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The Melanosome: Dark Pigment Granule Shines Bright Light On Vesicle Biogenesis And More

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Melanin pigment in mammalian skin is found primarily in keratinocytes and melanocytes. While melanin serves its primary function as photoprotective pigment in the keratinocytes, its biosynthesis occurs in melanocytes within specialized organelles known as melanosomes. Human skin pigmentation is, in essence, a collective manifestation of a complex cellular function of individual melanocytes that begins with formation of tissue-specific precursor organelles, synthesis and ordered accumulation of melanin polymer, directional transport of pigmented melanosomes to the dendritic tips and their eventual export to keratinocytes. Significant progress has been made in understanding the molecular mechanisms involved in these events, most notably in enzymatic regulation of melanin synthesis, and in transport of melanosomes to periphery. These topics have been reviewed elsewhere and will not be discussed here (Hearing 1999; Wu and Hammer 2000; Westbroek et al. 2001; Marks and Seabra 2001). More recently, new exciting developments have also been reported in the area of melanosome transfer to keratinocyte (Scott et al. 2001; Sieberg 2001; Minwalla et al. 2001).

Within melanocytes, four distinct morphological stages of melanosome maturation can be seen (Figure 1). Membrane vesicles containing no visible pigment but irregular internal membrane structures are defined as stage I or premelanosomes. Maturation of premelanosomes to stage II melanosomes results in elongation of the vesicles, and ordering of the internal membranes into parallel structures. Stage III melanosomes can be distinguished by the presence of ordered deposition of melanin (sometimes appearing as beads on a string) on the internal fibers. In mature, stage IV melanosomes filled with melanin pigment no luminal structures are distinguishable. This review will provide a brief account of the history and continuing evolution of ideas on the biogenesis of melanosomes, and provide a detailed discussion of studies on the trafficking of individual melanosomal membrane proteins, and ongoing molecular genetic and biochemical studies on mouse coat color mutations and human pigmentary disorders that are contributing to a better understanding of not only melanosome biogenesis in health and disease but also intracellular membrane dynamics in general.

The Nature and Origin of Melanosome

In this year of 50th anniversary of publication of DNA structure (Watson and Crick 1953), it is of historical significance to note that although the dark melanin pigment granules were morphologically identified and studied for long, the term “melanosome”, “a distinctive subcellular particle of mammalian melanocytes and the site of melanogenesis”, to describe the membrane bound pigment granules was not proposed until 1961 (Seiji et al. 1961). While classical light and electron microscopic studies helped to define the distinct stages in morphological maturation of the melanosome (Mishima 1962), biochemical composition of the limiting membrane and its biogenesis remained controversial. This is illustrated by scattered reports that suggested association of DNA with pigment granules and their relationship to mitochondria (De Buy et al. 1949; Baker et al. 1960). Application of subcellular fractionation and

Abbreviations: APPL: adaptor protein with pleckstrin homology, phosphotyrosine binding, and leucine-zipper domains; CHS: Chediak-Higashi syndrome; GAIP: G-protein interacting protein; GIPC: GAIP interacting protein; HPS: Hermansky-Pudlak syndrome; LAMP-1: lysosome associated membrane protein-1; OA1: ocular albinism1; OCA: oculocutaneous albinism; TGN: *trans*-Golgi network TRP: tyrosinase related protein (TRP-1 and TRP-2)

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enzyme histochemical methods provided early indications of the relationship of melanosomes to Golgi and lysosomes (Maul 1969), prompting Wolff and Honigsmann (1971) to raise the question "Are melanosome complexes lysosomes?" over three decades ago. Analysis of lysosomal function in inbred mice with coat color mutations revealed that lysosomal dysfunctions are associated with pigment gene mutations and led to the suggestion that "…there are several critical genes in mammals that control the biogenesis, processing and/or function of related classes of subcellular organelles" (Novak and Swank 1979). Two lines of investigation have contributed significantly to our current understanding of melanosome biogenesis, and its relationship to other lysosomes-related organelles. These are a) cell biological studies on intracellular sorting and transport of melanosomal membrane proteins, specifically tyrosinase, tyrosinase-related protein-1(Tyrp), p-protein and Pmel17 (reviewed in Raposo and Marks 2002 and Setaluri 2001), and b) molecular genetic analyses of mouse coat color mutations and a group of human pigmentary disorders, specifically Chediak-Higashi syndrome (CHS) and Hermansky-Pudlak syndrome (HPS) (Spritz 2000; Starcevic et al. 2002; Ward et al. 2002). The availability of mouse and human melanoma cell lines that retain differentiated phenotype and the ease of culturing mouse and human melanocytes have made melanosome an excellent model for understanding biogenesis of lysosome-related organelles.

Melanosome biogenesis: More than skin deep

Unraveling melanosome biogenesis has broader implications than simply understanding how melanin pigment is packaged. In mammals, the cellular machinery for biogenesis of melanosomes has emerged primarily in two groups of pigment cells i.e., cutaneous and uveal melanocytes derived from the neural crest, and the retinal pigment epithelial (RPE) cells derived form the neuroectoderm. Although these two groups of melanocytes are generally thought to be derived from different embryonic tissues (Dupin and Le Douarin 2003; Schraermeyer and Heimann 1999), it has been argued that a single progenitor in the neural tube could give rise to both neural crest and optic neuroectoderm melanoblasts (Boissy 1998). Defining precise embryonic origin of these two types of pigment cells has implications for understanding molecular regulation of melanosome biogenesis during development. Regardless of their origin, the limited and transient ability to produce melanosomes distinguishes ocular pigment cells (both uveal and RPE) from their cutaneous counterparts, which produce melanosomes continually throughout life (Feeney-Burns 1980). This difference between ocular and cutaneous pigment cells might hold clues to master genetic switches that regulate melanosome biogenesis. In addition to ocular pigment abnormalities associated with the well characterized oculocutaneous albinsms, mutations in genes encoding melanosomal proteins have recently been shown to be associated with other ocular diseases such as pigmentary glaucoma (Anderson et al. 2002; Libby et al. 2003).

Presence of melanosomes and/or expression of melanosomal antigens is used in diagnosis of pigmented lesions and abnormal differentiation of amelanotic lesions and occasionally for prognosis of certain melanomas (Yen et al. 2000; Suzuki et al. 2001). Moreover, in patients with autoimmune vitilgo and malignant melanoma, melanosomal proteins are frequent targets for both humoral and cellular immune responses (Kemp et al. 2001; Ramirez-Montagut et al. 2003; Raposo et al. 2002; Sakai et al. 1997). Involvement of endosomes in melanosome biogenesis, and intersection of melanosomal protein trafficking with intracellular pathways of antigen processing seems to facilitate preferential immune responses to these proteins (Wang et al. 1999). Melanosome is also considered a perfect model for cellular responses to environment (Hearing 2000).

Pathways to melanosome biogenesis

The earliest theory on the pathway of melanosome biogenesis was formulated by exploiting the ability to localize tyrosinase activity in various cellular compartments by ultrastructural enzyme histochemical methods (Seiji et al. 1961a, 1963). It was proposed that premelanosomal (stage I) vesicles containing luminal matrix proteins bud from smooth endoplasmic reticulum (ER), and mature to form elongated stage II melanosomes with the characteristic luminal parallel arrays of fibers. Fusion of *trans*-Golgi derived clathrin-coated, tyrosinase-containing coated vesicles with stage II melanosomes was proposed to initiate synthesis and deposition of melanin and produce mature melanosomes. This view gained support from the biochemical characterization of Pmel17/gp100, a melanosomal membrane protein encoded by the mouse *silver* locus. Based on the absence of Golgi-modifications of Asn-linked sugars of Pmel17, it was argued that this luminal protein and vesicles enriched with this protein, presumably premelanosomes, arise directly from the ER (Kobayashi et al. 1994). However, unlike its mouse counterpart, human Pmel17 is glycosylated and undergoes maturation in the Golgi (Berson et al. 2001). This obviously raises the question whether Pmel17 is a valid marker of premelanosmes. However, detailed ultrastructural studies on Pmel17conatining vesicles have provided an alternative view of the nature and origin of premelanosomes (Raposo et al. 2001). Pmel17 is found

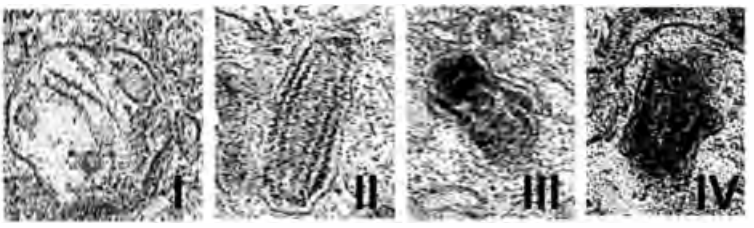

Figure 1. Morphology of melanosome maturation. Transmission electron micrograph of pigmented human melanoma cell line SK-MEL-19 showing different stages in melanosome biogenesis and maturation. Endosome-like structures containing internal vesicles and nascent fibrils are considered melanosomal precursors or stage I melanosomes. Reorganization and/or structured deposition of fibrils and elongation of the spherical vesicles into stage II melanosmes is followed by ordered deposition melanin (stage III) and its accumulation that eventually fills in the lumen (stage IV) can be clearly demonstrated.

enriched in intraluminal vesicles of multivesicular compartment with dense clathrin coat, but absent in *trans*-Golgi network (TGN) and clathrin-coated vesicles. Conversely, tyrosinase and tyrosinase related protein 1 (TRP-1/gp75), which are present in TGN and clathrin-coated vesicles, are absent in Pmel17 enriched vesicles. This Pmel17 enriched, multivesicular, coated endosomal compartment at the intersection of TGN, the early and late endosomes was proposed to represent pre-/stage I melanosome. Reorganization of the luminal fibrillar structures into parallel arrays seems to produce the characteristic stage II melanosmes. Other melanosomal proteins including tyrosinase and TRP-1 are then appear to be delivered to stage II melanosomes directly from the TGN via clathrin-coated vesicles. Interestingly, expression of Pmel17 in non-melanocytic cells is sufficient to produce multivesicular endosomes with internal structures similar to fibrils in stage II melanosomes, suggesting that Pmel17 plays a critical role in the biogenesis of luminal structures of melanosomes (Berson et al. 2001). Although a role for multivesicular bodies in melanosome formation in fish was reported earlier (Turner et al. 1975), it now appears that similar multivesicular bodies play a role in melanosome biogenesis in mammals. Using free-flow electrophoresis, a method to separate various subcellular organelles, Kushimoto et al. (2002) reported that whereas Pmel17/gp100 is clearly present in stage I melanosomes, tyrosinase and other melanosomal proteins are also detectable in such vesicles. The highly proteolytic environment in these vesicles seems to degrade or partially cleave these proteins and impair their enzymatic activity, whereas this very environment may be necessary to cleave Pmel17 and restructure premelanosomes. The reasons for this discrepancy between these studies on the presence of tyrosinase and TRP-1 in early stage I melanosomes are not yet understood.

We have been investigating trafficking of human TRP-1 and its role in melanosome biogenesis (Vijayasaradhi et al. 1991, 1995, Liu et al. 2001, 2002). Although TRP-1 is known to influence melanosome morphology, its exact role in the biogenesis of this organelle is not clear. Based on ultrastructural studies of hair bulb melanocytes in brown coat color mice expressing mutant alleles of *b*-locus, it was proposed that TRP-1 influences the size and shape of the pigment granules, specifically the internal structure in stage III melanosomes (reviewed in Silvers 1979; Sarangarajan and Boissy 2001). Melanin pigment in b-granules is flocculent and coarsely granular compared to homogeneous and finely granular appearance in black granules (Silvers 1979). In humans, loss of expression of TRP-1 due to a single base pair deletion is known to cause type 3 oculocutaneous albinism (OCA3), identified in a newborn hypopigmented African-American twin (Boissy et al. 1996). The affect of this mutation on melanosome biogenesis has not been investigated.

We exploited a system of selective and reversible inhibition of endogenous TRP-1 transcription in human melanoma cells to investigate the role of TRP-1 in melanosome biogenesis (Vijayasaradhi et al. 1995a; Fang et al. 2002). In pigmented human melanoma cell lines treated with the pharmacological compound, hexamethylene bisacetamide (HMBA), TRP-1 expression is completely suppressed and melanosomes exhibit many structural abnormalities (Figure 2, Setaluri *unpublished observations*). In control untreated cells, most melanosomes appear elliptical in longitudinal cross-section (LC), and circular in cross-sectional (CS) view, and melanin is deposited in uniform fashion. In HMBA treated cells, TRP-1 depleted melanosomes are curved and distended in LC, and dented in CS. Melanin is deposited in an uneven fashion. Defects in melanosome biogenesis are also evident. In light of the observation that TRP-1 is the most abundant melanosomal protein, these observations suggest a role for TRP-1 in the maintenance of melanosome membrane (Vijayasaradhi et al. 1991; Sarangarajan and Boissy 2001).

Melanosomal protein sorting: Signals and adaptors

The TGN is a key sorting station from where proteins traveling along the secretory pathway are sorted to various cellular destinations *via* transport vesicles. A large body of evidence accumulated to date shows that sorting of integral membrane proteins in the TGN is dependent on short stretches of amino acid residues within the cytoplasmic domains of proteins (reviewed in Kirchhausen et al. 1999). The most commonly found signals are tyrosine-based signals and di-leucine motif signals (Williams and Fukuda 1991; Ogata and Fukuda 1994; Vijayasaradhi et al. 1995). A di-leucine containing motif and a glutamic acid residue located 3-4 residues

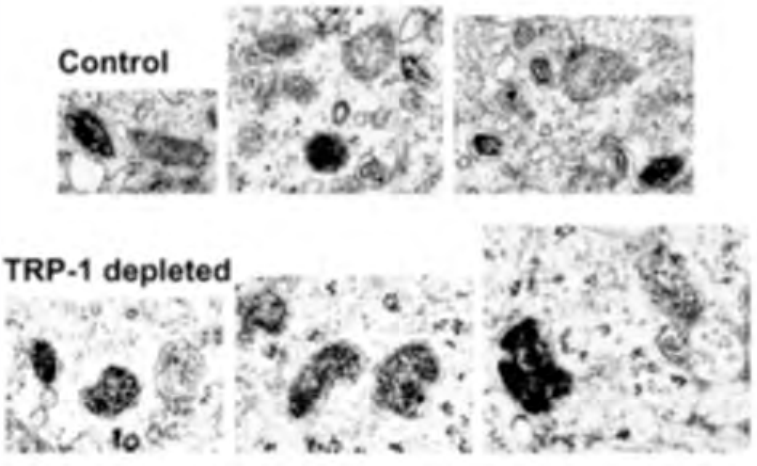

Figure 2. Melanosome structure and role of TRP-1. Ultrastructure of melanosomes in TRP-1 positive and TRP-1 depleted human pigmented melanoma cell line. In control cells, characteristic structures representing various stages of melanosome maturation can be seen. Cells treated with hexamethylene bisacetamide, which completely extinguishes TRP-1 expression, show accumulation of small vesicles near stage II and III melanosomes, and melanosomal abnormalities such as “sickle-like” appearance and uneven melanin deposition.

amino terminal to the di-leucine motif are conserved not only among other type I melanosomal proteins but also in a type III melanosomal protein (Vijayasaradhi et al. 1995). A role for these signals in the cytoplasmic tails in sorting melanosomal proteins in vivo is supported by two observations. First, the *platinum* mutant allele of mouse tyrosinase gene, which codes for truncated protein lacking the di-leucine motif, causes improper targeting of tyrosinase (Chintamaneni et al. 1991 and Beerman et al. 1995). Second, mutations in subunits of adaptor complexes AP-3, which bind the di-leucine motif (see below) produce coat color mutations *mocha* and *pearl*, and are responsible for the hypopigmentary disorder in a subset of individuals with Hermansky-Pudlak like syndrome (Kantheti et al. 1998; Feng et al. 1997; Dell'Angelica et al. 1999; Spritz 2000).

Although the di-leucine signal is both necessary and sufficient for intracellular sorting of tyrosinase and TRP-1, tyr-based signals (specifically Y-X-X-Ø where Ø is bulky hydrophobic amino acid), present in TRP-1 and TRP-2 may also play a role in melanosomal protein sorting (Simmen et al. 1999). This raises the possibility that melanosomal proteins may follow alternative paths to the melanosome by utilizing tyr-based sorting signals and clathrin adaptor complex AP-1 recognition for sorting in the TGN, and AP-2 mediated sorting for internalization from plasma membrane (Ohno et al. 1996, 1998). Another possibility is that the tyr-based signals are utilized to retrieve mis-targeted melanosomal protein (Vijayasaradhi et al. 1995). Melanosomal proteins may also utilize signals different from tyr- and di-leucine signals is indicated by the absence of di-leucine motifs in TRP-2, and the OA1protein (a type III membrane protein encoded by the ocular albinism type I gene (Schiaffino et al. 1996). Conversely, presence of di-leucine motif does not appear to completely prevent targeting melanosomal proteins to other cellular locations including plasma membrane (Xu et al. 1997).

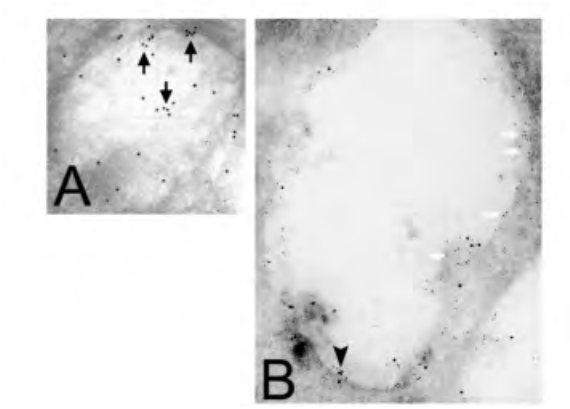

Figure 3. Segregation of lysosomal and melanosomal proteins. Immunoelectron microscopic localization of TRP-1 and LAMP-1. Ultrathin cryosections of mouse fibroblasts transfected with human TRP-1 were incubated with anti-TRP-1 mAb conjugated to 10nm gold particles and anti-LAMP-1 mAb conjugated to 5nm gold particles. Endosomal vesicle enriched with TRP-1 (**A**) or enriched with LAMP-1(*arrow* in **B**) could be seen. *Arrows* in **A** show fusion of TRP-1 enriched vesicles, internalization and intraluminal TRP-1. *Arrowhead* in **B** shows segregation of TRP-1 from LAMP-1 enriched vesicles, suggesting presence of mechanisms to target TRP-1 to specialized endosomes in non-melanocytic cells.

Clathrin-coated vesicles play a critical role in the transport of cellular cargo from the TGN and between endosomal compartments in all cell types (reviewed in Brodsky et al. 2001). So, what mechanisms do melanocytes utilize to enrich melanosomal cargo in clathrin-coated vesicles? How are such vesicles targeted to fuse selectively with pre- and/or stage II-melanosomes? An insight into these issues came from molecular genetic analyses of mouse coat color mutations and human hypopigmentary disorders.

It in now established that coat colors of *mocha* and *pearl* mutant mice and hypopigmentary disorder in a subset of patients with the Hermansky-Pudlak syndrome (HPS2) (Kantheti et al. 1998; Feng et al. 1997; Dell'Angelica et al. 1999; Spritz 2000) (and also a pigmentary defect in the eyes of fruit fly *Drosophila*) are caused by defects in specific subunits of the adaptor complex AP3. This tetrameric complex, expressed ubiquitously, binds to clathrin as well as the di-leucine motif in the cytoplasmic tails of melanosomal and lysosomal proteins. In both human HPS and mouse models, mislocalization of lysosomal proteins and tyrosinase is also accompanied by defects in platelet dense granules (Huizing et al. 2000; Swank et al. 2000). While localization of the misrouted tyrosinase in AP-3 deficient, HPS melanocytes to large multivesicular compartment is consistent with a defect in endosomal sorting, it is not clear whether sorting from the TGN is also affected. The complexity of these sorting events and the possible role of additional cytoplasmic proteins in early sorting steps are highlighted by the following observations. First, although AP3 is expected to bind TRP-1, in which the canonical di-leucine motif was first identified, in AP-3 deficient cells, sorting and targeting of TRP-1 to endosomes/melanosomes is not affected (Huizing et al. 2001). Second, newly synthesized TRP-1, but not tyrosinase, transiently binds to a cytoplasmic PDZ domain protein, GIPC, suggesting a role for this interaction in TRP-1 sorting (Liu et al. 2001). However, it is not clear whether the TRP-1-GIPC interaction occurs in the TGN or the endosomes or whether this interaction precedes or follows the di-leucine mediated sorting step. Interestingly, GIPC also interacts with GAIP, a clathrin-vesicle resident regulator of G_{ia} (De Vries et al. 1998), suggesting a possible role for G-protein signaling in early melanosomal protein sorting events (see below). These observations highlight the role specific protein-protein interactions between melanosomal membrane proteins and cytosolic proteins play at different sorting and targeting steps.

Melanosome biogenesis: The lysosome connection

The involvement of endosomes in melanosome biogenesis places these organelles in the midst of a highly dynamic biosynthetic and endocytic vesicular traffic that shuttles cargo to a variety of subcellular compartments including lysosomes (Sachse et al. 2002). But, what is the relationship of melanosomes to lysosomes? Are melanosomes specialized lysosomes? Can lysosomes be distinguished from melanosomes in melanocytes? Earliest indications of a relationship between these organelles came from the morphological

(enzyme cytochemistry) and biochemical (subcellular fractionation) studies that showed presence of several lysosomal hydrolases in melanosomes. Co-localization of melanosomal proteins and lysosomal membrane proteins in non-melanocytic cells transfected with melanosomal membrane proteins, specifically tyrosinase and TRP-1, appeared to support the view that melanosmes are modified lysosomes (Orlow 1995). Although melanosomes are lysosome-related organelles, they are readily distinguished from lysosomes, based on their unique morphology and enrichment of specific membrane proteins (Raposo et al. 2001). Indeed, it appears that mechanisms to segregate melanosomal membrane protein TRP-1from lysosomal membrane protein LAMP-1 exist in both melanocytic and non-melanocytic (Fujita et al. 2001 and Fig. 3, Setaluri *unpublished observations*). Immunoelectron microscopic studies of TRP-1 expressed in non-melanocytic cells showed the presence of three distinct types of vesicles based on the distribution of different size gold particles (5 and 10-nm) representing LAMP-1 and TRP-1. Type I vesicles containing both 5-and 10-nm gold particles, hence both TRP-1 and LAMP-1, represented a majority of labeled vesicles. Type II vesicles contained almost exclusively 5-nm gold particles (LAMP-1), while type III structures had mostly 10-nm gold particles (TRP-1) (Table I in Setaluri 2000). LAMP-1 enriched, type II structures were also distinguishable by an electron-dense core. These observations suggest that while TRP-1 and LAMP-1 (an endosome/lysosome marker) are colocalized in several cytoplasmic structures, presumably sorting endosomes, vesicles that are enriched for TRP-1 are present even in non-melanocytic cells. This is consistent with a view that following initial export of melanosomal and lysosomal proteins from the TGN to a common endosomal compartment by AP-1/AP-3 mediated sorting, additional signals and sorting mechanisms then segregate melanosomal proteins from late endosome/lysosome traffic. In melanocytic cells, budding of vesicles selectively enriched for melanosomal or lysosomal proteins, and fusion of such vesicles with melanosomes and late endosome/prelysosome, respectively, could generate and maintain the integrity of these specialized organelles (Figure 4). It is also possible that selective export of lysosomal proteins out of a common endosome, allows maturation of melanosomal protein-enriched structures to stage II melanosomes. Melanosomal proteins that are mistargeted could be retrieved into the coated endosomes by retrograde transport mechanisms similar to the Golgi-to-ER transport. Alternatively, mistargeted melanosomal proteins could be exchanged between late endosomes/lysosomes and stage II melanosomes. For example, in secretory cells, mistargeted mannose 6- phosphate receptors are retrieved from secretory granules by AP-1 mediated sorting mechanisms and then delivered to endosomes (Klumperman et al. 1998).

Defects in any one of these steps would be expected to result in failure to maintain integrity of melansomes and lysosomes. Chediak-Higashi syndrome (CHS), an autosomal recessive pigmentary disorder in humans, and *beige* coat color mutation of mice, appear to represent such scenario. A defect in secretory lysosome function produces abnormally enlarged or abnormally small lysosomes in the affected cell types, specifically lymphocytes and melanocytes. Although the gene and its protein product associated with CHS/*beige* have been identified, no specific function in vesicular transport has been assigned (Burkhardt et al. 1993; Baetz et al. 1995; Barbosa et al. 1999; Perou et al. 1996). Recently,Tchernev et al. (2002) reported that the CHS protein interacts with several proteins, such as SNARE complex (see below), important in vesicular transport and signal transduction

Two observations point to a possible function(s) of Lyst protein in endosomal/lysosomal protein transport: a) Overexpression of wild type protein in CHS1 mutant fibroblasts results production of abnormally small lysosomes, suggesting that lyst protein is involved in vesicle budding from endosomes/lysosome (Perou et al. 1996). b) Although the function of specialized secretory lysosomes is impaired in cells affected by CHS, endocytic and degradative functions of endosomes/lysosomes appears to be intact (Perou et al. 1997). It is possible that, in CHS/beige melanocytes, defects in budding mechanisms prevent endosomal sorting of melanosomal and lysosomal proteins resulting in large melano-lysosomes and pigment dilution (Spritz 1998). Interaction of CHS protein with signal transduction components raises the possibility that this protein provides a link between melanosome biogenesis and signaling pathways.

How to assemble a melanosome? By Rabs and SNAREs, of course!

The precise biochemical details of how vesicles enriched in melanosomal proteins are targeted to fuse selectively with melanosomal structures? However, it is now clear that all eukaryotes, yeast to man, utilize a combination of COPs (vesicle coat associated proteins), SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) and a family of small GTPases of the Ras superfamily to orchestrate vesicle budding and fusion events between different membrane compartments (Whyte and Munro 2002; Seabra et al. 2002). Therefore, it is reasonable to assume that same general principles that regulate vesicular trafficking in vacuolar system also control melanosome biogenesis. For example, in mouse coat color mutant *gunmetal*, a coat color phenotype and a mouse model of HPS with accompanying platelet dense granule defects, is associated with mutations in Rab geranylgeranyl transferase (Detter et al. 2000), and in *chocolate* mutant mice, mutation of Rab38 results in impaired targeting of TRP-1 (Loftus et al. 2002). Interestingly, Rab7, which is localized to late endosomes, may also be involved in trafficking of tyrosinase and TRP-1 from TGN to melanosomes (Gomez et al. 2001; Hirosaki et al. 2002)

A role for SNAREs in melanosome biogenesis is also emerging. According to the SNARE hypothesis, the specificity of vesicle fusion is regulated by recognition between SNAREs present on the transport vesicle (v-SNAREs) and their cognate SNAREs (t-SNAREs) on the target membrane compartments (Banfield 2001; Chen and Scheller 2001; Pelham 2001). The SNARE hypothesis was originally proposed based on the cycling of synaptic vesicles (Rizo and Sudhof 2002). SNARE complexes typically consist of a syntaxin, a vesicle-associated membrane protein (VAMP) and two synaptosome-associated proteins (SNAPs) These SNARE complex proteins, which are expressed in all eukaryotic

cells, also appear to play a role in melanosome biogenesis. For example, the product of *pallidin*, the mouse gene associated with pigmentation defects, platelet dense granule deficiency and emphysematous lungs in adult animals, binds to synatxin 13, a member of the syntaxin family of SNARE proteins (McGarry et al. 1999; Huang et al. 1999). Synatxin 13 is localized to tubular extensions of early endosomes, recycling endosomes and clathrin-coated endosomes (Prekeris et al. 1998). Based on the morphological similarity of endosomal intermediates involved in melanosome biogenesis with coated endosomes, and the requirement of syntaxin 13 in homotypic endosome-endosome fusion, it is conceivable the syntaxin 13-pallidin could function as a premelanosmal t-SNARE (McBride et al. 1999; Falcon-Perez and Dell'angelica 2002). Other melanosome-associated SNAREs include syntaxin 4, SNAP-23 and SNAP-25 (Scott and Zhao 2001).

In addition to the general SNARE-like biochemical machinery for fusion of melanosomal cargo vesicles with premelanosome, other melanocyte-specific proteins may also be involved in regulation of these steps. P protein, which is a 12-transmembrane domain containing transporter protein, may play such regulatory role. Mutations in the gene encoding this protein are responsible for human OCA2 and the mouse pink-eyed dilute phenotype. Brilliant (2001) proposed that a defect in the anion transport function of the P protein, which results in failure of acidification of transport vesicles, causes accumulation and/or mistargeting of melanosomal proteins to lysosomal compartments. The exact biochemical mechanisms through which P protein regulates melanosome biogenesis remains to be defined.

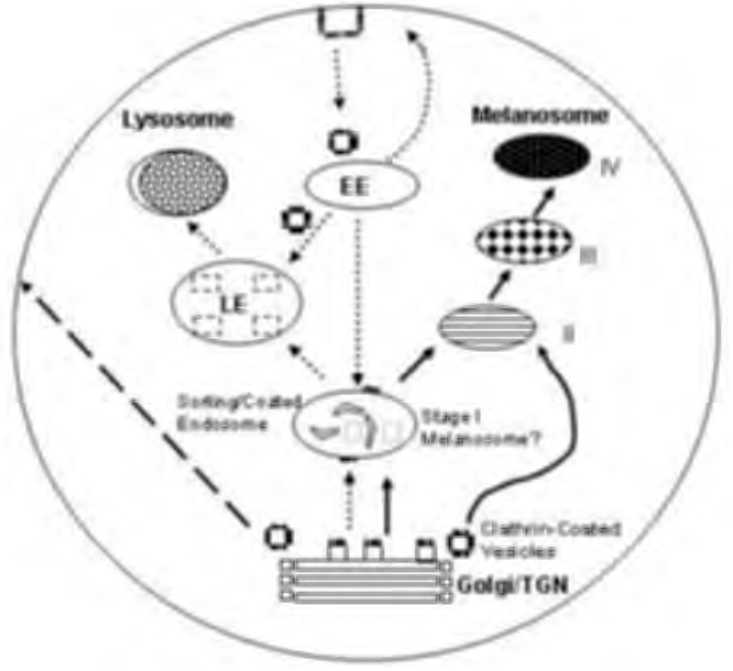

Figure 4. Melanosome biogenesis and its relationship to endosomes. A simplified scheme of primary pathways of protein sorting and transport, and the involvement of shared endosomal compartments in the biogenesis of lysosomes and melanosomes. EE: early endosomes; LE: late endosomes; TGN: trans-Golgi network. *Solid arrows* indicate pathways of melanosomal protein transport and progressive maturation of melanosomes. *Arrows with dotted lines* show biosynthetic and endocytic protein transport of lysosomal proteins through sorting and early endosomes to prelysosomes and lysosomes. *Arrow with dashed line* indicates transport of proteins targeted to the cell surface. Although the vesicular transport steps are shown as unidirectional, bidirectional transport can occur to facilitate membrane recycling and retrieval of mistargeted proteins. The multivesicular endosomal compartment immediately distal to the TGN is shown to represent pre- or stage I melanosome with characteristic fibrils shown in Fig.1.

Hermansky-Pudlak Syndrome: A treasure trove for vesicle biogenesis

Molecular identification of genes and their protein products associated with of HPS, a collection of autosomal recessive genetic disorders characterized by oculocutaneous albinism, ceroid accumulation in lysosomes and prolonged bleeding, is helping unravel the biochemical machinery involved in the biogenesis of lysosome-related organelles (Huizing et al. 2002; Starcevic 2002). In mice, there are at least 16 distinct genetic loci that produce coat color mutant phenotypes and associated HPS-like platelet deficiencies (Swank et al. 1998). As mentioned earlier, HPS type 2 and the mouse mutation *pearl* is associated with b3A subunit of AP-3 complex (Feng et al. 1999). Genes associated with HPS-1, -3, -4, -5 and -6 and their mouse orthologues *pale ear, cocoa, light ear, ruby eye 2, and ruby eye* respectively have been identified (Gwynn et al. 2000; Suzuki et al. 2001a, 2002; Zhang et al. 2003). Proteins encoded by these genes do not have any recognizable motifs that suggest their biochemical functions. Recent data shows that these proteins occur as complexes, termed BLOCs (biogenesis of lysosome-related organelles complex). BLOC-1 complex consists of the HPS related mouse pallid (pa), muted (mu) and cappucino (cno) proteins (Falcon-Perez et al. 2002), whereas HPS5 and HPS6 proteins form BLOC-2, and HPS1 and HPS4 proteins appear to be components of BLOC-3 and BLOC-4 (Chiang et al. 2003). Based on the observation that BLOC-1 is able to associate with actin filaments, it was proposed that this interaction with actin may be involved in the mechanism of BLOC-1 action in the biogenesis of lysosome related organelles. The specific roles played by the other BLOCs in the biogenesis of melanosomes remain to be determined. Are BLOCs novel SNARES? By virtue of their association with the cytoplasmic face, do BLOCs mediate communication with cellular signaling pathways that regulate vesicular traffic?

Cellular signaling and melanosome biogenesis

A variety of cellular signaling pathways including cyclic AMP, protein kinase C, and phosphatidylinositol 3-kinase (PI3K) are known to influence melanin pigment synthesis (Abdel-Malek et al. 1992; Berlotto et al. 1998; Park et

al. 1999; Oka et al. 2002). Whereas activation of cyclic AMP and protein kinase C pathways are generally known to activate or increase the expression of tyrosinase, PI3K signaling may be involved in melanosome biogenesis. PI3K is a member of a family of kinases that phosphorylate the membrane phospholipid, phosphatidylinositol. Phosphatidyl- inositol 3-phosphate (PI3P) generated by PI3K, which is localized in the cytoplasm, has been shown to influence endosomal membrane trafficking. PI3P appears to function by recruiting cytsolic effector proteins to the endosomal membrane (Odorizzi et al. 2000). Interestingly, in yeast the single isoform of PI3K enzyme is coded by the gene *vps34*, a member of a large family of more than 50 genes that are involved in vacuolar protein sorting (reviewed in Katzman et al. 2002). In mammalian cells, PI3K activity is required for protein trafficking and endosomal vesicular biogenesis. This is illustrated by the observation that injection of anti-human PI3K antibodies into cultured human cells or treatment with wortmannin, an inhibitor of PI3K activity, inhibits biogenesis of multivesicular bodies (Futter et al. 2001). Based on the intracellular distribution of TRP-1 in human melanoma cells treated with wortmannin, an inhibitor of PI3K, Chen et al. (2001) suggested that PI3K plays a role in trafficking of TRP-1 from late endosomes to melanosomes. Our preliminary observations that the PDZ protein GIPC, which binds to newly synthesized TRP-1, also interacts with a cytosolic signaling adaptor APPL (Mitsuuchi et al. 1999; Liu et al. 2002), containing multiple protein domains, including a phosphotyrosine binding (PTB) domain indicates a possible link between PI3K signaling and melanosome. In melanocytes and melanoma cell lines, inactive Akt2, the primary downstream target for activation by PI3K, is bound to the PTB domain of APPL (Liu et al. 2002a). This raises the possibility that an interaction of the TRP-1 cytoplasmic tail with GIPC-APPL-Akt2 complex might mediate regulation of TRP-1 trafficking by PI3K.

Additionally, interaction of GIPC with GAIP (a regulator of G-protein Gai3), which is localized to clathrin-coated vesicles, implicates GIPC in linking melanosome biogenesis to G-protein signaling. The notion that G-protein signaling may regulate melanosome biogenesis is supported by the findings that mutations in OA1, a melanosome-resident G-protein-coupled receptor, result in enlarged melanosomes and cause ocular albinism (OA) in both mice and humans (Schiaffino et al. 1999; Incerti et al. 2000; Shen et al. 2001). Biochemical mechanisms through which G-protein signaling influence melanosome biogenesis remain to be identified.

Apart from the melanocyte intracellular factors described above, several other factors including neuropeptides and certain therapeutic agents also influence melanosome biogenesis. Calcitonin gene-related peptide, a neuropeptide, seems to increase melanosome number, presumably by affecting their biogenesis, in skin organ cultures but not in isolated culture melanocyte (Toyoda et al. 1999). In mice, cyclophosphamide, a cancer chemotherapeutic alkylating agent, has been reported to induce disordered formation of melanosomes in hair bulb melanocytes (Slominski et al. 1996). Treatment of B16 mouse melanoma cells with paclitaxel, a microtubule stabilizing chemotherapeutic agent, on the other hand, increases number of melanosomes (Bicamumpaka and Page 1998).

Concluding Remarks

Melanosomes, long thought to represent merely membrane sacs that contain and transport pigment, are now attracting unprecedented attention of investigators from a wide range of basic and clinical disciplines including cell biology, molecular genetics, hematology, and tumor immunology. A collection of human oculocutaneous syndromes associated with a wide range of other clinical features, and their orthologues in a seemingly unending resource of mouse coat color mutants have proved valuable. This has led to the identification of an unprecedented number of novel genes involved in the biogenesis of lysosome-related organelles. The exact functions of many these proteins and their mechanisms of action remain to be discovered. Although melanin pigment granules are related to lysosomes, as they were long suspected, it is now established that melanosomes are distinct organelles. Melanocyte-specific proteins originally thought to be primarily involved in melanin pigment synthesis are now being assigned novel functions in vesicle biogenesis and roles in other ocular diseases. Many other pieces are required to complete the melanosome puzzle. For example, are there master regulatory switches, genetic and/or epigenetic, that regulate melanosome biogenesis? What are the mechanisms that link melanosome biogenesis to intracellular signaling? The recent attention these dark pigment granules have received is a cause for optimism that it will continue to shine brighter light on these and many other aspects of melanocyte biology in health and disease.

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