

Mechanisms of Protein Delivery to Melanosomes in Pigment Cells

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Vertebrate pigment cells in the eye and skin are useful models for cell types that use specialized endosomal trafficking pathways to partition cargo proteins to unique lysosome-related organelles such as melanosomes. This review describes current models of protein trafficking required for melanosome biogenesis in mammalian melanocytes.

The secretory and endocytic systems of all eukaryotic cells are subdivided into unique membranous organelles, each with its own set of functions and molecular components. Cells must maintain organelle identity despite constant membrane flux and transfer of materials among organelles. Eukaryotes have evolved elaborate vesicular trafficking mechanisms that accomplish this feat by ensuring 1) proper macromolecule sorting and delivery to, or retention within, the appropriate destination, and 2) proper maturation of secretory and endocytic organelles (15). These basic mechanisms are conserved among all eukaryotic cell types.

Other than ubiquitous secretory and endocytic organelles, certain cell types in multicellular organisms require additional unique organelles to perform specific physiological functions. One class of such organelles are the lysosome-related organelles (LROs; some of which are also referred to as secretory lysosomes; Ref. 11), a group of tissue-specific membranous structures that share some features with conventional lysosomes, such as derivation from the endocytic system, acidic luminal pH, and a cohort of lysosomal resident proteins (38, 148). Although some LROs such as cytolytic granules of cytotoxic T cells and natural killer cells appear to be modified lysosomes, other LROs are distinct and coexist with lysosomes within their respective cell type (148). Examples of such LROs with important physiological functions are dense granules in platelets, lamellar bodies in lung epithelial type II cells, signaling endosomes in plasmacytoid dendritic cells (10, 157), and melanosomes in pigment cells. Cells that harbor these LROs must exploit specific mechanisms to divert cargoes from conventional endocytic organelles and deliver them to nascent LROs. Failure of these mechanisms can lead to LRO deficiencies and consequent disease. The best-studied example of such an LRO is the mammalian melanosome, an organelle found in melanocytes of the eye and skin and pigmented epithelial cells of the retina, iris, and ciliary body of the eye, in which melanin pigments are synthesized and stored (120, 139). Because the same cellular machinery is used to sort cargoes to

LROs in diverse cell types, insights gained from studying trafficking in pigment cells such as mammalian melanocytes apply to multiple physiological systems and inform us regarding disease etiology. This review describes current models of melanosomal protein trafficking in melanocytes.

Melanin Synthesis and Melanosome Biogenesis

Melanin is a heterogeneous, insoluble nonprotein polymer composed of subunits that are products of enzymatically modified tyrosine (97). Mammals produce two types of melanins, black/brown eumelanins and red/yellow pheomelanins. The rate-limiting initial steps in all melanin synthesis (the hydroxylation of tyrosine to L-DOPA and oxidation of L-DOPA to DOPAquinone) are catalyzed by the pigment cell-specific enzyme tyrosinase (TYR; Ref. 108). Subsequent spontaneous oxidation and isomerization steps yield a mixture of eumelanin intermediates. Two other enzymes, DOPACHrome tautomerase (DCT) and tyrosinase-related protein 1 (TYRP1), influence the nature of the intermediates and properties of the eumelanins (166). Mutations in the genes encoding these and other melanosomal constituents result in oculocutaneous albinism (OCA; Table 1), characterized by partial or complete loss of eumelanin synthesis. If sufficient cysteine is present, DOPAquinone is modified to cysteinyl L-DOPA, and downstream steps yield pheomelanins (166). Melanosomes that predominantly make pheomelanins have distinct properties from those that predominantly make eumelanins (53, 100, 126) and will not be discussed further.

To sequester oxidative melanin intermediates, melanin synthesis is limited to the lumen of melanosomes. Melanosomes mature through four morphologically distinct stages (FIGURE 1), such that melanins synthesized in the last two stages are deposited on a “melanosome matrix” formed within the first two stages (161). *Stage I* melanosomes are vacuolar domains of early endosomes that harbor intraluminal vesicles (ILVs; Ref. 149);

they are distinguished from early endosomes in other cells by irregular arrays of amyloid fibrils, composed of the pigment cell-specific protein PMEL (8, 9, 102, 149), that emanate from the ILVs and eventually form the melanosome matrix (Refs. 87, 161; **FIGURE 1**). *Stage II* melanosomes are functionally segregated from the endocytic pathway and are

characterized by a fully formed melanosome matrix in which the fibrils are organized into arrays of parallel sheets (87, 106, 149). Melanin synthesis and deposition are initiated by the delivery to *stage II* melanosomes of enzymes such as TYR and TYRP1 and other proteins such as the copper transporter ATP7A and the putative

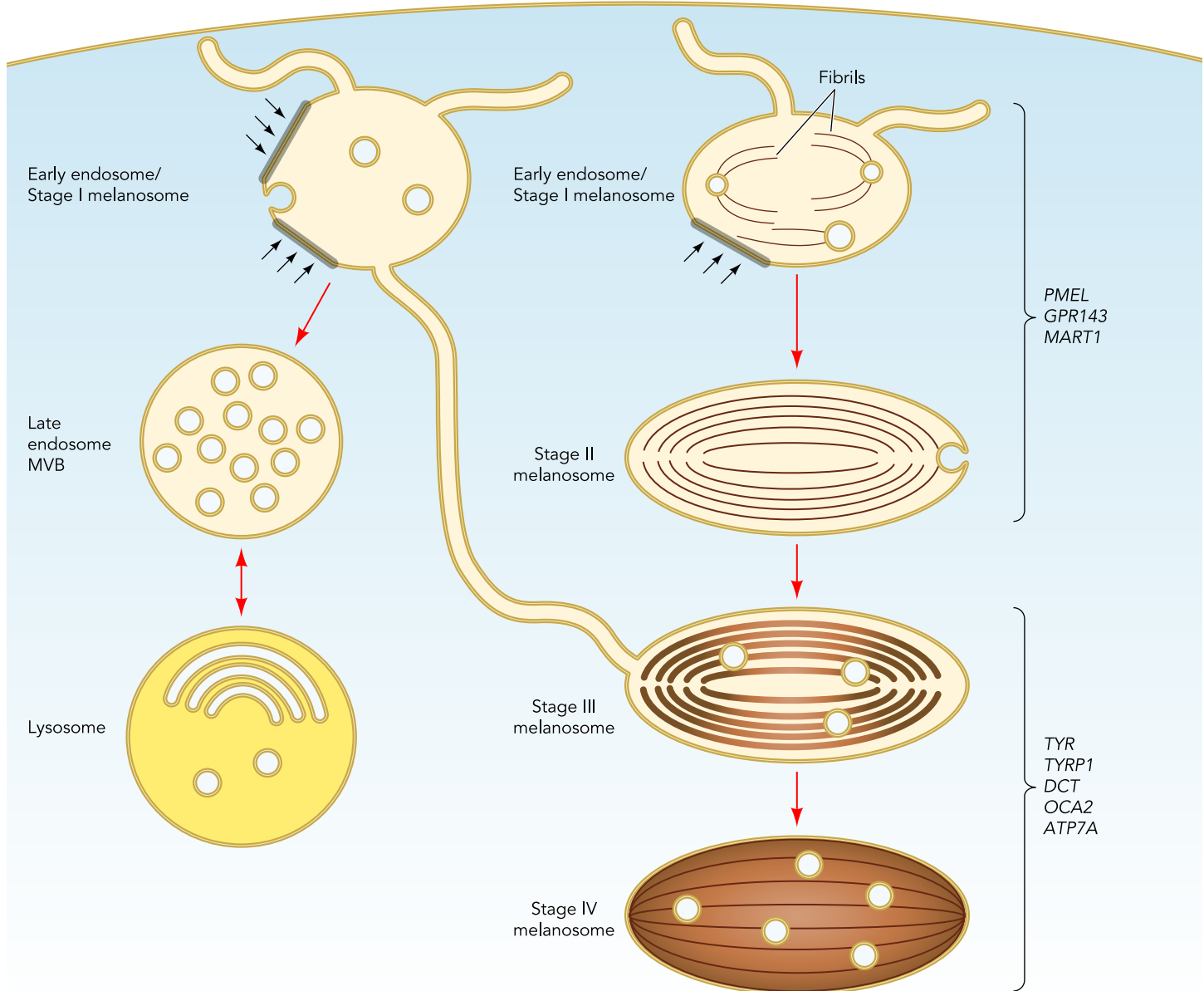


FIGURE 1. Melanosome maturation and segregation from endocytic organelles

Represented are maturation stages of endosomes (*left*) and melanosomes (*right*) from early endosomes (*top*) and their putative derivation by maturation from earlier compartments (red arrows). Protein contents of melanosomes as described in the text are indicated in italics to the right. Early endosomes (*top*) form by coalescence of membranes derived by internalization from the plasma membrane and by input from the secretory pathway. They consist of tubular domains and vacuolar domains; within the vacuolar domains, internal vesicles begin to form by invagination of the limiting membrane in regions rich in bilayered clathrin- and Hrs-containing coats (black arrows). The vacuolar domains mature into late endosomal multivesicular bodies (MVB) as in other cell types by exchange with other compartments via tubular connections. Luminal contents of late endosomes are degraded upon fusion with lysosomes. In pigment cells, the vacuolar domains of early endosomes also correspond to *stage I* melanosomes but are distinguished from vacuolar early endosomes of other cells by the presence of fibrils emanating from the internal vesicles as indicated. These fibrils elongate and assemble into sheets in *stage II* melanosomes. Delivery of contents to *stage II* melanosomes by vesicular traffic (not shown) or by tubular intermediates from early endosomes (as shown) results in the deposition of melanins on the fibrils in *stage III* melanosomes. Continued melanin deposition masks internal structure in *stage IV* melanosomes.

transporter OCA2. This causes darkening and thickening of the fibers in *stage III* melanosomes until all internal structure is obscured in *stage IV* melanosomes (Ref. 161; FIGURE 1). In mammals, mature melanosomes are transferred from skin melanocytes to neighboring keratinocytes, whereas melanosomes generated in eye pigment cells are retained.

Melanosome Resident Proteins Transit the Early Secretory Pathway

Most known protein constituents of *stage II–IV* melanosomes are integral membrane glycoproteins. Like all such glycoproteins, melanosomal proteins are synthesized by ribosomes associated with the endoplasmic reticulum (ER), become integrated in the ER membrane and translocate their luminal portions across the ER membrane via the ER translocation channel (translocon), are N-glycosylated by the translocon-associated oligosaccharyltransferase complex, and are folded and assembled by ER resident chaperone proteins and enzymes (47). TYR, TYRP1, and DCT are heavily glycosylated and modified by numerous disulfide bonds; their proper folding is thus highly reliant on ER redox conditions and calnexin, an integral membrane chaperone that binds to monoglucosylated N-linked oligosaccharides (67, 129, 130). TYR in particular is modified by six or seven N-linked oligosaccharides, and its folding and assembly are under tight control; we refer readers to an excellent review by Wang and Hebert for details (191). Failure to fold properly, as occurs in a number of albinism-associated forms of TYR, TYRP1, and OAI, results in retrotranslocation from the ER and degradation by the proteasome (19, 33, 66, 67, 182, 183). Some evidence suggests that TYRP1, OCA2, and the putative transporter SLC45A2 regulate the folding and ER exit of TYR in ways that are poorly understood (27, 31, 99, 184). Mutations in the genes encoding these proteins thus result in various isoforms of OCA (Table 1).

Melanosomal proteins that are properly folded in the ER transit through the Golgi complex en route to melanosomes, where their N-linked oligosaccharides are modified by resident glycosyltransferases, and in some cases, as for PMEL, O-linked oligosaccharides are added and modified. TYR first acquires enzymatic activity in the *trans*-Golgi network (TGN) (136), likely because this is where it first encounters copper (162), which is a critical cofactor (109). How melanosomal proteins are targeted from the Golgi to endosomal intermediates en route to melanosomes (see below) is not known, but GAIP COOH-terminus interacting protein (GIPC), a PDZ domain-containing protein of

unknown function, might facilitate TYRP1 trafficking through or from the Golgi. GIPC binds to the COOH terminus of newly synthesized TYRP1 within the Golgi or closely associated compartments (112). Knockdown of GIPC or of the associated APPL adaptor protein in melanocytes results in impaired melanogenesis and decreased TYR activity (95), suggesting that this interaction is functionally critical for trafficking of TYRP1 and perhaps other melanogenic enzymes. Where or how GIPC and APPL exert their functions, however, is not yet clear.

There has been some debate regarding whether PMEL traverses the Golgi en route to *stage II* melanosomes. The debate was sparked by the cofractionation of *stage II* melanosome-enriched subcellular fractions of a human pigmented melanoma cell line with PMEL isoforms containing N-linked glycans that lacked Golgi modifications (102). A number of subsequent publications purported to identify similarly unprocessed forms of PMEL at the plasma membrane or in endosomal compartments (186, 187). However, it is now clear that only PMEL forms with Golgi modifications, such as complex O-linked and sialylated N- and O-linked oligosaccharides, are detected in post-Golgi compartments and mature PMEL fibrils (68, 149); indeed, complex O-linked glycans appear to be required for formation of mature PMEL fibrils (68, 187). The earlier results likely reflected contamination of melanosome-enriched subcellular fractions with ER membranes (178), poorly characterized antibody epitopes, and low-resolution techniques.

Generation of Early Stage Melanosomes

To generate functional melanosomes, melanosome contents must be diverted from classical endocytic compartments within pigment cells. PMEL is diverted to early stage melanosomes by mechanisms that are incompletely understood. PMEL is synthesized as an integral membrane protein with a large luminal domain that has amyloidogenic properties, a single membrane-spanning domain, and a short cytoplasmic domain (178, 193). The latter bears a COOH-terminal ER exit signal that initiates transport through the secretory pathway and a conventional dileucine-based endocytic signal (see below) that facilitates internalization from the plasma membrane; a cytoplasmic domain truncation that disrupts these signals underlies the mild hypopigmentation observed in the *silver* mouse (121, 180) and essentially phenocopies a PMEL-null mutation (72), indicating that these are essential steps in PMEL maturation. Following endocytosis, PMEL is delivered via the early endosomal system

to *stage I* melanosomes, where the luminal domain becomes incorporated into ILVs that invaginate from the limiting membrane (8, 149). Two concomitant proteolytic cleavages within the PMEL luminal domain liberate the fibrillogenic peptide (8, 9, 101, 179), although some of these cleavages might also occur in the TGN (107). PMEL sorting to ILVs depends on a luminal determinant (78, 179) that overlaps with the fibrillogenic determinant (194) and is required for amyloid formation (179, 188). Unlike most other proteins that are sorted to ILVs, PMEL does not require ubiquitylation or the activity of the ESCRT machinery for its sorting (179) but rather requires the tetraspanin CD63 (188). PMEL fibril formation initiates from the ILVs, and the fibrils ultimately assemble into sheets that characterize *stage II* melanosomes (87). Although ILV formation and fibrillogenesis are initiated in *stage I* melanosomes that are accessible to endocytic tracers, it is not known how these compartments mature into separate late endosomal and *stage II* melanosomal compartments (FIGURE 1).

Two other pigment cell-specific proteins are found in early stage melanosomes but also in other compartments. GPR143, also known as OA1, is a G-protein-coupled receptor that is mutated in ocular albinism type 1 (Refs. 5, 158; Table 1). GPR143 is detected in late endosomes, lysosomes, and melanosomes, including especially early stage melanosomes (61, 143), and some evidence suggests that intraluminal L-DOPA can serve as its ligand (118). Two sorting signals in the cytoplasmic COOH-terminal domain, an unusual dileucine-based signal and a tryptophan-glutamate doublet, are together necessary and sufficient for lysosomal and melanosomal localization (143), but the effectors that recognize these signals and direct GPR143 delivery are not known. GPR143 is downregulated by ubiquitylation and ESCRT-dependent sorting to ILVs of late endosomes, and inhibition of this process leads to enhanced localization to mature melanosomes (60), indicating that the ubiquitylation state regulates GPR143 distribution between melanosomes and lysosomal degradation. MART1 (also called melan-a) is an unusual small integral membrane protein with no signal sequence (94). Like GPR143, MART1 localizes to multiple compartments, including late endosomes and lysosomes (34), to which it is targeted by ubiquitylation and ESCRT-dependent partitioning to lysosome-bound ILVs (110, 179). MART1 can be co-immunoprecipitated with GPR143 (61) and with PMEL (61, 77), but the functional consequences of these interactions are not clear. Gene targeting of the *Mart1* gene in the mouse results in modest hypopigmentation and abnormalities in melanosome morphology (3).

Protein Sorting to Mature Melanosomes

The maturation of *stage II* melanosomes to pigmented *stages III* and *IV* requires the delivery of melanogenic enzymes and transporters, such as TYR, TYRP1, and OCA2, from early endosomes/*stage I* melanosomes. These integral membrane proteins are recognized as melanosome cargoes in part by conventional cytoplasmically exposed endosomal sorting signals (7, 19, 165, 168, 181, 189), likely functioning in concert with luminal determinants (63, 168). The sorting signals in TYR, TYRP1, and OCA2 conform to the acidic dileucine-based consensus sequence of [D/E]XXXL[L/I] (14). Similar signals found in the cytoplasmic domains of many endosomal and lysosomal integral membrane proteins serve as independent, transferable sorting signals for endocytosis and/or subsequent endosomal sorting. Likewise, the acidic dileucine motifs in TYR, TYRP1, and OCA2 confer protein localization to late endosomes and lysosomes in non-melanocytes (19, 165, 168, 189) and are necessary for melanosome localization in melanocytes (7, 168, 189).

Acidic dileucine signals effect protein sorting by binding to conserved sites on heterotetrameric adaptor protein (AP) complexes. APs are a family of five related complexes that function as soluble sorting adaptors. They are recruited to membranes at least in part by binding to phosphoinositides (54) and/or Arf GTPases (45, 205), and then bind integral membrane cargo proteins that contain AP binding sites and cluster them into coated membrane domains. Membrane-bound AP-1, AP-2, and AP-3 also recruit clathrin and thus facilitate cargo incorporation into clathrin-coated vesicles that bud from these domains at the *trans*-Golgi network, cell surface, or endosomes, respectively (12, 14, 153). These vesicles are then directed to target membranes. AP-4 does not engage clathrin and sorts distinct sets of cargoes at the TGN toward endosomes (17). AP-5 was recently described and likely functions in receptor recycling out of endosomes (75).

In melanocytes, AP-1, AP-2, and AP-3 have been implicated in cargo trafficking to melanosomes. Signals in the OCA2 and TYR cytoplasmic domains interact with AP-1, AP-2, and AP-3 *in vitro* (23, 80, 168, 181). TYR can be localized to endosomal buds and tubules that are decorated with AP-1 or AP-3 (141, 181), and significant cohorts of OCA2 and TYR are mislocalized in melanocytes that are genetically deficient in AP-3 (Refs. 84, 151, 181; Sitaram A, unpublished observations). By contrast, the TYRP1 sorting signal interacts with AP-1 but not AP-3 *in vitro* (181), and TYRP1 can be cross-linked to and

co-immunoprecipitated with AP-1 in melanoma cells (35). Consistently, TYRP1 is correctly localized to melanosomes in AP-3-deficient melanocytes (84, 163) but is mislocalized to vacuolar early endosomal domains in a human melanoma cell line in which AP-1 is depleted by siRNA (35). Besides its adaptor function, AP-1 is also required to position endosomes in the melanocyte periphery near maturing melanosomes by recruiting the microtubule motor KIF13A; this likely facilitates the formation of transient connections between endosomes and melanosomes for cargo transfer (Ref. 35; FIGURE 2). AP-2 is required for efficient PMEL delivery to endosomes and for melanosomal accumulation in melanocytes (152), likely by interacting with the acidic dileucine signal in the PMEL cytoplasmic domain (180) (note this signal is not necessary for post-endocytic sorting to early stage melanosomes; Refs. 179, 180). From these results, it is clear that dileucine motifs and AP complexes play major roles in melanosomal trafficking. Interestingly, the melanin biosynthetic enzyme DCT lacks a cytoplasmic dileucine-based signal but harbors a sequence conforming to AP-binding tyrosine-based motifs (14) that likely serves a similar function.

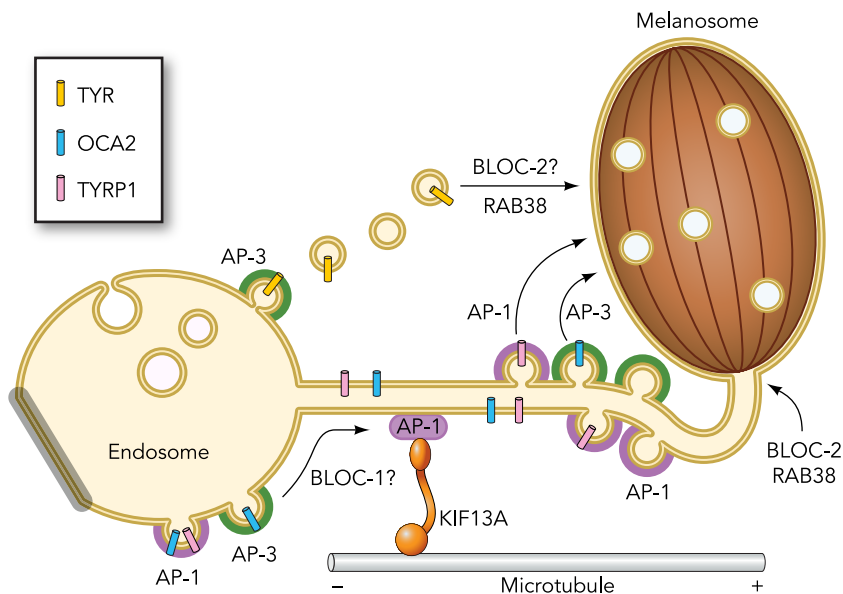


FIGURE 2. Model of delivery of selected cargoes to late-stage melanosomes

Adaptor proteins AP-1 and AP-3 concentrate specific melanosomal integral membrane cargo proteins such as OCA2 (blue bars), TYRP1 (pink bars), and tyrosinase (gold bars) within early endosomes. AP-1 also engages the microtubule plus-end directed kinesin KIF13A to establish tubules that emanate from recycling endosomal domains in the cell periphery and fuse with the membrane of nearby melanosomes. AP-1 and perhaps AP-3 direct cargoes such as OCA2 and TYRP1 into this pathway. BLOC-1 also functions on this pathway during cargo entry into the tubules. BLOC-2 and RAB38 appear to act downstream, perhaps to specify the targeting of these tubules to melanosomes. Engagement of cargo proteins on the tubules with AP-1 or AP-3 might be required for efficient delivery of cargoes from the tubules to the melanosome. AP-3 also functions from a distinct domain of early endosomes to direct BLOC-1-independent vesicular trafficking of TYR to the melanosome. RAB38 and perhaps BLOC-2 facilitate melanosomal targeting in this pathway. This figure is modified from Fig. 8 of Ref. 35 and used with permission from *Journal of Cell Biology*.

Diseases of Melanosome Biogenesis and Protein Trafficking

A number of components of the sorting machinery required for the proper targeting of melanosomal proteins have been identified through the discovery of human patients in whom LRO function is disrupted, resulting in oculocutaneous albinism and other functional disorders (Refs. 83, 147; Table 1). Griscelli Syndrome is one well characterized genetic disease of LROs, but it affects organelle motility and not maturation and will be briefly discussed later. Genetic diseases of LRO biogenesis include Chediak-Higashi Syndrome and Hermansky-Pudlak Syndrome (HPS; Ref. 83). Chediak-Higashi syndrome is a lethal, multisystem disorder that results from defects in the gene encoding a large protein called CHS1 or Lyst (93). Lyst deficiency results in decreased fission of lysosomes and presumably LROs (46), but how Lyst functions in this process is not known. HPS is characterized minimally by oculocutaneous albinism and prolonged bleeding due, respectively, to the malformation of melanosomes and platelet-dense granules; most patients additionally suffer from lung fibroses that most likely result from defective lamellar body function, leading to death in midlife (195), and some patients also exhibit immune deficiencies because of defects in LRO biogenesis or specific protein-sorting defects in cells of the adaptive and innate immune system (10, 30, 52, 157, 171). There are at least nine subtypes of HPS, each caused by defects in a different gene (32, 41, 83, 195). Mouse models exist for each HPS subtype, and at least six additional mouse models show similar symptoms (Refs. 41, 111; Table 1).

Although the clinical phenotypes of HPS arise specifically within tissues that harbor LROs, each of the affected genes is ubiquitously expressed. The proteins encoded by the 9 human and 13 of the 15 mouse HPS-associated genes are subunits of five distinct multisubunit complexes: the Class C Vps complex (a subcomplex of the homotypic fusion and vacuole sorting, or HOPS, complex), AP-3, and the biogenesis of lysosome-related organelles complex-1, -2, and -3 (BLOC-1, -2, and -3; Table 1). Each of these complexes has been implicated in membrane trafficking events that are required for melanosome biogenesis, as discussed above (for AP-3) and below.

HPS-Associated Multisubunit Complexes

Class C Vps complex

A point mutation in the gene encoding the VPS33A subunit of the four-subunit Class C Vps complex

underlies the HPS phenotype of the *buff* mouse (173). The Class C Vps complex has been best described in the yeast *Saccharomyces cerevisiae*, in which it is a core component of HOPS (160) and CORVET (142). HOPS and CORVET are effectors of endosomal Rab GTPases and function as “tethering” complexes that promote SNARE complex assembly and selectivity (reviewed in Ref. 196). The mammalian HOPS complex also functions in endosomal and lysosomal fusion events (20, 96, 144, 150). Mutations in genes encoding several Class C Vps subunits cause LRO defects in mouse, fly, and nematode models (73, 113, 145, 164, 169, 173). How this complex functions during melanosome biogenesis is not clear. The yeast HOPS complex facilitates docking of AP-3-coated vesicles with the vacuole (2), suggesting that the VPS33A-containing HOPS complex in mammals might regulate fusion of AP-3-dependent transport intermediates with nascent melanosomes in melanocytes. Interestingly, a mutation in the human gene encoding a homologous Vps-C subunit, VPS33B, causes arthrogyrosis-renal dysfunction-cholestasis syndrome, which is associated with defects in neuronal, kidney, and bile duct dysfunction as well as bleeding diathesis, but not with albinism (62). This suggests that distinct mammalian Vps-C complexes function in different membrane trafficking steps.

AP-3

The ubiquitously expressed heterotetrameric AP-3 complex consists of β 3A, μ 3A, σ 3, and δ subunits, whereas β 3B and μ 3B subunits replace the respective ubiquitous subunits in a neuron-specific AP-3B complex (37). The *AP3B1* gene encoding the β 3A subunit is mutated in HPS2 patients and the *pearl* mouse (51, 201, 204). The *Ap3d* gene encoding the δ subunit is mutated in the *mocha* mouse (92), which suffers from neurological disorders in addition to HPS (103, 135, 175) due to the loss of both the ubiquitous and neuron-specific complexes. Nematode and fly models of AP-3 deficiency also have LRO defects (73, 127, 128, 138, 167). In melanocytes, AP-3 is required for the efficient delivery of TYR (84, 181) and OCA2 (Ref. 81; Sitaram A, unpublished observations) to nascent melanosomes. AP-3 localizes predominantly to clathrin-coated buds on early endosomal tubules in melanocytic cells and is thought to function by recruiting cargoes such as TYR into buds and eventually vesicles that are destined for melanosomes (181). AP-3 might also function from recycling endosomal tubules for some cargoes (141).

BLOCs

The majority of the HPS-affected genes encode subunits of BLOC-1, -2, and -3, which are well conserved

in metazoans; distant homologs of BLOC-1 and BLOC-3 can be found in *Dictyostelium* and *S. cerevisiae* (24, 70). Each complex is an obligate oligomer, such that most subunits of the corresponding BLOC are destabilized by known HPS-associated mutations in any one BLOC subunit. BLOC subunits lack homology to proteins of known function, and the molecular functions of the BLOCs are not yet known. However, clues to their functions have been deduced from the phenotypes of cells lacking BLOC subunits and from genetic and biochemical interactions of BLOC subunits with other effectors.

BLOC-1. BLOC-1 comprises at least eight subunits, and mutations in the genes encoding three and five of them, respectively, underlie human and mouse HPS models (32, 41, 124). Among the mouse HPS models, BLOC-1-deficient mice are the most severely hypopigmented (36). This is due at least in part to a requirement for BLOC-1 in the delivery of the copper transporter, ATP7A, to melanosomes; copper is an obligate cofactor for TYR, and BLOC-1-deficient melanocytes thus lack TYR activity within melanosomes despite only a modest deficiency in TYR localization to melanosomes (162). Analyses of TYRP1 trafficking have shown that BLOC-1 facilitates the exit of selected melanosomal cargoes from recycling endosomal domains toward melanosomes (32, 163), consistent with genetic interactions between BLOC-1 and the recycling endosomal Rab11 GTPase in *Drosophila* (25) and in a bioinformatics screen (154). Similarly, in neurons, BLOC-1 functions in effecting cargo transport from early endosomes to synaptic vesicles (131, 156). In neurons, this BLOC-1 activity functions in concert with AP-3 (131, 156), and consistently a cohort of BLOC-1 physically associates with AP-3 in several cell types (42). Nevertheless, in melanocytes AP-3 and BLOC-1 localize at steady state to non-overlapping domains of early endosomes (42) and regulate melanosome localization of distinct cargoes: TYR delivery is highly dependent on AP-3 but less so on BLOC-1, whereas TYRP1 and ATP7A delivery is highly dependent on BLOC-1 but independent of AP-3 (32, 84, 162, 163, 181). Although some cargoes might require both (Sitaram A, unpublished observations), this suggests that AP-3 is not an obligate BLOC-1 partner.

How BLOC-1 functions at the molecular level is unclear. Physical interactions between BLOC-1 subunits or intact BLOC-1 with the endosomal t-SNARE component syntaxin 13 (59, 82, 125) and the exocytic tSNARE components SNAP23 and SNAP25 (59, 89, 190) suggest that BLOC-1 might influence either endosomal membrane fusion events or sorting of endosomal SNAREs, consistent with observations of SNARE missorting in BLOC-1-deficient neurons (156). Intriguingly, the phenotype

of melanocytes from BLOC-1-deficient animals or patients resembles that of a pigmented human melanoma line in which AP-1 was knocked down by short interfering RNAs, in that cells are highly hypopigmented, TYRP1 is mislocalized, and endosomal vacuoles accumulate (35, 162, 163). To date, however, interactions between BLOC-1 and AP-1 have not been reported.

BLOC-2. BLOC-2 comprises three large subunits, each the product of human and mouse HPS-associated genes (41), and an HPS5 ortholog is defective in a *Drosophila* eye color mutant (49). BLOC-2-deficient mice have a more modest coat color dilution than BLOC-1 mutants, but melanosomes in choroidal melanocytes uniquely clump into multi-melanosomal structures (58, 203). In primary melanocytes from BLOC-2-deficient HPS patients, TYR and TYRP1 are mislocalized (13, 71, 85, 151); similarly, TYRP1 in BLOC-2-deficient mouse melanocytes is mislocalized to endosomal structures but does not accumulate in vacuolar endosomal domains, suggesting that BLOC-2 might function downstream of BLOC-1 (163). Consistent with a function in the BLOC-1 pathway, BLOC-2 localizes to endosomal tubules in melanocytes and physically interacts with a cohort of BLOC-1 (42, 156). Skin melanocytes from BLOC-2-deficient patients accumulate TYR-containing vesicular structures in the cytoplasm (13, 71), suggesting that BLOC-2 might play a role in fusion of BLOC-1-dependent transport intermediates with maturing melanosomes.

BLOC-3. BLOC-3 is composed of two subunits that are defective in HPS patients and mouse models, including the most prevalent HPS, type 1 (39). BLOC-3-deficient patients have the most dramatic lung fibrosis (1, 57, 64), making this subset of patients the most clinically important, yet among the BLOCs we understand the least about BLOC-3. Loss of BLOC-3 in mouse models has a mild effect on coat and eye color but a more dramatic effect on the hairless skin of the ears and tail, indicating differences in melanosome biogenesis and development in follicular vs. interfollicular melanocytes (57, 133), and results in melanosome enlargement in choroidal melanocytes (50, 56, 174). The heightened severity of LRO phenotypes in double mutants lacking both BLOC-3 and either BLOC-2 or AP-3 suggests that BLOC-3 works in a separate pathway from those complexes and perhaps from BLOC-1 as well (42, 50, 57). The molecular function of BLOC-3, however, is not clear. Knockdown of BLOC-3 subunit expression in fibroblasts results in altered distribution of late endosomes/lysosomes, suggesting a potential primary role for BLOC-3 in organelle motility (48). This is consistent with defects in melanosome transfer observed in hair

follicles of BLOC-3-deficient mice (132, 134). Intriguingly, BLOC-3 in insect cells appears to be required for optimal gene silencing mediated by small RNAs (69, 105), suggesting that some of the effects of BLOC-3 deficiency might reflect ineffective gene silencing rather than a direct activity of BLOC-3 on membrane trafficking.

Other Effectors of Melanosome Biogenesis

Rab proteins

Among additional known effectors of melanosome biogenesis are several members of the Rab family of small GTPases. Rabs function as molecular switches; in their GTP-bound state, they bind to membranes and recruit effector proteins, whereas in their GDP-bound state they generally fail to bind to effectors (170). Among known effectors are tethering complexes, fusion proteins, cytoskeletal motors, phosphoinositide kinases and phosphatases, and GTPase activating proteins or guanine nucleotide exchange factors for other Rabs. The HPS model *gunmetal* mouse is deficient in the α subunit of the ubiquitously expressed Rab geranylgeranyltransferase II [needed to modify target Rab proteins with a COOH-terminal prenyl group to allow for membrane-anchoring (40, 202)] but displays phenotypes primarily in platelets and melanocytes (159). One of the targets of RABGGTA is RAB27A, a tissue-restricted Rab family member that is expressed in melanocytes and a number of other cell types (26, 146). In melanocytes, activated RAB27A associates with mature melanosomes and, together with its effector protein melanophilin, recruits the actin motor myosin VA (86, 198, 199). This enables transfer of melanosomes from microtubules onto the peripheral actin network of skin melanocytes, allowing for subsequent transfer to keratinocytes. Mutations in the genes encoding either RAB27A, melanophilin, or myosin VA lead to abnormal perinuclear clustering of melanosomes (4) and give rise, respectively, to the hypopigmentation phenotype of *ashen*, *leaden*, and *dilute* mice, models of three subtypes of Griscelli Syndrome (Refs. 122, 123, 140, 197; Table 1). A similar RAB27A-containing tripartite complex, with a distinct adaptor (MyRIP) and myosin motor (MyoVIIa), maintains peripheral accumulation of melanosomes in the apical domain of retinal pigment epithelia (117). Although RAB27A deficiency affects primarily melanosome positioning, the reduction in melanosome number seen in *gunmetal* mice and other data implicates additional Rabs in melanosome biogenesis, including potentially RAB11, as discussed earlier (25, 35, 154, 202). For the sake of brevity, only a few examples are discussed here.

Mutations in the *Rab38* gene in mice are associated with modest diminution in coat color (114) but a dramatic defect in melanosome maturation in the retinal pigment epithelia (116), and a mutation in rat *Rab38* is associated with an HPS-like phenotype (137). The modest effect on coat color in the *chocolate Rab38* mutant mouse appears to reflect functional redundancy in skin melanocytes between RAB38 and the closely related RAB32 (192). A RAB38/32 ortholog in *Drosophila* is also required for pigment granule formation (119). RAB38 localizes to melanosomes and to transport intermediates en route to melanosomes, and in RAB38-/RAB32-deficient melanocytes both TYR and TYRP1 are mislocalized to the TGN and/or degraded in lysosomes (192). Similar functional outcomes result from the depletion of VARP, a RAB38/RAB32 effector (177), and this defect can be rescued by wild-type VARP but not by a mutant that fails to interact with RAB38/32 (176). This suggests that RAB38 (and RAB32 in melanocytes), at least in part via recruiting VARP, functions in specifying the delivery of transport intermediates from either the Golgi or endosomes to melanosomes and that in its absence at least TYRP1 is largely degraded in lysosomes. Interestingly, VARP also binds to VAMP7 (also called TI-VAMP; Ref. 16), a vSNARE involved in late endosomal maturation and late endosome-lysosome fusion in other cell types (22). Interference with the VARP-VAMP7 interaction also disrupts TYRP1 trafficking (176), suggesting that the RAB38-VARP complex might regulate the formation of trans-SNARE complexes required for cargo delivery to melanosomes.

Other Rab GTPases likely play additional roles in melanosome biogenesis but have been less well characterized. For example, the ubiquitous RAB7A appears to regulate motility of early stage melanosomes through its ability to recruit cytoplasmic dynein, a minus-end-directed microtubule motor (91), with potential downstream effects on recruitment of melanosome markers (74). The ubiquitous RAB9 physically interacts with BLOC3 when in its active GTP-bound state, suggesting that BLOC3 might be an effector of RAB9 (98), although functional data supporting this contention are lacking. RAB17 is coregulated with melanosomal proteins in melanocytes, and together with RAB8 (21) influences peripheral actin dynamics and melanosome secretion (6). A number of less well characterized Rab proteins, including RAB31, RAB32, and RAB33A in addition to RAB27A and RAB38, are downregulated during melanocyte transformation to melanoma (79), and PMEL and TYRP1 levels are reduced in cells expressing a putative dominant active RAB33A variant (28). Elucidating how these Rab proteins

function with other trafficking machinery will be important to understand the regulation of melanosome biogenesis.

ESCRTs

ESCRTs are a series of highly conserved multisubunit complexes that have well defined roles in the formation of and cargo entry to the internal vesicles of endosomes in all eukaryotes, as well as in viral budding, exosome formation, and cytokinesis (reviewed in Ref. 88). The ESCRT-0, ESCRT-I, and ESCRT-II complexes concentrate cargoes that are ubiquitylated or recognized by other means into clathrin-coated membrane patches on maturing early endosomal vacuoles and drive these cargoes into invaginating membranes; ESCRT-III and the AAA ATPase VPS4 then function to release these vesicles into the endosomal lumen. In pigment cells, ESCRTs function as expected in the lysosomal sorting and degradation of MART-1 and GPR143 (60, 110, 179, 185), but they also appear to play additional roles in melanosome maturation. For example, in cells that are depleted of the ESCRT-I component Tsg101 or in which ESCRT function is inhibited by expression of a dominant negative form of VPS4B, TYRP1 is missorted into autophagosome-like structures (185). Whether this reflects a direct role for ESCRT-I in sorting of melanosomal cargoes or an indirect role in regulating endosomal morphology required for melanosome maturation is not yet clear, but it points to a non-classical function for ESCRT-I and perhaps other ESCRTs in pigment cells. Interestingly, *stage III* and *IV* melanosomes, like late endosomes, harbor intraluminal vesicles on which TYR accumulates (181). Whether these vesicles form in an ESCRT-dependent fashion or whether ubiquitylation is required for cargo sorting into these vesicles is not yet known.

Syntaxin 17

The distinctive age-related coat depigmentation and high incidence of melanoma in the widely bred Gray horse were found to correlate with a duplication of an intronic region within the gene for syntaxin 17 (STX17; Ref. 155). Within melanoma tissue, the mutation causes increased expression of STX17 and the neighboring nuclear receptor protein NR4A3 due to the duplication of a binding site for the pigment cell-specific transcription factor MITF (172). STX17 is poorly characterized within the syntaxin family of SNAREs, and STX17 protein levels and function were not investigated; thus it is unclear whether altered trafficking to melanosomes plays a role in the phenotype. Alternatively, age-related depigmentation might be caused by reduced hair bulb melanocyte survival, as in the

autoimmune disease vitiligo (104) and potentially in PMEL-deficient mice (72).

TRAPP

TRAPPI and TRAPPII are cytosolic multisubunit complexes in yeast that act as both Rab GEFs and vesicle tethers in ER-Golgi and intra-Golgi membrane trafficking, respectively (18). Both TRAPPs share a common core of seven subunits, and TRAPPII contains an additional three. TRAPP subunits are conserved in mammals and are expressed in many tissues (115), and mammalian TRAPP also acts as a Rab GEF and a tether in the secretory pathway (200). Importantly, TRAPP has been implicated in melanosome biogenesis. Disruption of murine *Trappc6a*, encoding the homolog of the Trs33p subunit of the yeast core TRAPP complex, in the *mhyp* mouse is associated with mosaic coat hypopigmentation and a reduction in RPE melanosomes (65). However, the structure of mammalian TRAPP is not identical to the yeast complex (206), and the precise mechanism of TRAPP involvement in melanosome biogenesis remains to be elucidated.

Phosphoinositides and autophagy regulators

Recent reports have revealed two unexpected sets of effectors of melanosome biogenesis. One is phosphatidylinositol(3,5)bisphosphate [PtdIns(3,5) P_2], a rare lipid found nearly exclusively in late endosomal organelles (43). The gene encoding FIG4, a phosphatase that removes the 5' phosphate from PtdIns(3,5) P_2 , is mutated in the *pale tremor* mouse and patients with Charcot-Marie Tooth syndrome type 4J (29). Both the *pale tremor* mouse and mutants in *Vac14*, a gene encoding a scaffolding protein in the FIG4 complex, are severely hypopigmented (29, 90). This suggests that PtdIns(3,5) P_2 plays a unique and important role in melanosome biogenesis. The second unsuspected pathway is autophagy. Several components of the autophagy pathway were identified in a siRNA silencing screen for genes required for pigmentation in a human melanoma cell line, and targeted knock-down of these and other components impaired melanogenesis (55). This appears to reflect a negative impact of mTOR kinase signaling in melanosome biogenesis (76). Intriguingly, both the PtdIns(3,5) P_2 and autophagy pathways might be linked by a single protein, WIPI1, which both engages the autophagy pathway (76) and is an effector of PtdIns(3,5) P_2 (44). Whether these effects are solely mediated at the transcriptional level or more directly influence melanosome biogenesis is not yet clear.

Perspectives and a Model for Membrane Trafficking in Melanosome Maturation

As described in this review, the routes through which cargo proteins access nascent melanosomes are numerous and complex and involve many regulators, but it remains unclear how these regulators are integrated to effect proper melanosome maturation. Based on the analyses of HPS models and several additional effectors, we propose a model whereby early stage melanosomes are matured to *stage III* and *IV* by a dual pathway of cargo delivery, one that is BLOC-1-dependent and one that is BLOC-1-independent (FIGURE 2). Based on observations of tubular connections between endosomes and melanosomes by both three-dimensional reconstruction of electron tomograms and live cell imaging (35), we propose that the BLOC-1-dependent cargoes are delivered via tubular intermediates between recycling endosomal domains and maturing melanosomes (FIGURES 1 AND 2). The integrity of this pathway requires AP-1 and its associated KIF13A. A second BLOC-1-independent pathway is likely mediated by vesicles that bud from sorting endosomal domains. How other effectors fit into this pathway remains unclear, but we speculate that RAB38, BLOC-2, and perhaps RAB33A and HOPS function during fusion of these transport intermediates with maturing melanosomes. Analyses of additional cargoes will be needed to better characterize these pathways and to test this model. The function of the BLOCs and the mechanism underlying their cargo selectivity remain an intriguing mystery and should be a major focus of future investigation. Finally, comparative studies of trafficking in other LRO-containing cells such as platelets and immune cells are yielding insights that may lead to a more unified and general model of trafficking specialization within the endosomal system. ■

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