

Cell type-specific Rab32 and Rab38 cooperate with the ubiquitous lysosome biogenesis machinery to synthesize specialized lysosome-related organelles

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Lysosome-related organelles (LROs) exist in specialized cells to serve specific functions and typically co-exist with conventional lysosomes. The biogenesis of LROs is known to utilize much of the common protein machinery used in the transport of integral membrane proteins to lysosomes. Consequently, an outstanding question in the field has been how specific cargoes are trafficked to LROs instead of lysosomes, particularly in cells that simultaneously produce both organelles. One LRO, the melanosome, is responsible for the production of the pigment melanin and has long been used as a model system to study the formation of specialized LROs. Importantly, melanocytes, where melanosomes are synthesized, are a cell type that also produces lysosomes and must therefore segregate traffic to each organelle. Two small GTPases, Rab32 and Rab38, are key proteins in the biogenesis of melanosomes and were recently shown to redirect the ubiquitous machinery—BLOC-2, AP-1 and AP-3—to traffic specialized cargoes to melanosomes in melanocytes. In addition, the study revealed Rab32 and Rab38 have both redundant and unique roles in the trafficking of melanin-producing enzymes and overall lysosome biogenesis. Here we review these findings, integrate them with previous knowledge on melanosome biogenesis and discuss their implications for biogenesis of other LROs.

Introduction

In humans, the pigment melanin is responsible for pigmentation of hair, skin and

eyes and serves to minimize the damage caused by exposure to the UV radiation from sunlight. Melanin is produced in a specialized organelle, the melanosome, which is found in melanocyte cells, in skin and hair follicles and retinal and iris pigmented epithelial cells in the eyes.¹⁻⁴ The formation of melanosomes has been heavily studied both because of disease implications caused by defects in melanosome formation and because the melanosome is a prototype of the specialized class of organelles called lysosome-related organelles (LROs).^{1,3-6} Lysosome-related organelles are found in specialized cell types such as melanocytes, platelets, lung alveolar type II cells and some innate and adaptive immune cells and have critical roles in pigment production, blood clotting, lung surfactant production, lytic activity of the innate immune system and antigen-processing of the adaptive immune system, respectively.⁴⁻⁸ LROs are so called because of shared acidic lumen, protein components and because LROs utilize similar biogenesis pathways as lysosomes.⁴⁻⁶

Melanosome maturation is characterized by four morphologically distinct phases as observed in electron micrographs.^{1,9} Stage I melanosomes are formed by the delivery of the transmembrane, structural protein Pmel17 to vacuolar early endosomes, most likely after rapid transit through the cell surface and subsequent sorting to intraluminal vesicles (Fig. 1).^{1,10} This Pmel17 sorting is independent of the endosomal sorting complex required for transport (ESCRT), the machinery that mediates formation of intraluminal vesicles in multi-vesicular bodies

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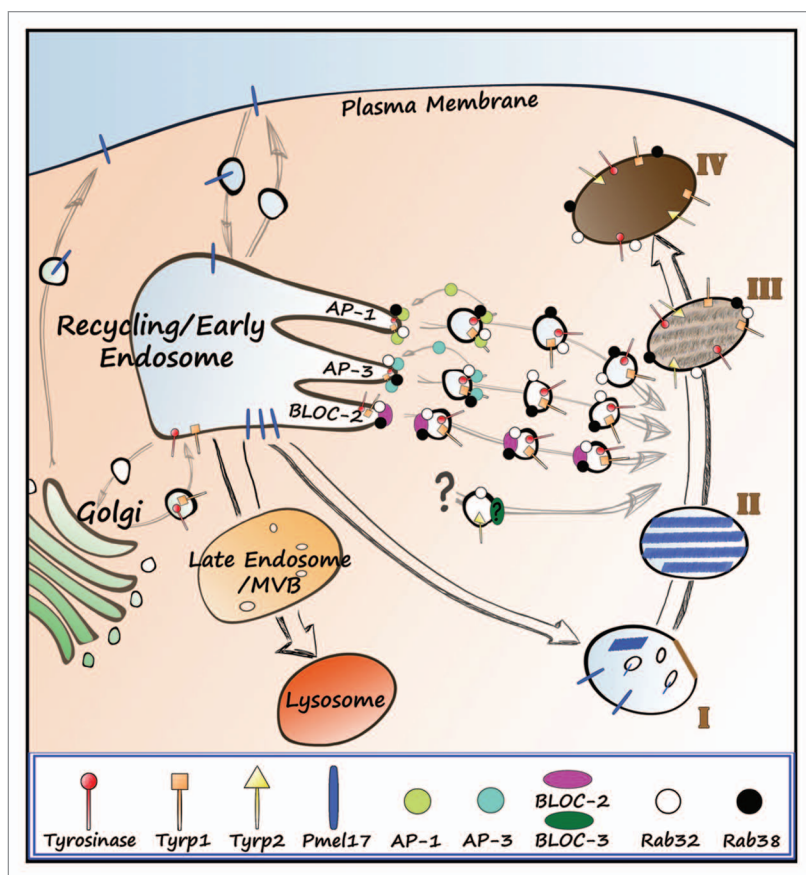


Figure 1. Model of melanosome biogenesis. Schematic diagram of the four stages of melanosome maturation (I–IV), endosomal organelles and biosynthetic transport pathways followed by the cargo integral membrane proteins Pmel17, tyrosinase, tyrosinase-related protein-1 (Tyrp-1) and Tyrp-2. Melanosomal cargoes derive from the Golgi complex and traverse early/recycling endosomal domains either directly or through the cell surface. Sorting of Pmel17 to intraluminal vesicles from the limiting membrane of vacuolar early endosomal domains mark Stage I melanosomes. This process initiates the segregation of pre-melanosomes from the degradative late endosome/multi-vesicular body (MVB) pathway to lysosomes. Formation of Pmel17 fibrils across the length of the organelle characterizes stage II melanosomes. Tyrosinase and Tyrp1 reach the maturing melanosome from specialized tubular domains of early/recycling endosomes and catalyze the synthesis of the melanin pigment observed in stage III and IV melanosomes. Rab32 and Rab38 interact with AP-1, AP-3 and BLOC-2 on early/recycling endosome tubules, where cargo such as tyrosinase and Tyrp1 are loaded into vesicles or transport intermediates. This trafficking machinery is organized into at least two parallel or alternate routes for transport of cargo to the maturing melanosome, such that deficiency of one component typically causes a partial defect rather than complete failure of melanosome biogenesis. Rab32, Rab38 and possibly BLOC-2 remain associated with the vesicles or transport intermediates to promote their motility, tethering and fusion with the maturing melanosome. The pathway taken by Tyrp2 is not known, but it is at least partially different from that of tyrosinase or Tyrp1 and it depends strictly on Rab32—not on Rab38—and BLOC-3.

(MVBs)/late endosomes and defines the ubiquitous degradative/lysosome pathway (Fig. 1).^{1,11} Stage I melanosomes are also differentiated from MVBs by the presence of large, flat, clathrin-containing coats on their limiting membrane.^{9,12} Pmel17 is then cleaved in the luminal region of the protein by a proprotein convertase and the luminal Pmel17 fragments form

amyloid fibrils across the length of the organelle, thus characterized as stage II melanosomes (Fig. 1).^{1,9,11,13} Stage I and II melanosomes are occasionally referred to as pre-melanosomes, and they do not yet contain the melanin pigment. Delivery of the transmembrane enzymes tyrosinase and tyrosinase-related proteins-1 and -2 (Tyrp1 and Tyrp2), the main proteins

responsible for melanin synthesis, is required to drive maturation from stage II to stage III melanosomes (Fig. 1).^{1,12} Tyrosinase, Tyrp1 and Tyrp2 form large melanin polymers that are deposited upon Pmel17 fibrils to form partially pigmented stage III melanosomes.^{1,9,14} Further melanin synthesis produces mature stage IV melanosomes, which are fully pigmented and are transported to the cell periphery for transfer to keratinocytes, in the case of skin melanocytes, or long-term storage, in the case of retinal pigmented epithelial cells in the eye.^{2,15}

Transport of newly synthesized tyrosinase and Tyrp1 to the maturing melanosome requires a sorting step at specialized tubular domains of early/recycling endosomes, rather than direct transport from the trans-Golgi network (Fig. 1).^{1,16–18} Packaging of the tyrosinases into transport vesicles at early/recycling endosome-associated tubules is dependent on ubiquitous adaptor protein complex (AP)-1 and AP-3, and biogenesis of lysosome-related organelles complex (BLOC)-1 and BLOC-2.^{16,17} Furthermore, AP-3 and BLOC-2 define parallel pathways for melanosome biogenesis, thus, deficiency of both complexes causes a more severe defect in Tyrp1 transport and overall pigmentation than either single deficiency (Fig. 1).¹⁷ Analogously, AP-1 and AP-3 provide alternate routes for transport of tyrosinase and possibly other cargoes to maturing melanosomes (Fig. 1).¹⁶ While it has not been established if the AP-3 independent pathways—i.e., BLOC-2- and AP-1-dependent, respectively—are separate or the same trafficking route, it is clear that the early/recycling endosomal system is a key sorting station for delivery of cargo to maturing melanosomes.¹ Remarkably, lysosome integral membrane proteins, such as LAMPs, reach the lysosome-limiting membrane from analogous early/recycling endosome tubules in vesicles formed by the same AP and BLOC complexes.^{17,19–21} It is, therefore, puzzling how specialized cells such as the melanocyte simultaneously produce and maintain lysosomes and specialized LROs such as the melanosome. How does the cell define separate early/recycling endosomal tubular domains and utilize at least partially overlapping machinery to

mediate distinct integral membrane protein transport to different organelles?^{1,6,21} What other factors are involved in the biogenesis to allow for the separate, but concurrent, existence of the two pathways and organelle types?

One possible mechanism to facilitate the production and existence of both lysosomes and LROs is the expression of cell type-specific proteins that function in LRO biogenesis and not in lysosome biogenesis.²² Two closely related small GTPases of the Rab family, Rab32 and Rab38, are expressed in selected cell types such as melanocytes and other cells where LROs are present.²³⁻²⁵ Rab32 and Rab38 have been shown to work on melanosome biogenesis and to mediate transport of tyrosinase and Tyrp1 by an unknown mechanism.²³ Rab38-deficient mice and rats are hypopigmented and Rab32 silencing in melanocytes isolated from Rab38 mutant mice further enhances the melanosome biogenesis defect.^{23,26,27} This cooperation between Rab32 and Rab38 for melanosome biogenesis resembles that of BLOC-2, AP-3 and AP-1 described above. These Rabs could therefore be the cell type-specific factors that interact with the ubiquitous trafficking machinery to mediate transport to maturing melanosomes.²² Such a scenario would be in agreement with the known functions of the Rab family proteins, which belong to the Ras superfamily and operate as elegant “switches” that regulate vesicular trafficking through interactions with effector proteins.²⁸⁻³⁰ However, the specific function and partners of Rab32 and Rab38 in the biogenesis of melanosomes or other LROs had remained poorly characterized.^{23,31}

Rab32 and Rab38 interact physically and colocalize with BLOC-2, AP-1 and AP-3. To study endogenous Rab32 and Rab38 using biochemical and immunofluorescence microscopy approaches, antibodies against the Rabs were produced and validated.³¹ In immunoprecipitation experiments, endogenous Rab32 and Rab38 were found to interact with BLOC-2, AP-1 and AP-3 in membrane, but not cytosolic fractions of MNT-1 melanocyte cells.³¹ These results suggest a specific interaction with BLOC-2, AP-1 and AP-3 on membranes, where the adaptors are known to function. In GST-Rab pulldown

assays, Rab32 and Rab38 showed preferential binding to BLOC-2, AP-1 and AP-3 when bound to GTP instead of GDP.³¹ This GTP-bound specificity is consistent with the idea that Rab32 and Rab38 have an active, functional role in the trafficking pathway mediated by BLOC-2, AP-1 and AP-3. The exciting possibility emerges that Rab32 and Rab38 may function with the ubiquitous machinery in trafficking cargoes to maturing melanosomes and could be the cell type-specific factors that differentiate trafficking pathways to melanosomes instead of lysosomes.

Adaptor proteins orchestrate the formation of membrane coats that mediate cargo selection and vesicle budding.³²⁻³⁴ Adaptor proteins are recruited to membranes through interactions with membrane lipids, the cytoplasmic tails of transmembrane cargoes and, at least in some cases, ARF proteins—another family of GTP-binding proteins of the Ras superfamily.³²⁻³⁴ While it was initially suspected that AP-3 may be clathrin-independent, subsequent research has shown that AP-3 and AP-1 act as clathrin-binding adaptor proteins, and it is also possible that BLOC-2 functions as a clathrin-binding adaptor.^{19,35-41} Clathrin is a structural component that defines one of the major classes of transport vesicle coats.³⁴ Adaptor proteins also help recruit a host of proteins involved in downstream vesicle functions such as motility through motor protein-cytoskeleton interactions, and vesicle tethering and fusion with the target organelle.³² An RNAi approach was used to determine if the recruitment or stabilization of Rab32 and Rab38 on membranes is dependent on the presence of AP-1, AP-3 or BLOC-2.³¹ Depletion of AP-3 or BLOC-2 causes a significant decrease in the percentage of Rab38 that is associated with membranes at steady-state, but has a modest effect on Rab32 membrane association. Depletion of AP-1 has no effect on either Rab32 or Rab38 membrane association. Furthermore, depletion of BLOC-2, but not AP-1 or AP-3, causes a large reduction in the total amount of Rab38 in cells, but modest reduction of total amounts of Rab32, which is likely due to Rab protein destabilization. Given the strong effect that depletion of BLOC-2 has on Rab38 membrane association and

stability, RNAi depletion of Rab32 and Rab38 was performed to determine if there is any effect on BLOC-2 membrane association. Depletion of Rab38 significantly decreases the membrane association of BLOC-2, but Rab32 depletion has no effect on BLOC-2 levels. These results indicate that while both Rab32 and Rab38 interact physically with BLOC-2, AP-1 and AP-3 on membranes, the association may be functionally stronger between Rab38 and AP-3 and even stronger between Rab38 and BLOC-2.³¹

Confocal immunofluorescence microscopy experiments show that a significant percentage of structures labeled with endogenous AP-1, AP-3 and BLOC-2 co-localize with endogenous Rab32 and Rab38 in many locations throughout MNT-1 melanocytes.³¹ A larger percentage of structures labeled by AP-1, AP-3 or BLOC-2 co-localize with Rab38 than with Rab32. This is consistent with the stronger membrane association defects observed with Rab38 compared with Rab32 upon depletion of AP-3 and BLOC-2. The localization of Rab32 and Rab38 is likely to specific tubular domains of early/recycling endosomes that contain AP-1, AP-3 or BLOC-2.^{16,17} In support of that idea, Rab32 and Rab38 do not colocalize with the early endosome vacuolar domain marker EEA1 or tubular domains involved in the retrieval pathway to the trans-Golgi network labeled by the retromer complex.³¹ Interestingly, both Rab38 and Rab32 partially co-localize with the coat protein clathrin.³¹ Upon vesicle budding, the clathrin coat and adaptors disassemble and return to the cytosolic pool so they can be reutilized in further rounds of traffic (Fig. 1). Therefore, Rab32 and Rab38 are probably loaded onto the transport vesicles on endosomal tubular domains during the budding process or soon after vesicle release, but before vesicle uncoating. Rab32 and Rab38 likely remain bound to vesicles upon disassembly of AP-1, AP-3 and clathrin from vesicles (Fig. 1). It has been suggested that BLOC-2 may also be present in downstream vesicles or transport intermediates and may not undergo quick dissociation as is expected for AP-1, AP-3 and clathrin.¹ This possibility would be consistent with the stronger Rab38-BLOC-2 membrane association described

above. Rabs are ideal candidates to serve as mediators of trafficking between endosomal tubules and downstream organelles through interactions with specific effector proteins.²⁸⁻³⁰ The specificity of Rab32 and Rab38 function in endosome to melanosome trafficking is demonstrated by their localization to stage III and IV melanosomes and not to lysosomes (Fig. 1).^{23,31} These results indicate that Rab32 and Rab38 operate in the same pathways previously defined for AP-1, AP-3 and BLOC-2 and suggest they are the specific proteins that divert AP-1, AP-3 and BLOC-2-dependent cargoes to maturing melanosomes and away from lysosomes.

Rab32 and Rab38 serve critical functions in the trafficking of melanin-producing enzymes. If Rab32 and Rab38 are redirecting protein trafficking from early/recycling endosomal domains to melanosomes instead of lysosomes, then depletion of these Rab proteins should cause mistrafficking of the cargo proteins. RNAi depletion of Rab32 or Rab38 causes incorrect trafficking of the melanin-producing enzyme Tyrp1 to the plasma membrane in MNT-1 melanocytes.³¹ The same phenotype is produced by deficiency of either of AP-1, AP-3 or BLOC-2,^{17,31} consistent with the idea that Rab32 and Rab38 participate in a transport step mediated by AP-1, AP-3 and BLOC-2. This mistrafficking likely represents a blockage in transport to compartments downstream the early/recycling endosomes, which results in accumulation of cargo in early endosomes and leakage into the recycling pathway to the plasma membrane (Fig. 1). Simultaneous depletion of Rab32 and Rab38 elicits a more severe trafficking defect for Tyrp1, thus suggesting partially redundant roles for Rab32 and Rab38.³¹

Examination of the total abundance of tyrosinase and Tyrp1 further demonstrates the importance of Rab32 and Rab38 for correct, functional trafficking. Independent depletion of either Rab32 or Rab38 causes a significant decrease in overall tyrosinase abundance, but only modest decrease in Tyrp1 abundance.³¹ However, simultaneous depletion of both Rab32 and Rab38 further reduces the abundance of tyrosinase and Tyrp1.³¹ Inhibition of lysosomal hydrolases partially restores

tyrosinase and Tyrp1 to steady-state levels even with simultaneous depletion of Rab32 and Rab38. These results demonstrate that Rab32 and Rab38 are critical to direct trafficking of melanosome-specific cargoes to maturing melanosomes, instead of the default trafficking to lysosomes (Fig. 1). These results also resemble those obtained with melanocytes deficient for AP-1, AP-3 or BLOC-2, reinforcing the notion of functional cooperation with Rab32 and Rab38 in the traffic of cargo to melanosomes.^{17,42,43}

It appears that Rab32 and Rab38 function in parallel roles for the trafficking of tyrosinase and Tyrp1 and are able to partially compensate for the loss of the other Rab. Such a result is also observed upon depletion of AP-1, AP-3 or BLOC-2, where each protein can partially compensate for the loss of the other.^{16,17} As mentioned above, this observation has been interpreted as evidence of parallel AP-1, AP-3 or BLOC-2-dependent pathways in the trafficking of tyrosinase and Tyrp1. Given the physical interaction and colocalization of Rab32 and Rab38 with AP-1, AP-3 and BLOC-2, these Rabs likely serve all of the AP-3, AP-1 and BLOC-2-dependent pathways in the trafficking of tyrosinase, Tyrp1 and possibly additional cargoes required for melanosome biogenesis (Fig. 1).³¹

Evidence for Rab32 unique roles in melanosome biogenesis. In contrast with the cooperation displayed between Rab32 and Rab38 in the transport of tyrosinase and Tyrp1, the Rabs do not appear to have redundant or compensatory roles in the trafficking of Tyrp2.³¹ Depletion of Rab32, but not Rab38, causes a dramatic loss of Tyrp2 within MNT-1 melanocytes, which is likely due to mistraffic to lysosomes.³¹ What is more, depletion of Rab32 or Rab38 causes a reduction in the total amount of melanin within MNT-1 melanocytes, an indication of deficient melanosome biogenesis.³¹ However, Rab32 depletion has a much more severe effect than depletion of Rab38, and simultaneous depletion of both Rabs elicits similar melanin levels as depletion of Rab32 alone. These results imply there are key Rab32 roles in melanosome biogenesis, such as Tyrp2 transport, that Rab38 cannot carry out. Rab32 and Rab38 appear to have partial functional redundancy, but

differ in the membrane-association dependency with BLOC-2 and AP-3, the extent of co-localization with AP-3 and AP-1, trafficking of Tyrp2 and overall function in melanin production. Both Rab32 and Rab38 are present at similar levels within MNT-1 cells, demonstrating that non-redundant functions of Rab32 are not secondary to overall protein abundance.³¹ Ultimately, Rab32 and Rab38 have some redundant functions, but some unique function of Rab32 is required to maintain normal pigmentation within melanocytes.

In stark contrast with the transport of tyrosinase and Tyrp1, the trafficking pathway followed by Tyrp2 to reach maturing melanosomes is unknown. The above results suggest it may use a distinct pathway that requires Rab32 but not Rab38. Provocatively, depletion of BLOC-3, but not BLOC-1, both components of the machinery involved in melanosome biogenesis, also causes substantial loss of Tyrp2 within MNT-1 melanocytes, suggesting a possible independent role for Rab32 in the trafficking of Tyrp2 through a BLOC-3-dependent pathway.³¹ The function of BLOC-3 and its localization in specialized LRO-producing cells are unknown, although it has been shown to impact the localization of late endosomes/lysosomes in fibroblasts⁴⁴ and to interact physically with Rab9.⁴⁵

Implications for other lysosome-related organelles and future directions. Several genetic disorders exist with simultaneous defects in the production of melanosomes and other LROs, consistent with the idea that LROs share a common biogenesis mechanism.^{1,3,4,6,46,47} For example, Hermansky-Pudlak Syndrome (HPS) patients and the corresponding animal models have abnormal melanosomes, platelet dense granules and lamellar bodies of lung type II epithelial cells. These defects produce partial oculocutaneous albinism, bleeding diathesis and lung disease, respectively.^{1,3,4,6,46-48} Mutations in subunits of AP-3, BLOC-1, BLOC-2 and BLOC-3 underlie many forms of HPS.^{4,20,49} Importantly, Rab38 deficiency in rodent disease models causes biogenesis defects in melanosomes, platelet dense granules and lamellar bodies.^{25-27,50-52} Therefore, it is likely that the cooperation between Rab38 and the ubiquitous

transport machinery uncovered in melanocytes also functions in the biogenesis of other LROs, such as platelet dense granules and lamellar bodies. In future studies, it will be important to assess the potential contribution of Rab32 to the biogenesis of other LROs, in addition to melanosomes.

Recruitment of the Rabs occurs at specific early/recycling endosome tubular domains, but they are also present on melanosomes, suggesting that they remain bound to vesicles until fusion with the downstream melanosome.^{23,31} These results constitute a step forward in our understanding of these pathways but also open other questions. What are the functions of Rab32 and Rab38? What are the effectors of Rab32 and Rab38 that facilitate trafficking to and fusion with melanosomes? What proteins are important for the regulation of Rab32 and Rab38? Little is known about the protein effectors of Rab32 and Rab38 beyond interaction with the protein Varp, which is implicated in the recruitment of the v-SNARE VAMP-7/TI-VAMP and Tyrp1 traffic.^{15,53,54} This Rab-Varp interaction is likely important for the vesicle-melanosome fusion event. Potential interactions of Rab32 and Rab38 with tethering proteins such as the Homotypic fusion and Protein Sorting (HOPS) complex could also facilitate trafficking or fusion of vesicles with melanosomes.⁵⁵⁻⁵⁷ Supporting this possibility, mutation of the HOPS complex subunit Vps33a results in deficient melanosome biogenesis in the buff mouse.⁵⁵ However, the precise mechanisms that control the movement and targeting of vesicles remain unclear. The mechanisms used by Rab32 and Rab38 in the trafficking of transmembrane cargoes to melanosomes, and perhaps other LROs, will doubtless be investigated in future studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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