosphingolipid/cholesterol rafts. However, so far, no difference in detergent solubility of tyrosinase (an indirect assay for raft association) was found between MEB4 and GM95 cells (unpublished data). Alternatively, an interaction of GlcCer with the proteins may be mediated by a lectin-type interaction in the lumen of the sorting compartment, either directly (29) or indirectly by lectins in the lumenal compartments (30). Finally, GlcCer may indirectly cause oligomerization of tyrosinase and Tyrp1 by changing the lumenal environment. For example, we have measured a difference in the lumenal pH of the TGN in MEB4 and GM95 cells (J. Wolthoorn, S. van der Poel, S. Neumann, D. van den Heuvel, M. Egmond, H. Gerritsen, H. Sprong and G. van Meer, manuscript preparation). The findings in this study identify a sorting switch between melanosomal and lysosomal proteins. The properties of this switch and the direct or indirect role of glycosphingolipids are the topics of our present investigations.

Materials and Methods

Antibodies and reagents

Chemicals, if not indicated otherwise, were from Sigma and used in the highest purity available. Silica thin-layer chromatography plates were from Merck, organic solvents were from Riedel de Haën and cell culture media and reagents were from Invitrogen. [^{14}C]serine (5.59 GBq/mmol) was from Amersham. Tran[^{35}S]label (>36 TBq/mmol) was from ICN. Rabbit antisera against the cytoplasmic tails of mouse Tyrp1 (anti-pep1) and mouse tyrosinase (anti-pep7) were generated as described (31,32). Anti-pep7 was affinity purified using the same peptide coupled to affigel-15 columns (Bio-rad). The rabbit anti-tyrosinase antibody used for electron microscopy was described previously (5). The rabbit antiserum against the lumenal domain of human CD25 was from M. Marks (University of Pennsylvania, Philadelphia, PA, USA). The mouse antibodies against the different subunits of the AP complexes were from L. Traub (University of Pittsburgh, Pittsburgh, PA, USA), except for the SA4 mouse anti-δAP3 antibody, which was from A. Peden (University of Cambridge, Cambridge, UK). The rabbit antiserum against phosphatidylinositol transfer protein β (PITPβ) was from G. Snoek (Utrecht University, Utrecht, The Netherlands). The mouse 9E10 antibody against the c-myc epitope, the mouse monoclonal antibody (mAb) 32M4 against the lumenal domain of human CD8 and the rat 1D4B antibody against the lumenal domain of mouse LAMP-1 were from Santa Cruz Biotechnology. The mAb ABL93 against the lumenal domain of LAMP-2 was from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). The mAb TA99 against the lumenal domain of Tyrp1 was from Abcam. Rabbit polyclonal antibodies against Hrs, early embryonic antigen-1, cathepsin D and 46 kD mannose 6-phosphate receptor were kind gifts of S. Urbé and M. Clague (University of Liverpool, Liverpool, UK), A. Hasilik (Philipps University, Marburg, Germany) and K. von Figura (University of Göttingen, Göttingen, Germany), respectively. Fluorescein isothiocyanate and Texas-red fluorescent secondary goat antibodies were obtained from Jackson. Rabbit anti-mouse immunoglobulin G and horseradish peroxidase-conjugated secondary goat antibodies were from DAKO.

Plasmids

The chimera having the lumenal and transmembrane domains of CD25 and the cytoplasmic tail of tyrosinase (CCY-pCDM8.1) was from M. Marks (33). Mouse Tyrp1-pCMV6 and the chimeras of CD8 and Tyrp1 (CCT- and CTT-pCDM8.1) were from A. Houghton (34). The construction of GCS-pCB7, myc-tagged Tyr, Tyr-TM6 and α2,6-sialyltransferase-pCB7 were described before (10). Complementary DNA (cDNA) of mouse LAMP-1 was from RZPD. The TLL construct having the lumenal domain of Tyrp1 (until E478) and the transmembrane domain (from M371) and the cytoplasmic tail of LAMP-1 was generated by site-directed mutagenesis by using two-step amplification. The polymerase chain reaction fragment was cloned in frame with a triple hemagglutinin (HA) tag present in the mammalian expression vector pcDNA3 on its cytosolic tail. Oligos used for RNAi of CERT, GCS, LacCerS, δAP-3 subunit and lamin (sequences available on request) were inserted between the *Bgl*II and *Hin*dIII sites of the RNAi plasmid pKoen (35). All synthetic cDNAs were verified by restriction analysis and dye termination sequencing.

Cell culture and transfection

GlcCer synthase-deficient GM95 cells and their parental MEB4 cells from the RIKEN Cell Bank (Tsukuba, Japan) were grown in DMEM containing 4.5 g/L glucose and 10% fetal calf serum (FCS) at 37°C with 5% CO_2. Cells were stably transfected with the ER form of GCS (GCS-pCB7) (10) or myc-tagged α2,6-sialyltransferase-pCB7 or transiently with CCY-, CTT- or CCT-pCDM8.1 using Lipofectamine 2000. Myc transfectants were screened with immunofluorescence using the 9E10 antibody. Stable transfectants of GM95–GCS were screened by assaying the GCS as before (36). MEB4 and GM95 cells were stably transfected with RNAi plasmids (pKoen) against CERT, GCS, LacCerS, lamin and the δAP-3 subunit using Lipofectamine 2000. Stable transfectants were selected in the presence of hygromycin B (200 U/mL) or G418 (0.6 mg/mL), and individual colonies were obtained by limiting dilution subcloning. Stable transfectants carrying the pKoen constructs were initially selected for green fluorescent signal in the nucleus and subsequently for their glycosphingolipid composition or protein expression levels of δAP-3. For this, subconfluent cells on 3-cm dishes were incubated overnight in MEMα medium, 5% FCS and 15 kBq/mL [^{14}C]serine. Cells were washed with PBS, and lipids were extracted and analyzed as described (37). Protein expression was induced by 5 mM sodium butyrate (Fluka) 14–16 h prior to all experiments.

Immunofluorescence and immunoelectron microscopy

Cells were fixed, permeabilized, labeled with fluorescent antibodies and viewed by confocal microscopy as before (38). A negative effect of melanin on fluorescent labeling of the CD8 and CD25 antigens when present in melanosomes cannot be fully excluded because of epitope inaccessibility

or fluorescence quenching by melanin (5). For electron microscopy, cells were fixed and frozen, and ultrathin sections were cut and labeled with antibodies and protein A-gold, all as before (38).

Antibody internalization

Confluent cells on 3-cm dishes were incubated for 3 h at 37°C with 1 mL medium containing 20 μg/mL leupeptin and ~50 μg antibodies against the lumenal domain of LAMP-1, LAMP-2, Tyrp1, CD8 or CD25. Cells were washed five times with ice-cold PBS and lysed in reducing Laemmli sample buffer. Equal amounts of protein were analyzed by SDS–PAGE, and the amount of internalized antibody was determined by Western blotting (36). As an internal standard, LAMP-1 or Tyrp1 was detected using the 1D4B antibody or anti-pep1, respectively. Films were scanned on a Bio-Rad GS 700 imaging densitometer equipped with an integrating program. The maximum signal on a film that was still linear was determined by using a concentration series of blotted mouse immunoglobulin Gs, detected with anti-mouse peroxidase. It was set at 100 arbitrary units (AU), and the time of film exposure in each experiment was optimized to yield a value of roughly 100 AU for the spot with the highest amount of label.

Binding of APs to membranes

Confluent cells on 3-cm dishes were washed three times with ice-cold PBS. To remove their cytosolic proteins, cells were then incubated with 1 mL PBS and/or 0.1% (w/v) saponin for 20 min on ice. The incubation medium with cytosol was removed, and the cells were washed with ice-cold PBS and lysed in reducing Laemmli sample buffer. Samples were analyzed by SDS–PAGE and Western blotting. Contrary to the membrane protein Tyrp1, the soluble protein PITPβ was almost completely removed by the saponin treatment.

Pulse–chase and cell surface biotinylation

Pulse–chase and cell surface biotinylation were performed as described for Tyrp1 (10) except that the cells were transiently transfected with TLL using Lipofectamine 2000 (Invitrogen). The expression of TLL was induced by 5 mM sodium butyrate 16 h prior to experiments. After the cell surface biotinylation, the construct was immunoprecipitated with anti-HA antibodies (Y11).

Acknowledgments

We are grateful to Michael Clague, Kurt von Figura, Andrej Hasilik, Vince Hearing, Alan Houghton, Michael Marks, Andrew Peden, Graça Raposo, Gerry Snoek, Linton Traub and Sylvie Urbé for their generosity in providing reagents. We also thank René Scriwanek and Marc van Peski for excellent photographic work. We thank our colleagues for critical reading of the manuscript and helpful discussions. A. C. T. and G. v. M. are particularly grateful for constructive discussions with the laboratories of Mickey Marks, Graça Raposo, Margaret Robinson and with the Traffic referees. This work was supported by VICI grant 918.56.611 of the Netherlands Organization for Scientific Research (NWO) to J. K., grant 902-23-197 from ZonMW with financial support from NWO to S. G.-D., J. W., S. M. v. D. and G. v. M., by the European Commission Research Training Network grant HPRN-CT-2000-00077 to G. v. M. and by SENTER, the Dutch Ministry of Economic Affairs (G. v. M.).

Supplementary Materials

Table S1: Subcellular distribution of endosomal/lysosomal markers in MEB4 and GM95 cells. [a]The morphology of all secretory and endocytotic organelles other than the melanosomes as well as the intracellular distribution of the late endosome/lysosome markers LAMP-1 and cathepsin D, the early endosome markers Hrs and early embryonic antigen-1 and the 46 kD mannose-6-phosphate receptor (CD-MPR), which cycles between TGN and endosomes, were comparable between GM95 and MEB4 cells. These data show that in GM95 cells, the compartments of the endocytic pathway are normally formed and that glycosphingolipid synthesis is not required for proper sorting of endosome–lysosome-associated proteins. Importantly, however, although tyrosinase and Tyrp1 reach endosomal compartments in GM95 cells, melanin pigments are not synthesized. [b]Proteins in ultrathin cryosections were detected by specific antibodies followed by protein A-gold labeling as described in *Materials and Methods*. The relative distribution of the proteins was determined by random screening of single-labeled sections from formaldehyde-fixed cells. All gold particles on or up to 25 nm from a membrane-bound compartment were scored as label on that organelle type, and percentages of the total amount of gold particles counted were calculated for each compartment. The relative frequency is expressed as '+++' for more than 25%, '++' for 15–25%, '+' for 5–15%, '±' for 1–5% or '−' for less than 1% of total label. Per cell line, 600 gold particles were scored for tyrosinase and 200–300 for the other proteins. [c]MVBs with striated bodies occur very rarely in MEB4 cells. EE, early endosome; LE, late endosome; Mel II, III and IV, melanosome stages II, III and IV; NA, not applicable.

Figure S1: GlcSph addition to GM95 cells corrects missorting of Tyrp1 and restores the plasma membrane component of transport of the CTT construct (lumenal domain of CD8 and transmembrane and cytoplasmic tail of Tyrp1) to the lysosomes. A) MEB4 and GM95 cells were grown in the presence or absence of GlcSph (20 μM) for 2 days. The cells were incubated with anti-Tyrp1 antibody for 3 h at 37°C, and internalized antibodies were visualized by Western blotting and quantified. Data were analyzed as described in the *Materials and Methods*. Numbers represent the means of three experiments in duplicate ± SD. B) Conversely, in a similar experiment using anti-CD8 antibody, GlcSph restored transport through the cell surface of CTT (lumenal domain of CD8 and transmembrane and cytoplasmic tail of Tyrp1) to the level of wild-type MEB4 melanocytes.

Figure S2: Antigenicity defect in GM95 cells unrelated to the lack of GlcCer. A) Cells were stained with HMB45 antibody. Pictures were taken with a confocal laser scanning microscopy using similar settings. Green intensity of GM95 and GM95–GCS is ×10 enhanced in Photoshop (bar, 20 μm). Remarkably, the GM95 cells could not be labeled with HMB45 anti-Pmel17 antibodies that decorate the fibrils of normal melanosomes, whereas an antibody recognizing the cytoplasmic tail of Pmel17 detected similar protein levels in MEB4 and GM95 cells by Western blotting (B). The band at ~100 000 kDa is shown. There was no difference in the intensities of the 25-kDa bands. The mouse mAb HMB45 against Pmel17 was from Lab Vision Corporation, and the anti-Pmel17 tail antibody was anti-PEP13 from Dr V. Hearing, National Institutes of Health (39). Tyrp1 was detected using anti-pep1. Unpigmented MEB4–GCS knockdown cells (MEB4-aGCS) still labeled with HMB45, and HMB45 antibody staining was not restored in the GM95–GCS cells, showing that the lack of HMB45 labeling was independent of GlcCer and independent of pigmentation. HMB45 recognizes a sialylated glycoconjugate on Pmel17 (40). GM95 is a phenotypic mutant line selected for lacking the sialylated glycolipid GM3, NeuAcα2,3Galβ1,4GlcCer (18). Indeed, a lectin recognizing NeuAcα2,3Gal moieties on glycoproteins bound to significantly fewer proteins in GM95 and GM95–GCS cells than in the parental MEB4 cells (not shown). Thus, independent of the lack of GCS activity, GM95 cells carry mutations that affect protein sialylation and Pmel17 staining by HMB45 antibodies. These mutations were irrelevant for melanosome assembly and pigmentation.

Figure S3: GlcCer-independent recovery of pigmentation of GM95 cells did not restore melanosomal protein sorting and melanosome assembly. A and C) When GM95 cells were transfected with a tyrosinase

construct with an elongated transmembrane domain containing a myc tag (Tyr-TM6), this tyrosinase was no longer confined to the Golgi area (10) and the GM95 cells turned black, as shown in the top row representing cell pellets of MEB4, GM95 and GM95–Tyr-TM6 (A). Independently, 3-cm dishes of the same cells were incubated with αTyrp1 antibody (TA99) for 3 h at 37°C. After washing, the internalized antibody was quantified in equal fractions of the samples by blotting with a horseradish peroxidase-conjugated secondary antibody where total Tyrp1 in the samples was assessed by Western blotting using anti-pep1. Tyrp1 traveling over the cell surface was not rescued by Tyr-TM6 transfection. In addition, electron microscopy (C) showed that GM95–Tyr-TM6 cells still lacked stages II–IV melanosomes. Instead, cells contained dark (arrows) as well as light (double arrow) MVBs and lysosomes occasionally containing a striated body (arrowhead; scale bar, 100 nm). B, D and E) Treatment of melanocytes with NH_4Cl or bafilomycin A stimulates pigmentation (41), and treating GM95 cells for 8 h with 10 mM NH_4Cl or 10 μM bafilomycin A (not shown) restored pigmentation of the cell pellet (B). A fraction of tyrosinase had escaped the perinuclear region as seen in cells fixed, labeled for tyrosinase (anti-pep1) and viewed by fluorescence microscopy (E; bar, 5 μm). However, also in the presence of pigmentation, missorting of Tyrp1 in GM95 cells, as measured by 3 h antibody uptake after 5-h pretreatment with NH_4Cl, was not corrected. Cells treated with NH_4Cl expressed more total Tyrp1 compared with the loading control calreticulin, probably because of reduced degradation in the lysosomes. Finally (D), GM95 cells treated with NH_4Cl had no true melanosomes but electron-dense endosomal vacuoles (arrows) that contained tyrosinase. The GM95 cells had been transfected with a Tyr–Myc construct (10) and were labeled by anti-myc antibody and 10-nm gold particles (bars, 100 nm). The black organelles resembled those in GM95–Tyr-TM6 cells (arrows and arrowheads, as above). The NH_4Cl effect on pigmentation was probably provoked by its stimulating effect on tyrosinase stability and activity because of pH neutralization of endosomes that contained a low level of tyrosinase (41). Together, these results demonstrate that GlcCer is required for melanosome biogenesis and reinforce the notion that the presence of tyrosinase and Tyrp1 in endosomal structures alone is not sufficient for melanosome formation.

Figure S4: LAMP-1 and -2 transport over the cell surface is increased in fibroblasts lacking AP-3. The uptake of anti-LAMP-1 and anti-LAMP-2 antibodies was measured in *mocha* (δ-deficient) mouse fibroblasts and *mocha* fibroblasts retransfected with δAP-3. *Mocha* fibroblasts [deficient in the δAP-3 subunit; (42)] and retransfected *mocha* fibroblasts were a gift from A. Peden (Genentech Inc.) and were cultured as described in Le Borgne et al. (7). Internalized antibodies were quantified as in Figure S1. Data are means of at least two independent experiments in duplicate ± SD.

Supplemental materials are available as part of the online article at http://www.blackwell-synergy.com

References

1. Raposo G, Tenza D, Murphy DM, Berson JF, Marks MS. Distinct protein sorting and localization to premelanosomes, melanosomes, and lysosomes in pigmented melanocytic cells. J Cell Biol 2001;152: 809–824.
2. Ni-Komatsu L, Orlow SJ. Heterologous expression of tyrosinase recapitulates the misprocessing and mistrafficking in oculocutaneous albinism type 2: effects of altering intracellular pH and pink-eyed dilution gene expression. Exp Eye Res 2006;82:519–528.
3. Dell'Angelica EC, Shotelersuk V, Aguilar RC, Gahl WA, Bonifacino JS. Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the β3A subunit of the AP-3 adaptor. Mol Cell 1999;3:11–21.
4. Höning S, Sandoval IV, von Figura K. A di-leucine-based motif in the cytoplasmic tail of LIMP-II and tyrosinase mediates selective binding of AP-3. EMBO J 1998;17:1304–1314.
5. Theos AC, Tenza D, Martina JA, Hurbain I, Peden AA, Sviderskaya EV, Stewart A, Robinson MS, Bennett DC, Cutler DF, Bonifacino JS, Marks MS, Raposo G. Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. Mol Biol Cell 2005;16:5356–5372.
6. Huizing M, Sarangarajan R, Strovel E, Zhao Y, Gahl WA, Boissy RE. AP-3 mediates tyrosinase but not TRP-1 trafficking in human melanocytes. Mol Biol Cell 2001;12:2075–2085.
7. Le Borgne R, Alconada A, Bauer U, Hoflack B. The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins. J Biol Chem 1998;273:29451–29461.
8. Peden AA, Rudge RE, Lui WW, Robinson MS. Assembly and function of AP-3 complexes in cells expressing mutant subunits. J Cell Biol 2002;156:327–336.
9. Janvier K, Bonifacino JS. Role of the endocytic machinery in the sorting of lysosome-associated membrane proteins. Mol Biol Cell 2005;16: 4231–4242.
10. Sprong H, Degroote S, Claessens T, van Drunen J, Oorschot V, Westerink BH, Hirabayashi Y, Klumperman J, van der Sluijs P, van Meer G. Glycosphingolipids are required for sorting melanosomal proteins in the Golgi complex. J Cell Biol 2001;155:369–380.
11. Diallo M, Arenz C, Schmitz K, Sandhoff K, Schepers U. Long endogenous dsRNAs can induce complete gene silencing in mammalian cells and primary cultures. Oligonucleotides 2003;13:381–392.
12. van Meer G, Voelker DR, Feigenson GW. The lipid composition and dynamic organization of cell membranes. Nat Rev Mol Cell Biol 2008;9: 112–124.
13. Yamashita T, Wada R, Sasaki T, Deng C, Bierfreund U, Sandhoff K, Proia RL. A vital role for glycosphingolipid synthesis during development and differentiation. Proc Natl Acad Sci U S A 1999;96:9142–9147.
14. Singh RD, Puri V, Valiyaveettil JT, Marks DL, Bittman R, Pagano RE. Selective caveolin-1-dependent endocytosis of glycosphingolipids. Mol Biol Cell 2003;14:3254–3265.
15. Simons K, van Meer G. Lipid sorting in epithelial cells. Biochemistry 1988;27:6197–6202.
16. Tooze SA, Martens GJ, Huttner WB. Secretory granule biogenesis: rafting to the SNARE. Trends Cell Biol 2001;11:116–122.
17. Rodriguez-Boulan E, Kreitzer G, Müsch A. Organization of vesicular trafficking in epithelia. Nat Rev Mol Cell Biol 2005;6:233–247.
18. Ichikawa S, Nakajo N, Sakiyama H, Hirabayashi Y. A mouse B16 melanoma mutant deficient in glycolipids. Proc Natl Acad Sci U S A 1994;91:2703–2707.
19. Hanada K, Kumagai K, Yasuda S, Miura Y, Kawano M, Fukasawa M, Nishijima M. Molecular machinery for non-vesicular trafficking of ceramide. Nature 2003;426:803–809.
20. Drecktrah D, Brown WJ. Phospholipase A2 antagonists inhibit nocodazole-induced Golgi ministack formation: evidence of an ER intermediate and constitutive cycling. Mol Biol Cell 1999;10:4021–4032.
21. Seiji M, Fitzpatrick TB, Simpson RT, Birbeck MSC. Chemical composition and terminology of specialized organelles (melanosomes and melanin granules) in mammalian melanocytes. Nature 1963;197:1082–1084.
22. Raposo G, Marks MS. The dark side of lysosome-related organelles: specialization of the endocytic pathway for melanosome biogenesis. Traffic 2002;3:237–248.
23. Di Pietro SM, Falcon-Perez JM, Tenza D, Setty SR, Marks MS, Raposo G, Dell'Angelica EC. BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. Mol Biol Cell 2006;17:4027–4038.

24. Setty SR, Tenza D, Truschel ST, Chou E, Sviderskaya EV, Theos AC, Lamoreux ML, Di Pietro SM, Starcevic M, Bennett DC, Dell'Angelica EC, Raposo G, Marks MS. BLOC-1 is required for cargo-specific sorting from vacuolar early endosomes toward lysosome-related organelles. Mol Biol Cell 2007;18:768–780.
25. Peden AA, Oorschot V, Hesser BA, Austin CD, Scheller RH, Klumperman J. Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. J Cell Biol 2004;164: 1065–1076.
26. Marks MS, Seabra MC. The melanosome: membrane dynamics in black and white. Nat Rev Mol Cell Biol 2001;2:738–748.
27. Dell'Angelica EC. Melanosome biogenesis: shedding light on the origin of an obscure organelle. Trends Cell Biol 2003;13:503–506.
28. Grass I, Thiel S, Honing S, Haucke V. Recognition of a basic AP-2 binding motif within the C2B domain of synaptotagmin is dependent on multimerization. J Biol Chem 2004;279:54872–54880.
29. Theos AC, Truschel ST, Tenza D, Hurbain I, Harper DC, Berson JF, Thomas PC, Raposo G, Marks MS. A lumenal domain-dependent pathway for sorting to intralumenal vesicles of multivesicular endosomes involved in organelle morphogenesis. Dev Cell 2006;10:343–354.
30. Schrag JD, Procopio DO, Cygler M, Thomas DY, Bergeron JJ. Lectin control of protein folding and sorting in the secretory pathway. Trends Biochem Sci 2003;28:49–57.
31. Jimenez M, Kameyama K, Maloy WL, Tomita Y, Hearing VJ. Mammalian tyrosinase: biosynthesis, processing, and modulation by melanocyte-stimulating hormone. Proc Natl Acad Sci U S A 1988;85:3830–3834.
32. Jimenez M, Tsukamoto K, Hearing VJ. Tyrosinases from two different loci are expressed by normal and by transformed melanocytes. J Biol Chem 1991;266:1147–1156.
33. Calvo PA, Frank DW, Bieler BM, Berson JF, Marks MS. A cytoplasmic sequence in human tyrosinase defines a second class of di-leucine-based sorting signals for late endosomal and lysosomal delivery. J Biol Chem 1999;274:12780–12789.
34. Vijayasaradhi S, Xu Y, Bouchard B, Houghton AN. Intracellular sorting and targeting of melanosomal membrane proteins: identification of signals for sorting of the human brown locus protein, gp75. J Cell Biol 1995;130:807–820.
35. Deneka M, Neeft M, Popa I, van Oort M, Sprong H, Oorschot V, Klumperman J, Schu P, van der Sluijs P. Rabaptin-5α/rabaptin-4 serves as a linker between rab4 and γ_1-adaptin in membrane recycling from endosomes. EMBO J 2003;22:2645–2657.
36. Sprong H, Kruithof B, Leijendekker R, Slot JW, van Meer G, van der Sluijs P. UDP-galactose:ceramide galactosyltransferase is a class I integral membrane protein of the endoplasmic reticulum. J Biol Chem 1998;273:25880–25888.
37. Sprong H, Degroote S, Nilsson T, Kawakita M, Ishida N, van der Sluijs P, van Meer G. Association of the Golgi UDP-galactose transporter with UDP-galactose:ceramide galactosyltransferase allows UDP-galactose import in the endoplasmic reticulum. Mol Biol Cell 2003; 14:3482–3493.
38. Halter D, Neumann S, van Dijk SM, Wolthoorn J, de Mazière AM, Vieira OV, Mattjus P, Klumperman J, van Meer G, Sprong H. Pre- and post-Golgi translocation of glucosylceramide in glycosphingolipid synthesis. J Cell Biol 2007;179:101–115.
39. Kobayashi T, Urabe K, Orlow SJ, Higashi K, Imokawa G, Kwon BS, Potterf B, Hearing VJ. The Pmel 17/silver locus protein. J Biol Chem 1994;269:29198–29205.
40. Harper DC, Theos AC, Herman KE, Tenza D, Raposo G, Marks MS. Premelanosome amyloid-like fibrils are composed of only Golgi-processed forms of Pmel17 that have been proteolytically processed in endosomes. J Biol Chem 2008;283:2307–2322.
41. Ancans J, Tobin DJ, Hoogduijn MJ, Smit NP, Wakamatsu K, Thody AJ. Melanosomal pH controls rate of melanogenesis, eumelanin/phaeomelanin ratio and melanosome maturation in melanocytes and melanoma cells. Exp Cell Res 2001;268:26–35.
42. Kantheti P, Qiao X, Diaz ME, Peden AA, Meyer GE, Carskadon SL, Kapfhamer D, Sufalko D, Robinson MS, Noebels JL, Burmeister M. Mutation in AP-3 δ in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. Neuron 1998;21:111–122.