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Melanocytes Derived from Patients with Hermansky–Pudlak Syndrome Types 1, 2, and 3 Have Distinct Defects in Cargo Trafficking

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Abstract

Hermansky–Pudlak Syndrome (HPS) is a genetically heterogeneous disorder in which mutations in one of several genes interrupts biogenesis of melanosomes, platelet dense bodies, and lysosomes. Affected patients have oculocutaneous albinism, a bleeding diathesis, and sometimes develop granulomatous colitis or pulmonary fibrosis. In order to assess the role of HPS genes in melanosome biogenesis, melanocytes cultured from patients with HPS subtypes 1, 2, or 3 were assessed for the localization of various melanocyte proteins. Tyrosinase, Tyrp1, and Dct/Tyrp2 were atypically and distinctly expressed in HPS-1 and HPS-3 melanocytes, whereas only tyrosinase showed an atypical distribution in HPS-2 melanocytes. The *HPS1* and *AP3B1* (i.e., HPS-2) gene products showed no expression in HPS-1 and HPS-2 melanocytes, respectively, whereas HPS-3 melanocytes exhibited normal expression for both proteins. In normal human melanocytes, the HPS1 protein was expressed as an approximately 80 kDa molecule with both granular and reticular intracellular profiles. In HPS-1, lysosome associated membrane protein 1 (LAMP1), and LAMP3 were localized to abnormal large granules; in HPS-2, all LAMPs exhibited a normal granular expression; and in HPS-3, LAMP1, and LAMP3 exhibited a distinct less granular and more floccular pattern. In contrast, the expressions of Rab 27, transferrin, and cKit were unaffected in all three HPS genotypes. These data demonstrate that the three initially identified subtypes of human HPS exhibit distinct defects in the trafficking of various melanocyte-specific proteins.

Keywords

pigmentation; albinism; tyrosinase; lysosomes; translocation

Abbreviations

HPS, Hermansky—Pudlak Syndrome; LAMP, lysosome associated membrane protein; NHM, normal human melanocytes

The biogenesis of functionally pigmented melanosomes is complex and poorly understood, but a few elements of this cellular process have been elucidated (Orlow, 1998; Raposo and

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Marks, 2002). Numerous structural proteins, melanogenic enzymes, and regulatory proteins need to be accurately trafficked from their site of synthesis to their melanosomal targets for efficient melanization to occur in this distinct organelle (Marks and Seabra, 2001). Specialized protein complexes function in the selective recruitment of melanosome-destined cargo at sites of synthesis, the translocation of cargo vesicles toward the melanosomes, either directly or via an intermediate organelle, and the efficient recognition, docking, fusion, and incorporation of these specific cargo into the melanosomes. Elucidating these trafficking molecules/chaperones in the melanocyte has been a challenging endeavor.

Hermansky–Pudlak Syndrome (HPS [MIM203300]) is a genetically heterogeneous, autosomal recessive disorder that provides an experiment of nature for understanding how trafficking molecules function in the biogenesis of melanosomes. To date, seven genetic types of the HPS have been identified in humans (Huizing *et al*, 2002; Li *et al*, 2003; Zhang *et al*, 2003) and approximately 15 genetically distinct HPS models exist in mice (Swank *et al*, 1998; Huizing *et al*, 2002). HPS presents with oculocutaneous albinism and a bleeding diathesis (Hermansky and Pudlak, 1959; King *et al*, 2001; Huizing *et al*, 2002). The albinism results from the inability of cutaneous and ocular melanocytes to create fully pigmented melanosomes due to mistrafficking of melanogenic proteins (Huizing *et al*, 2002). The bleeding diathesis results from the absence of dense bodies within platelets, compromising clot formation (Witkop *et al*, 1987; Gahl *et al*, 1998). Some HPS patients develop lysosomal ceroid storage (Bednar *et al*, 1964), granulomatous colitis (Schinella *et al*, 1980), or a fatal pulmonary fibrosis (Harmon *et al*, 1994; Brantly *et al*, 2000). Each individual HPS gene is considered to regulate a molecular aspect critical for the biosynthesis of melanosomes, platelet dense bodies, and lysosomes.

HPS-1, HPS-2, and HPS-3 are the three genetic forms of human HPS initially identified. The *HPS1* gene was originally mapped and isolated in a population of patients residing in northwestern Puerto Rico (Wildenberg *et al*, 1995; Oh *et al*, 1996). HPS1 localizes to chromosome 10q23 and encodes a predicted 79.3 kDa protein with no homology to known proteins, although it contains a short segment predicted to form coiled-coil structures (Oh *et al*, 1996). The HPS1 protein, whose function remains unknown, is distributed in melanotic cells among uncoated vesicles, early-stage melanosomes, and the cytoplasm as part of a large complex of molecular weight ~ 200 kDa in the cytoplasm and >500 kDa in a membrane-bound form (Oh *et al*, 2000). Melanocytes from HPS-1 patients are hypopigmented and exhibit unique membranous complexes with tyrosinase-containing compartments (Boissy *et al*, 1998).

Mutations in the *AP3B1* gene cause HPS-2 (Dell'Angelica *et al*, 1999). *AP3B1* is localized to chromosome 5q14.1 and encodes the 140 kDa β3A-subunit of adaptor complex-3 (AP3) involved in vesicle/membrane formation and trafficking (Dell'Angelica *et al*, 1997, 1998). Melanocytes from HPS-2 patients are hypopigmented and exhibit sequestration of tyrosinase to large endosome/multivesicular body-like structures (Huizing *et al*, 2001b).

The *HPS3* gene was initially identified in a central Puerto Rican HPS isolate (Anikster *et al*, 2001) and subsequently in other populations (Huizing *et al*, 2001a). *HPS3* is located on chromosome 3q24 and encodes a predicted 113.7 kDa protein with no apparent homology to other proteins (Anikster *et al*, 2001; Suzuki *et al*, 2001). The HPS3 protein, however, does contain a putative clathrin binding region (Anikster *et al*, 2001), suggesting participation in intracellular trafficking. We have recently demonstrated that 50 nm vesicles containing tyrosinase are extensively distributed throughout HPS-3 melanocytes rather than being confined to the trans-Golgi network as in normal human melanocytes (NHM) (Boissy *et al*, 2004).

Specialized protein complexes function in series along a cellular pathway for trafficking. Multimeric components within these various complexes coordinate cargo recruitment, vesicle

budding, cytoskeletal interaction, target recognition, and vesicle fusion (Bonifacino and Glick, 2004). HPS gene products are components of a group of complexes termed BLOC (i.e., biogenesis of lysosome-related organelles complexes) (Falcon-Perez *et al*, 2002; Chiang *et al*, 2003; Ciciotte *et al*, 2003; Li *et al*, 2003; Martina *et al*, 2003; Gautam *et al*, 2004). BLOC facilitate the trafficking of cargo to melanosomes, lysosomes, and platelet dense bodies. But the molecular functions and sites of action of BLOC remain unknown.

In this report, we compare the trafficking patterns of various melanosomal proteins in melanocytes cultured from patients with HPS-1, HPS-2, and HPS-3 to further characterize the various pathways required for correct protein trafficking and assembly of a functional melanosome. The abnormal distribution patterns of proteins transported via endolysosomal pathways distinguish these HPS subtypes and implicate distinct sites of action for the three HPS gene products.

Results

Expression of tyrosinase gene family proteins

Melanocytes from patients with HPS-1, HPS-2, and HPS-3 were evaluated by immunocytochemistry for the expression pattern of tyrosinase gene family proteins [i.e., tyrosinase, tyrosinase-related protein-1 (Tryp1), and DOPAchrome tautomerase/tyrosinase-related protein-2 (Dct/Tyrp2)] (Fig 1). In NHM, all three proteins exhibited intense staining around the perinuclear area with marked granularity. Throughout the lateral areas of the cell body and along the dendrites, the staining remained granular but less dense. In HPS-1 melanocytes, in addition to this normal staining pattern, all three proteins were localized to large granules distributed predominantly throughout the cell body and occasionally within the dendrites (Fig 1).

In HPS-2 melanocytes, tyrosinase was predominantly restricted in its expression to the cell body with minimal expression along the dendrites (Fig 1). In contrast, the localization patterns for both Tyrp1 and Dct/Tyrp2 appeared normal, with marked expression in the dendrites (Fig 1). In HPS-3 melanocytes, both tyrosinase and Tyrp1 exhibited an expression pattern subtlety different from NHM. Although expression of these proteins occurred in both the cell body and dendrites, the pattern was less granular and more floccular compared with that in NHM (Fig 1). This differential stain pattern in HPS-3 melanocytes was most noticeable throughout the dendrites where the immunofluorescence appeared more homogeneously distributed in HPS-3 melanocytes and heterogeneous (i.e., granular) in NHM.

The co-localization of tyrosinase and Tyrp1 in large granules, characteristic of HPS-1 melanocytes, was quite heterogeneous (Figs 2*a–c*). Some large granules contained one protein selectively, but more frequently a large granule contained both proteins. Occasionally, tyrosinase and Tyrp1 exhibited discrete, separate areas of localization within the granules, with partial overlap. As previously described, the granules represent large membranous complexes containing melanosomes (Boissy *et al*, 1998). Dihydroxyphenylalanine (DOPA) histochemistry of HPS-1 melanocytes revealed melanin reaction product in a subset of cisternae of the membranous profiles and within some of the associated melanosomes (Fig 2*d*), consistent with the heterogeneity in localization of tyrosinase and Tyrp1 observed on light microscopy.

Expression of HPS gene family proteins

Melanocytes from patients with HPS-1, HPS-2, and HPS-3 were evaluated by immunocytochemistry for expression of the HPS1 protein and the β3A and μ3A subunits of AP3 (Fig 3). In NHM, HPS1 showed a distinct perinuclear pattern with dramatically reduced expression along the dendrites (Fig 3). HPS-2 and HPS-3 melanocytes exhibited a similar

pattern of expression. In contrast, HPS1 was absent from melanocytes of HPS-1 patients bearing either the 16 base pair duplication (Fig 3) or the S396 del C (not shown) mutations in *HPS1*.

The expression of the β3A and μ3A subunits of AP3 in NHM occurred with a distinct localized area in the cell body (Fig 3). The μ3A subunit exhibited a compact, tubular-network profile, unilateral to the nucleus. In contrast, the β3A subunit exhibited a more diffuse staining pattern, with a more intense focus localized unilateral to the nucleus. Similarly, HPS-1 and HPS-3 melanocytes displayed normal expression for both β3A and μ3A (Fig 3). In contrast, HPS-2 melanocytes exhibited an absent or dramatically reduced expression of β3A and the μ3A, respectively.

Expression of the HPS1 protein in NHM was further evaluated (Fig 4). Western blotting showed that HPS1 antibodies recognized an approximately 80 kDa protein in melanocytes derived from both a Caucasian and an African-American donor; this protein was absent from melanocytes of an HPS-1 patient (Fig 4*a*). In addition, the HPS1 antibodies exhibited, by routine fluorescent microscopy, prominent cell body staining with reduced dendritic staining in NHM (Fig 4*b*) that was absent from HPS-1 melanocytes (Fig 4*c*). Further analysis of HPS1 protein in NHM by confocal microscopy demonstrated prominent expression in the perinuclear area of the cell body (Fig 4*d*) with a relative reduction of staining in the Golgi area and the dendrites (Fig 4*d*). The perinuclear staining appeared predominantly granular (Fig 4*d*, *arrows*) with a reticular staining pattern apparent among the granular profiles (Fig 4*d*, *arrowheads*).

Expression of various melanocyte-expressed proteins

Melanocytes of patients with HPS-1, HPS-2, and HPS-3 were evaluated by immunocytochemistry for the expression pattern of LAMP 1–3, Rab27, transferrin, and cKit (Fig 5). In NHM, LAMP 1–3 exhibited a granular staining pattern within the cell body and throughout the dendrites. HPS-1 melanocytes exhibited normal localization for LAMP2, but LAMP1 and LAMP3 showed additional expression in large granules similar to that observed for the tyrosinase gene family members. HPS-2 melanocytes displayed normal localization of LAMP 1–3. HPS-3 melanocytes exhibited a floccular distribution pattern for LAMP1 and LAMP3, similar to that observed for tyrosinase, Tyrp1, and Dct/Tyrp2, and normal expression for LAMP2. Expression of Rab27, transferrin, and cKit exhibited normal profiles in melanocytes of the three HPS subtypes.

Discussion

The cellular pathways through which cargo proteins are directed, from their site of synthesis to melanosomes, have yet to be clearly delineated. The most prominent such cargos are the tyrosinase gene family proteins tyrosinase, Tyrp1 and Dct/Tyrp2. Tyrosinase is a glycoprotein synthesized in the rough endoplasmic reticulum that travels through the Golgi for carbohydrate modification (Ujvari *et al*, 2001; Francis *et al*, 2003; Watabe *et al*, 2004). Although it is already enzymatically mature by the time it reaches the trans-Golgi, tyrosinase has been shown with DOPA histochemistry and electron microscopy to bud off the trans-Golgi into vesicles that can be clathrin coated and travel a short distance to premelanosomes (Novikoff *et al*, 1968; Maul, 1969; Maul and Brumbaugh, 1971). Tyrosinase may also traffic through structures resembling multivesicular bodies en route to the premelanosome (Turner *et al*, 1976). Tyrp1 and Dct/Tyrp2 are also glycoproteins that traverse the Golgi apparatus (Tsukamoto *et al*, 1992; Jimenez-Cervantes *et al*, 1994); Tyrp1 may also travel through an organelle resembling a large multivesicular body (Orlow *et al*, 1993). It is unknown whether tyrosinase, Tyrp1, and Dct/Tyrp2 travel together in the same or independent pathways to early-stage melanosomes, defined as containing the melanofilament molecule Silver/Pmel17 (Berson *et al*, 2001, 2003).

Analysis of melanocytes cultured from patients with HPS-1, HPS-2, and HPS-3 revealed a normal distribution for proteins that do not reside in melanosomes (i.e., transferrin and cKit). In addition, a normal distribution was observed for Rab27, a family of molecules that regulate exocytosis of various cell-specific organelles (Izumi *et al*, 2003), in which Rab27a associates with the melanosome to facilitate its transport to and capture at the terminal ends of dendrites (Strom *et al*, 2002). In contrast, mutations in *HPS1* and *HPS3* did cause a distinctly aberrant localization of the tyrosinase family proteins. In the absence of HPS1, the tyrosinase-related proteins, as well as LAMP1 and LAMP3, were sequestered in large membranous complexes resembling macroautophagosomes (Boissy *et al*, 1998).

Macroautophagy is a dynamic process involving rearrangement of subcellular membranes to sequester cytoplasm and organelles for delivery to the lysosome or vacuole where the sequestered cargo is degraded and recycled (Dunn, 1990; Seglen and Bohley, 1992; Klionsky and Emr, 2000). The incorporation of tyrosinase, Tyrp1, and Dct/ Tyrp2 into macroautophagosomes in HPS-1 melanocytes suggests that the HPS1 protein targets these cargos to melanosomes, and the cargos move by default to macroautophagosomes when HPS1 is dysfunctional. Precedent for such default pathways is provided by the mutant huntingtin protein, which becomes aberrantly localized to large autophagosomes in Huntington's disease (Kegel *et al*, 2000). In addition, the endogenous antigenic proteins presented by MHC class II traffic through autophagosomes (Nimmerjahn *et al*, 2003).

Consistent with the role of HPS-1 in melanosomal targeting of cargo is the ultrastructural localization of HPS1 to the cytoplasmic face of melanosomes (Oh *et al*, 2000) (Boissy, personal observation). HPS1 appears to be a predominantly cytosolic protein that can be associated with membranes, possibly as a multimeric complex (Dell'Angelica *et al*, 2000; Oh *et al*, 2000). HPS1 also appears to interact with the HPS4 protein (Anderson *et al*, 2003) in a transient or indirect manner (Suzuki *et al*, 2002) as part of the biogenesis of lysosome-related organelles complex-3 (BLOC-3) (Chiang *et al*, 2003; Martina *et al*, 2003).

Mutations in *HPS3* also result in aberrant localization of all three tyrosinase family members but with a different distribution than what occurs in HPS-1 melanocytes. This suggests distinct roles for the HPS3 and HPS1 proteins. HPS-3 melanocytes exhibit a diffuse, less granular distribution for the three tyrosinase gene family proteins, as well as for LAMP1 and LAMP3. Recent evidence indicates that tyrosinase is localized in 50 nm vesicles that are abnormally scattered throughout the cell body and dendrites of HPS3 melanocytes (Boissy *et al*, 2004), as if by default of a normal, HPS3-mediated pathway. This suggests that the HPS3 protein regulates the targeting of vesicles to a recipient site and, in the absence of the HPS3 protein, these cargo vesicles are widely distributed throughout the cell body and dendrites of the mutant cells. In addition, LAMP1 and LAMP2 exhibit a greater perinuclear concentration in HPS-3 fibroblasts compared with normal cells and frequently fail to extend into the cell periphery (Helip-Wooley *et al*, 2004). The recognition step in trafficking regulated by HPS3 appears to be independent of HPS1, since the morphologies of the two HPS subtypes differ.

The HPS3 protein contains a putative clathrin binding domain, a possible tyrosine phosphorylation site at codon 295, and several putative tyrosine-based sorting signals, but no potential di-leucine sorting signals (Anikster *et al*, 2001) that could function in trafficking cargo. In normal murine melanocytes, the HPS3 protein exhibits a cytoplasmic distribution and is not associated with the type 1 IP3 receptor, Rab3A, or the AP3 complex (Suzuki *et al*, 2001). In NHM, the HPS3 protein interacts with clathrin (Helip-Wooley *et al*, 2004), and may function in the BLOC-2 complex (Di Pietro *et al*, 2004; Gautam *et al*, 2004) that also contains the HPS5 and HPS6 proteins (Zhang *et al*, 2003).

In contrast to *HPS1* and *HPS3*, the *AP3B1* gene (associated with HPS-2) affects only tyrosinase without any apparent influence on the cellular distribution of Tyrp1 or Dct/ Tyrp2. AP3 is one of four adaptor complexes, i.e., heterotetrameric complexes involved in vesicle/membrane formation and trafficking (Dell'Angelica *et al*, 1999; Robinson and Bonifacino, 2001). Adaptor complexes play a role in the formation of coated vesicles, as well as in the selection of cargo for these vesicles. AP3 contains δ, μ3, and σ3subunits, in addition to β3A. The known mutations in the *AP3B1* gene cause loss of the β3A, destabilization of the δ, μ3, and σ3 subunits (Fig 3) (Huizing *et al*, 2001b), and thus loss of functional AP3. The β3A subunit binds clathrin, whose rigid triskelion structure causes outpouching from an existing membrane (Dell'Angelica *et al*, 1998). In addition, AP3 contains putative di-leucine and tyrosine-based signal motifs for sorting in the β3A and the μ3A subunits, respectively (Robinson and Bonifacino, 2001). Tyrosinase interacts with AP3 via its di-leucine motif (Honing *et al*, 1998) and is retained in multivesicular body/late endosome-like structures in HPS-2 melanocytes (Huizing *et al*, 2001b). Apparently, neither Tyrp1 nor Dct/Tyrp2 express the requisite di-leucine motif required to bind AP3, accounting for their normal distribution in HPS-2 melanocytes. In contrast, *AP3B1* gene mutations result in impaired trafficking of tyrosinase to melanosomes. HPS-2 fibroblasts also traffic LAMP 1–3 through the plasma membrane in an enhanced fashion, suggesting that the plasma membrane provides a default pathway that operates when normal AP3 function is blocked in this cell type (Dell'Angelica *et al*, 1999). This default pathway does not appear to occur in HPS-2 melanocytes, suggesting that an AP3-independent route via the plasma membrane is not an alternate for tyrosinase in melanocytes.

Taken together, these data suggest that the gene products of *HPS1*, AP3, and *HPS3* regulate distinctly different steps in the trafficking pathway for tyrosinase gene family proteins from the Golgi to melanosomes. This proposed assessment for the cellular roles of these proteins is based primarily on morphologic data; however, biochemical analysis is required to substantiate this model. The HPS1 protein is essential for the accurate deposition of these proteins to the melanosomes. In the absence of HPS1, tyrosinase, Tyrp1, and Dct/Tyrp2 are trafficked to macrophagosomes, presumably for default degradation. The HPS1 protein is primarily a cytosolic protein that can be associated with the limiting membrane of the melanosome (Oh *et al*, 2000) (Boissy, personal observations). This positions HPS1, along with its partner HPS4, at the recognition/docking/fusion interface between incoming cargo vesicles and melanosomes (Fig 6). The HPS3 protein also regulates the accurate trafficking of tyrosinase gene family members to melanosomes. As described above, however, the HPS3-regulated step is distinct from that regulated by the HPS1/HPS4 complex. We propose that HPS3, along with its complex partners HPS5 and HPS6, functions upstream of HPS1/HPS4 (Fig 6). In contrast, the protein defective in HPS-2 (i.e., the β3A subunit of AP3) regulates the trafficking of tyrosinase only. We propose that this selective step occurs within an endosome/multivesicular-like sorting body used for transit between the Golgi and melanosomes (Fig 6). It is uncertain whether Tyrp1 and Dct/Tyrp2 travel through a similar intermediate compartment and whether they use an alternate adaptor-like molecule to do so. In addition, it is uncertain whether Tyrp1 and Dct/Tyrp2 are sorted through similar or distinct pathways with respect to each other.

The comparative analysis of the patterns of melanocyte protein distribution in HPS-1, HPS-2, and HPS-3 described herein provides a glimpse into the elaborate mechanism responsible for targeting cargo from the Golgi apparatus to melanosomes. Some HPS molecules regulate trafficking of tyrosinase gene family members coordinately en route to the melanosomes, whereas other HPS molecules assist the trafficking of selective melanocyte proteins. Melanin synthesis generates several toxic intermediates. Segregation of tyrosinase gene family members during post-translational translocation may be important to prevent inappropriate melanization from occurring in non-melanosomal structures prior to accumulation in the melanosomal microdomain.

Materials and Methods

Cell culture

Cultures of melanocytes were developed from neonatal foreskins or skin biopsies. Normal human neonatal foreskins (from African/American and Caucasian infants) were obtained from the nursery of University Hospital in Cincinnati after routine circumcision using a protocol approved by the University of Cincinnati Institutional Review Board. Three HPS-1 patients, two HPS-2 patients, and three HPS-3 patients were enrolled in a protocol approved by the National Institute of Child Health and Human Development and the National Human Genome Research Institute Institutional Review Boards to study the clinical and molecular aspects of HPS. All procedures for obtaining human tissue were conducted according to Declaration of Helsinki principles. Patient numbers corresponded to those of a master list of enrolled subjects. Two of the HPS-1 patients were homozygous for the 16 base pair duplication and one for the S396 del C mutation in the *HPS1* gene. The two HPS-2 patients were brothers and compound heterozygous for a 63 base pair deletion and an L580R mutation in the β*3A* gene (Dell'Angelica *et al*, 1999). Two of the three HPS-3 patients were homozygous for a 3.9 kb deletion encompassing exon 1 of the *HPS3* gene, and the third HPS-3 patient was compound heterozygous for the 3.9 kb deletion and a I243insA. After written informed consent was obtained, a 4 mm punch biopsy was taken from each patient, and half was placed immediately in melanocyte growth medium with 2 × antibiotic/antimycotic solution and shipped by express mail to Cincinnati, Ohio. All skin samples were placed in trypsin (2.5 mg per L) and incubated for 2 h at 37°C. The trypsin was replaced with MCDB-153 medium, and the tissue was gently vortexed for 30 s to separate the dermis as a single piece and to produce an epidermal cell suspension. The epidermal cells were seeded in a T-25 cm^2 flask (for foreskins) or a 2 cm^2 well (for skin biopsies) in MCDB-153 medium (Irvine Scientific, Santa Anna, California) as previously described (Medrano and Nordlund, 1990). The MCDB-153 growth medium was supplemented with 0.6 ng per mL basic fibroblast growth factor, 8 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 5 μg per mL insulin, 5 μg per mL transferrin, 1.0 μg per mL α-tocopherol, 30 μg per mL crude pituitary extract (Clonetic Laboratories, San Diego, California), 0.5 μg per mL hydrocortisone, 20 μg per mL catalase from bovine liver, and 10% heat-inactivated fetal calf serum. Cultures were fed with fresh medium twice weekly. Catalase was omitted from the medium after Day 6. Fibroblasts were eliminated by incubating cultures for 3–4 d in the presence of 100 μg per mL geneticin (G418 sulfate) (Halaban and Alfano, 1984). Cultures from the second to the fifth passage were used for the experiments described herein.

Immunofluorescent microscopy

Established cultures of melanocytes, maintained in regular MCDB-153 growth media, were plated on gelatin-coated Lab-Tek (Nunc, Naperville, Illinois) chamber slides at 2.5×10^4 cell per 0.9 cm^2 well and processed for indirect immunofluorescence the next day. The cells were fixed in 2% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, blocked in 1% bovine serum albumin (BSA) in PBS for 5 min at room temperature, and incubated in primary antibody solution diluted in 0.2% Saponin in PBS containing 0.1% BSA for 60 min at room temperature. The primary antibodies used in this study included rabbit polyclonal antiserum to tyrosinase provided by R. King and W.S. Oetting (hPEP1; University of Minnesota, Minneapolis) (1:300), mouse monoclonal antiserum to Tyrp1 (Mel-5; Signet Laboratories, Dedham, Massachusetts), (1:50), rabbit polyclonal antiserum to Dct/Tyrp2 provided by V. Hearing (PEP8; National Institutes of Health), (1:200), mouse monoclonals antisera to LAMP1 (H4A3), LAMP2 (H4B4), and LAMP3 (H5C6) (Developmental Studies Hybridoma Bank (Iowa City, Iowa) (1:300), rabbit polyclonal antiserum to the HPS1 protein (Dell'Angelica *et al*, 2000) (1:300), rabbit polyclonal antisera to β3A and μ3A provided by M. S. Robinson (Cambridge Institute for Medical Research, Cambridge, Great Britain) (both

1:300), rabbit polyclonal antiserum to Rab27 (610595; BD Transduction Laboratories, Lexington, Kentucky) (1:300), rabbit polyclonal antiserum to transferrin (0061; Dako, Santa Barbara, CA) (1:300), and mouse monoclonal antiserum to c-Kit (CD117; Novocastra Laboratories, Newcastle upon Tyne, UK) (1:50). Cells were then washed and incubated in secondary antibody solution diluted in 0.2% Saponin in PBS containing 0.1% BSA for 60 min at room temperature. The secondary antibodies consisted of Cy-2-conjugated donkey anti-mouse and Cy-3-conjugated donkey anti-rabbit (Jackson Immunoresearch, West Grove, Pennsylvania). After washing, coverslips were applied to the slides using Fluoromount G (Southern Biotechnologies, Birmingham, Alabama) and observed and digitally photographed with either a Leitz (Rockleigh, New Jersey) Dialu Fluorescent microscopy or a Zeiss (Thornwood, New York) LSM 510 confocal microscope.

DOPA histochemistry and electron microscopy

Established cultures of HPS-1 melanocytes, maintained in regular MCDB-153 growth medium, were seeded into Lab-Tek chamber slides (Nunc) coated with 1% pig gelatin, and grown to 90% confluence. Cultured melanocytes were fixed in wells with half-strength Karnovsky's fixative (Karnovsky, 1965) in 0.2 M sodium cacodylate buffer at pH 7.2 for 30 min at room temperature. For DOPA histochemistry, fixed cells were incubated in a 0.1% solution of L-DOPA twice for 2.5 h. The cells were washed three times in buffer and treated with 1.0% osmium tetroxide containing 1.5% potassium ferrocyanide (Karnovsky, 1971) for 30 min. The cells were washed, stained *en bloc* with 0.5% uranyl acetate for 30 min, dehydrated, and embedded in Eponate 12. Areas of the Epon cast were cut out and mounted on Epon pegs and sectioned on an RMC MT 6000-XL ultramicrotome. Ultrathin sections were then stained with aqueous solutions of uranyl acetate (2%) and lead citrate (0.3%) for 15 min each and then viewed and digitally photographed in a ZEOL 1230 transmission electron microscope. (All tissue-processing supplies were purchased from Ted Pella, Tustin, California.)

Western blot analysis

Total cellular proteins, from an NHM line and a line of melanocytes derived from a patient with HPS-1 (homozygous for a 16-base pair duplication in *HPS1*), were extracted using RIPA buffer. Equal amounts of protein were fractionated on a 10% sodium dodecyl sulfate polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and incubated with 10% non-fat dry milk in PBS and Tween-20 (PBST, pH 7.4 with 0.2% Tween-20) for 1 h at room temperature. The membrane was probed with the rabbit polyclonal antiserum to the HPS1 protein (Dell'Angelica *et al*, 2000) (1:1000) or a monoclonal antibody to actin (Santa Cruz Biotechnology, Santa Cruz, California) (1:500). Primary antiserum/antibody was diluted in 2% nonfat milk/PBST and incubated at 4°C for 3 h. The bands of interest were visualized by indirect immuno-enzymatic staining using an alkaline phosphatase-labeled secondary antiserum followed by BCIP/NBT substrate (Kirkegaard and Perry, Gaithersburg, Maryland).

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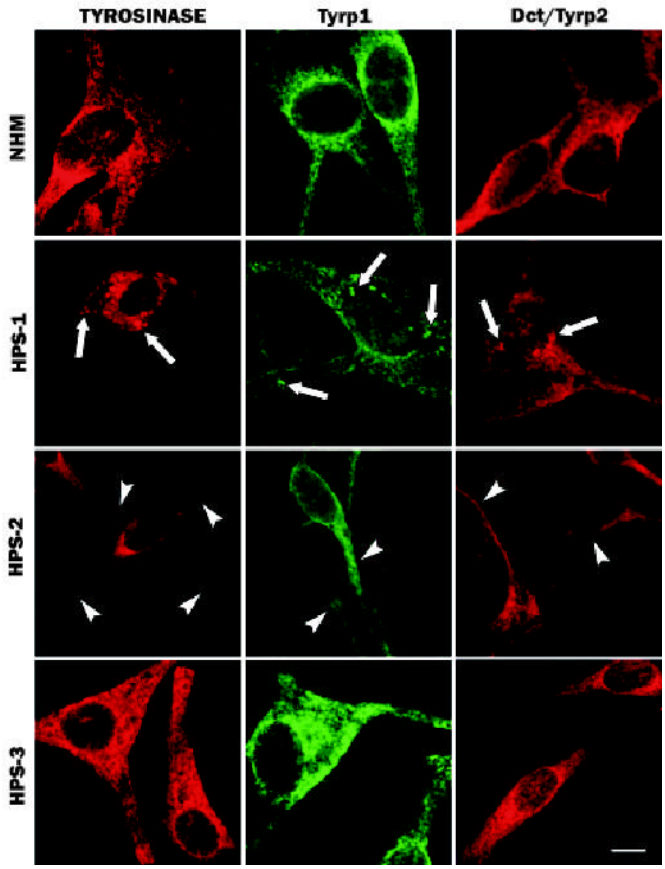

Figure 1. Melanocytes cultured from patients with Hermansky–Pudlak Syndrome (HPS)-1, HPS-2, and HPS-3 exhibit specific alterations in the distribution of tyrosinase gene family members

Cultures of melanocytes derived from an unaffected individual (normal human melanocytes (NHM)) and from patients with HPS-1, HPS-2, or HPS-3 were immunostained for tyrosinase, tyrosinase-related protein-1 (Tyrp1), or DOPAchrome tautomerase/tyrosinase-related protein-2 (Dct/Tyrp2). The NHM exhibited a granular staining pattern with prominent perinuclear localization for all three proteins. HPS-1 melanocytes exhibited a similar granular staining pattern with additional large aggregates (*arrows*) for all three proteins throughout the melanocytes. HPS-2 melanocytes exhibited a relative restriction of tyrosinase to the cell body with limited or no expression in the dendrites (*arrowheads*); Tyrp1 and Dct/Tyrp2 exhibited normal expression patterns. HPS-3 melanocytes displayed a floccular staining pattern within the cell body and dendrites for tyrosinase, Tyrp1, and Dct/Tyrp2 expression. *Scale bar* =10 μm.

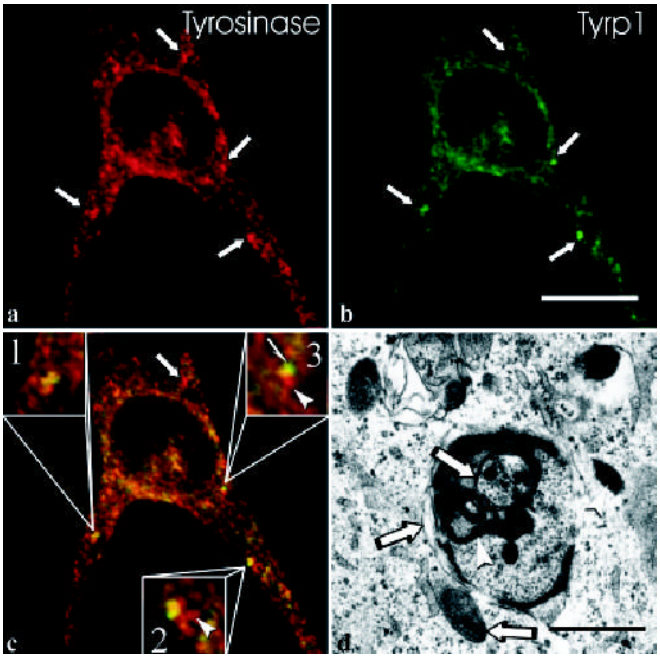

Figure 2. Hermansky–Pudlak Syndrome (HPS)-1 melanocytes exhibit a variation in co-localization of tyrosinase and tyrosinase-related protein-1 (Tyrp1) within characteristic large granules/ membranous complexes

In HPS-1 melanocytes, the characteristic large granules (*arrows*) are positive for tyrosinase [red in (*a*)] and Tyrp1 [green in (*b*)]. The merged image (*c*) demonstrates that some large granules contained tyrosinase only (*arrow*), some show equal co-localization of tyrosinase and Tyrp1 (*inset* #1), some display prominent co-localization of tyrosinase and Tyrp1 with areas containing tyrosinase only (*arrowhead*, *inset* #2), and some show polar expression of tyrosinase (*arrowhead*, *inset* #3) and Tyrp1 (*half-arrow*) with an intervening area of co-localization. Electron microscopic evaluation of HPS-1 melanocytes treated for DOPA histochemistry (*d*) demonstrates that, within a membranous complex, some cisternae and melanosomes express (*arrowheads*) or lack (*arrows*) reaction product, indicating the presence and absence of tyrosinase, respectively. *Scale bars* =10 (*b*) and 1.5 (*d*) μm.

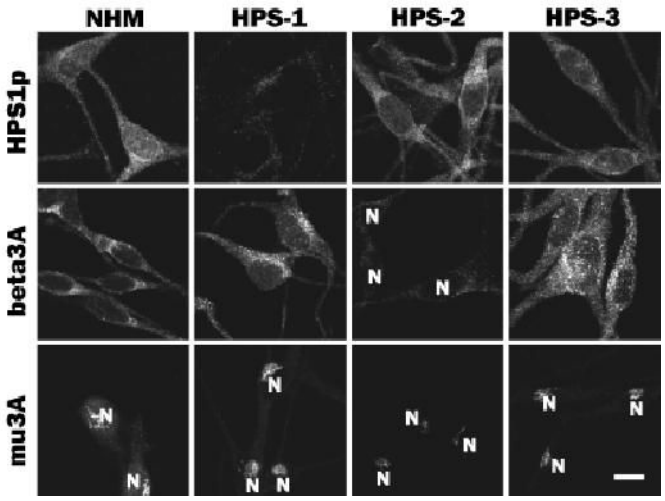

Figure 3. Melanocytes cultured from patients with Hermansky–Pudlak Syndrome (HPS)-1, HPS-2, and HPS-3 exhibit specific alterations in the distribution of HPS gene products

Cultures of melanocytes derived from an unaffected individual (normal human melanocytes (NHM)) and patients with HPS-1, HPS-2, or HPS-3 were immunostained for HPS1 (HPS-p) and the β3A (beta3A) and μ3A (mu3A) subunits of AP3. Expression of HPS1 protein (HPS1p) was similar in NHM, HPS-2 melanocytes, and HPS-3 melanocytes but markedly diminished in HPS-1 melanocytes. Expressions of both β3A and μ3A were normal in NHM, HPS-1 melanocytes, and HPS-3 melanocytes but markedly diminished in HPS-2 melanocytes. N, nucleus. *Scale bar* =10 μm.

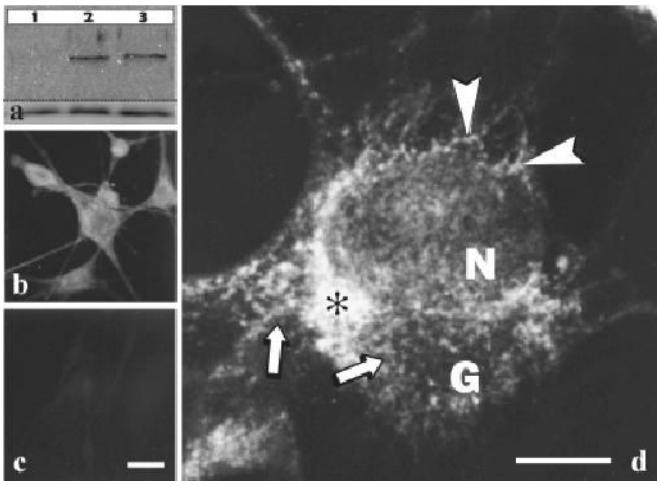

Figure 4. The Hermansky–Pudlak Syndrome (HPS)1 protein is expressed in normal human melanocytes (NHM) and absent in HPS-1 melanocytes

(*a*) Lysates of cultured melanocytes derived from an HPS-1 patient with the 16 bp duplication (*lane 1*), a normal Caucasian (*lane 2*), and a normal African/American (*lane 3*) individual were processed for western blot analysis using HPS1 antiserum (*upper blot*) and actin antibody (lower blot). A band of ~ 80 kDa was present in both controls and absent in the HPS-1 samples. Cultured melanocytes derived from a Caucasian individual (*b* and *d*) and an HPS-1 patient with the 16 bp duplication (*c*) were processed for indirect immunofluorescent cytochemistry using HPS1 antiserum. Immunostaining as observed by routine fluorescent microscopy was present predominantly in the cell body of control melanocytes (*b*) and absent in the HPS-1 melanocytes (*c*). As observed by confocal microscopy (*d*), the staining pattern for the HPS-1 protein in NHM was prominent in the perinuclear area of the cell body (*asterisk*) with a relative reduction in staining in the Golgi area (G) and the dendrites. The perinuclear staining appeared predominantly granular (*arrows*) with a reticular staining pattern (*arrowheads*) apparent among the granular profiles. N, nucleus. *Scale bars* =20 (*c*) and 7.5 (*d*) μm.

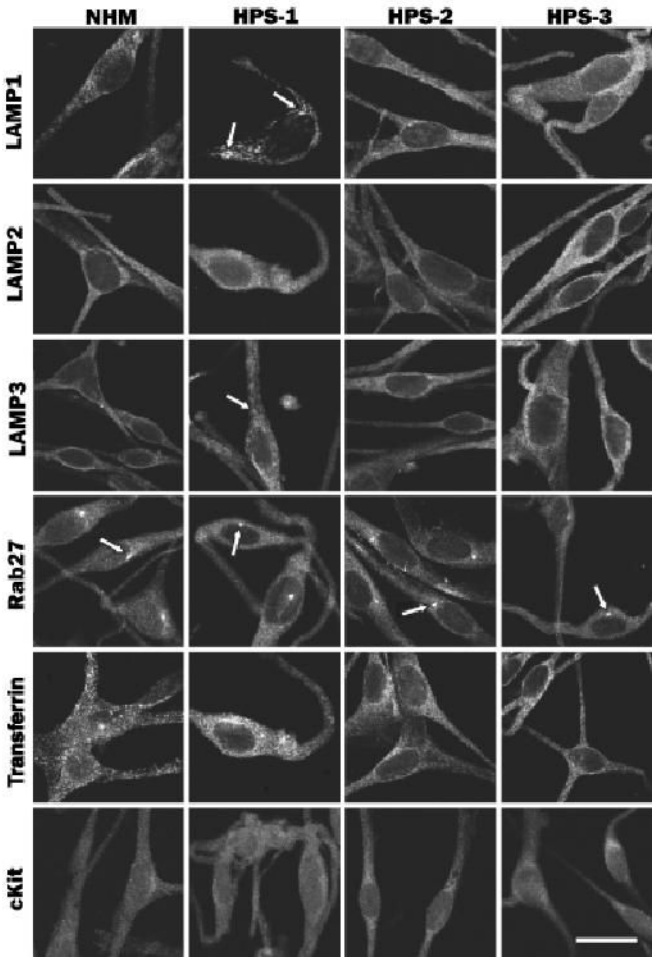

Figure 5. Melanocytes cultured from patients with Hermansky–Pudlak Syndrome (HPS)-1, HPS-2 and HPS-3 were evaluated for expression of various proteins

Normal human melanocytes (NHM) and HPS-1, HPS-2, or HPS-3 melanocytes were immunostained for LAMP 1–3, Rab 27, transferrin, and cKit. Expression of LAMP1 and LAMP3 was granular and that of LAMP2 was diffuse throughout the NHM. HPS-1 melanocytes exhibited, in addition to the normal localization for LAMP 1–3, localization to large granules (*arrows*) for LAMP1 and LAMP3. HPS-2 melanocytes exhibited normal localization for LAMP 1–3. HPS-3 melanocytes exhibited a more floccular distribution pattern for LAMP1 and LAMP3 and a normal distribution of LAMP2. Expression of Rab27, transferrin, and cKit in NHM exhibited a uniform pattern throughout the melanocytes with a distinct centriole localization (*arrows*), a punctate pattern throughout the melanocytes, and a diffuse pattern throughout the melanocytes, respectively. HPS-1, HPS-2, and HPS-3 melanocytes exhibit a normal staining pattern for Rab27, transferrin, and cKit. *Scale bar* =20 μm.

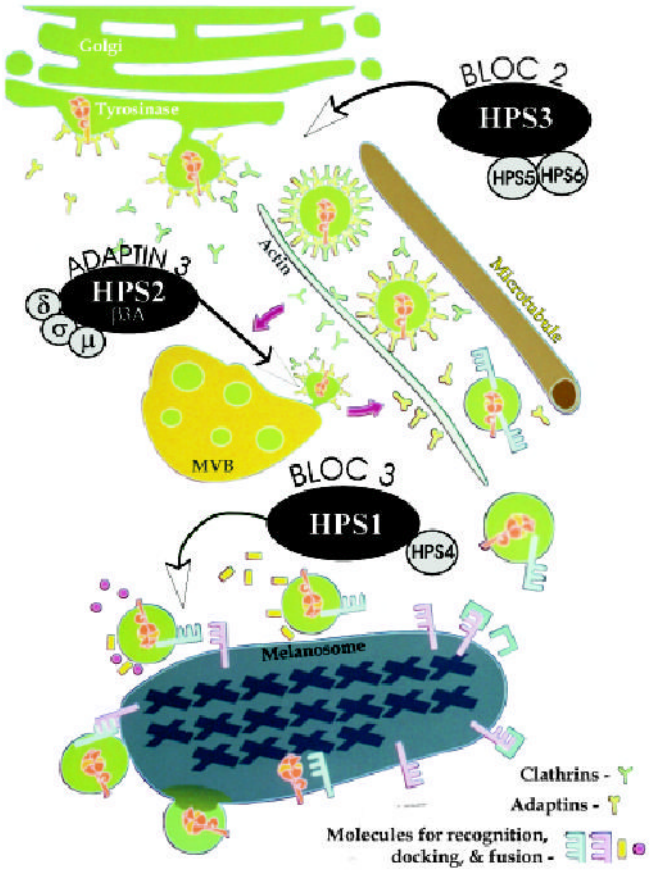

Figure 6. Model for involvement of Hermansky–Pudlak Syndrome (HPS1), HPS2, and HPS3 in the trafficking of tyrosinase gene family members from the Golgi to the melanosome

Tyrosinase, tyrosinase-related protein-1 (Tyrp1), and DOPAchrome tautomerase/tyrosinase-related protein-2 (Dct/Tyrp2) are recruited from the Golgi apparatus into clathrin-coated vesicles to be trafficked along cytoskeletal element to Stage II melanosomes in the perinuclear area. Biogenesis of lysosome-related organelles complexes (BLOC)-2, composed in part of HPS3, HPS5, and HPS6, facilitates this early cargo trafficking event. In the absence of HPS3, cargo vesicles are aberrantly trafficked beyond the perinuclear area. Tyrosinase, and possibly Tyrp1 and Dct/Tyrp2, transit through multivesicular body (MVB)/late endosome-like structures on route to melanosomes. Adaptin3 facilitates the recruitment of tyrosinase from the MVB. In the absence of the β3A subunit of AP3, tyrosinase is aberrantly retained in the MVB. Ultimately, vesicles containing tyrosinase, Tyrp-1, and Dct/Tyrp2 recognize, dock, and fuse with Stage II melanosomes. BLOC-3, composed in part of HPS1 and HPS4, facilitates this later cargo trafficking event. In the absence of HPS1, cargo vesicles are aberrantly trafficked to macroautophagosomes.