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Molecular Pathogenesis of Genetic and Inherited Diseases

Melanocyte-Specific Proteins Are Aberrantly Trafficked in Melanocytes of Hermansky-Pudlak Syndrome-Type 3

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Hermansky-Pudlak Syndrome-type 3 (HPS-3) is a relatively mild subtype of HPS with minimal cutaneous and ocular depigmentation. The *HPS-3* gene encodes a novel protein of unknown function with a predicted molecular weight of 114 kd. To assess the role of the HPS3 protein in melanization, cultured melanocytes developed from HPS-3 patients were evaluated biochemically and histologically for activity and localization of melanocyte-specific proteins. Endogenous tyrosinase activity of HPS-3 melanocytes was substantial, but tyrosinase activity and melanin synthesis was suppressed in intact melanocytes. However, the level of suppression, as well as extent to which up-regulation by isobutylmethylxanthine and cholera toxin was muted, was less that in HPS-1 melanocytes. Ultrastructurally, HPS-3 melanocytes contained morphologically normal melanosomes, predominantly of stage I and II with minimal stage III and few stage IV melanosomes. Dihydroxyphenylalanine (DOPA) histochemistry demonstrated an increase in melanization of melanosomes. Unique to HPS-3 melanocytes were numerous DOPA-positive 50-nm vesicles and tubular elements present throughout the cell body and dendrites. Tyrosinase, tyrosinase-related protein-1 (Tyrp1), dopachrome tautomerase (Dct), and LAMP1 and 3 localization in HPS-3 melanocytes, as evaluated by immunocytochemistry and confocal microscopy, demonstrated a fine, floccular distribution in contrast to the coarse, granular distribution characteristic of control melanocytes. The localization profile of other proteins expressed by melanocytes (ie, Silver/Pmel17, Melan-A/MART-1, LAMP2, Rab 27, transferrin, c-kit, adaptin-3, and the HPS1 protein) appeared normal. These results suggest that a specific subset of melanocyte proteins are aberrantly trafficked throughout the HPS-3 melanocyte and may be responsible for the reduction in melanin synthesis. *(Am J Pathol 2005, 165:231–240)*

Hermansky-Pudlak Syndrome (HPS [MIM203300]) is a congenital disorder that presents initially with oculocutaneous albinism and a bleeding diathesis.[1–3] The oculocutaneous albinism results from the reduced ability of cutaneous and ocular melanocytes to synthesize a pigmented melanosome due to mistrafficking of melanocyte-specific proteins to this organelle.[2] The bleeding diathesis results from the absence of dense bodies within platelets that subsequently compromises clotting efficiency.[4,5] Patients with some subtypes of HPS can develop a ceroid storage disease and pulmonary fibrosis that may result from impairment of the lysosomal system in stromal cells of various tissues of the body.[6,7] Defective biosynthesis of the melanosome, the platelet dense body, and the lysosome in HPS suggest that these organelles are derived via a common mechanism.

HPS is a genetically heterogeneous disease. To date, seven genetic subtypes of HPS have been identified in humans.[2,8] All but one of these seven causative genes encodes novel proteins with functions that have yet to be discerned.[9–13] However one type of HPS, ie, HPS2, encodes the β3A subunit of the heterotetrameric AP-3 complex.[14,15] Adaptin-3 is a member of the adaptin family of proteins that function to recruit cargo proteins into clathrin vesicles for transport away from a donor membrane within either the Golgi apparatus, plasma membrane, and/or endosomal compartments.[16] Although the specific functions of the novel HPS proteins are unknown,

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they appear to also participate in trafficking events within the target cells. In mice, there are approximately 15 models for HPS.[2,17] Functions for several of the murine HPS genes have been identified and the gene products also appear to regulate trafficking events. Specifically, the murine HPS gene products include several adaptin subunits,[18,19] Rab27a,[20] the vacuolar protein sorting 33a molecule,[21] and the α subunit of a Rab geranylgeranyl transferase.[22]

Hermansky-Pudlak Syndrome type 1 (HPS-1) was the first genotype to be identified, initially in a genetic isolate of patients residing in northwest Puerto Rico.[9,23,24] Subsequently, Hermansky-Pudlak Syndrome type 3 (HPS-3) was identified initially in a genetic isolate of patients residing in central Puerto Rico.[25,26] Patients with HPS-3 have since been ascertained throughout the globe.[27] HPS-3 is a relatively mild form of the disease in that both the oculocutaneous albinism and the bleeding diathesis are mild and pulmonary fibrosis generally does not develop.[27] The *HPS3* gene is located on chromosome 3q24 and has 17 exons and a 3015-bp open reading frame. The molecule encoded by the *HPS3* locus is a 114-kd protein with no apparent homologies with other proteins.[25,26] However, the HPS3 protein does contain a putative clathrin binding region and an ER-retention signal,[25,26] again suggesting participation in trafficking.

In this report we describe the biochemical and cellular features unique to melanocytes cultured from several patients with HPS-3. Some of the identified features are distinct to this form of HPS and clearly differentiate it from HPS-1 and HPS-2.

Materials and Methods

Cell Culture

Separate cultures of melanocytes were developed from individual neonatal foreskins or forearm skin biopsies. Normal human neonatal foreskins [from dark (NHM_D) and light (NHM_L) skin infants] were obtained from the nursery of University Hospital in Cincinnati after routine circumcision. An OCA-1 individual (age = 6 months) was enrolled in a protocol approved by the University of Cincinnati Institutional Review Board to study the cellular and molecular aspects of albinism and a written informed consent obtained before obtaining a 4-mm punch biopsy. HPS-1 (n = 3; ages = 3, 6, and 7) and HPS-3 (n = 3; ages = 5, 8, and 12) 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.

Two of the three HPS-1 individuals (patients 1 and 3) were of Puerto Rican ancestry and represent the genotype with the 16-bp duplication in the *HPS1* gene[9] and one patient (2) of Caucasian ancestry was either homozygous or hemizygous for S396 del C, ie, a cytosine deletion in serine codon 396.[28] Both mutations result in loss of expression of *HPS1*.[29] For HPS-3, patients 1 and 2 were homozygous for the 3.9-kb deletion encompassing exon 1 and its surrounding introns in the HPS3 gene that arose in central Puerto Rico.[25] Patient 3 was compound heterozygous for the 3.9-kb deletion and a I243insA mutation. These mutations result in undetectable levels of the 4.4-kb HPS3 mRNA in affected cells.[25,27]

After written informed consent was obtained, a 2-mm punch biopsy was taken from each HPS patient, placed immediately in melanocyte growth medium with 2X antibiotic/antimycotic solution, and shipped by express mail to Cincinnati, OH. All skin samples were placed in trypsin (2.5 mg/L) and incubated for 2 hours at 37°C. The trypsin was replaced with MCDB-153 medium, and the tissue was gently vortexed for 30 seconds 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, CA) as previously described.[30] The MCDB-153 growth medium was supplemented with 0.6 ng/ml basic fibroblast growth factor, 8 nmol/L 12-O-tetradecanoylphorbol-13-acetate (TPA), 5 μg/ml insulin, 5 μg/ml transferrin, 1.0 μg/ml α-tocopherol, 30 μg/ml crude pituitary extract (Clonetic Laboratories, San Diego, CA), 0.5 μg/ml hydrocortisone, 20 μg/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 to 4 days in the presence of 100 μg/ml geneticin (G418 sulfate).[31] Cultures from the second to the fifth passage were used for the experiments described herein.

The medium used to up-regulate melanin synthesis consisted of the above-described MCDB-153 growth medium with the following modifications: TPA was omitted, basic fibroblast growth factor was increased twofold (1.2 ng/ml), and cholera toxin (CT) at 2.0 ng/ml and isobutyl methylxanthine (IBMX) at 0.1 mmol/L were added.[32,33]

Assays

To quantitatively measure melanin content, 10 μg of protein (as determined by the bicinchoninic acid protein assay; Pierce Chemical Company, Rockford, IL) from cell lysates was solubilized in 0.5 ml of 0.1 N NaOH. The optical density of supernatant at 475 nm was compared with a standard curve by using known concentrations of synthetic melanin, and results were expressed as micrograms of melanin/milligram of protein.

To quantitatively measure and compare endogenous versus functional tyrosinase activity, two assay protocols were performed.[34] In intact cells, melanocytes were incubated in medium containing 1 μCi/ml [^{3}H] tyrosine (specific activity, 54.2 Ci/mmole) for 24 hours. In cell lysates, solubilized melanocytes were incubated in 250 μl of reaction mixture containing 10 to 25 μg of protein, 0.08 mmol/L L-DOPA, 0.08 mmol/L tyrosine, and 8 μCi/ml of [^{3}H] tyrosine at 37°C for 1 hour. Duplicate aliquots of medium (1 ml) or lysate/reaction mixture (250 μl) were mixed with an equal volume of 10% (w/v) solution of activated charcoal in 0.2 N citric acid. The samples were

centrifuged at 2500 rpm, and the supernatants were passed over a Dowex ion-exchange column followed by a wash of 0.1 N citric acid. Radioactivity of the eluate was counted in a Packard 1900 CA liquid scintillation analyzer. Tysosinase activity was expressed as dpm/protein.

DOPA Histochemistry and Electron Microscopy

Established cultures of melanocytes from three control and the three HPS-3 individuals, maintained in regular MCDB-153 growth medium, were seeded into Lab-Tek chamber slides (Nunc, Inc., Naperville, IL) coated with 1% pig gelatin and grown to 90% confluence. Cultured melanocytes were fixed in wells with half-strength Karnovsky's fixative[35] in 0.2 mol/L sodium cacodylate buffer at pH 7.2 for 30 minutes at room temperature. For DOPA histochemistry, fixed cells were incubated in a 0.1% solution of L-DOPA twice for 2.5 hours. The cells were washed three times in buffer and treated with 1.0% osmium tetroxide containing 1.5% potassium ferrocyanide[36] for 30 minutes. The cells were washed, stained *en bloc* with 0.5% uranyl acetate for 30 minutes, 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. Ultra-thin sections were then stained with aqueous solutions of uranyl acetate (2%) and lead citrate (0.3%) for 15 minutes each and then viewed and photographed in a ZEOL 1230 transmission electron microscope. All tissues processing supplies were purchased from Ted Pella, Inc., Tustin, CA.

Immunofluorescence

Established cultures of melanocytes from three control and the three HPS-3 individuals, maintained in regular MCDB-153 growth media, were plated on gelatin-coated Lab-Tek (Nunc, Inc.) chamber slides at 2.5×10^4 cell/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 minutes at room temperature, blocked in 1% bovine serum albumin (BSA) in PBS for 5 minutes at room temperature, and incubated in primary antibody solution diluted in 0.2% Saponin in PBS containing 0.1% BSA for 60 minutes at room temperature. The primary antibodies used in this study included rabbit polyclonal antiserum to human tyrosinase provided by R. King and W.S. Oetting (hPEP7; University of Minnesota, MN) [1:300],[37] mouse monoclonal antibody to Tyrp1 (Mel-5; Signet Laboratories, Dedham, MA), [1:50], rabbit polyclonal antiserum to Dct provided by V. Hearing (PEP8; National Institutes of Health), [1:200], mouse monoclonal antibody to the Silver protein (HMB45; DAKO Corp., Santa Barbara, CA) [1:100], mouse monoclonal antibody to Melan-A/MART-1 (NeoMarkers, Inc., Fremont, CA) [1:100], mouse monoclonal antibodies to LAMP1 (H4A3), LAMP2 (H4B4), and LAMP3 (H5C6) (Developmental Studies Hybridoma Bank, Iowa City, IA) [1:300], rabbit polyclonal antiserum to the HPS1 protein[10] [1:300], rabbit polyclonal antiserum to β adaptin-3 provided by M.S. Robinson (Cambridge Institute for Medical Research, Cambridge, UK) [1:300], rabbit polyclonal antiserum to Rab27 (610595; BD Transduction Laboratories, Lexington, KY) [1:300], rabbit polyclonal antiserum to transferrin (0061; Dako Corp.) [1:300], and mouse monoclonal antibody to c-Kit (CD117; Novocastra Laboratories Ltd, Newcastle on 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 minutes 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, PA). After washing, coverslips were applied to the slides using Fluoromount G (Southern Biotechnologies, Birmingham, AL) and observed with a LSM 510 confocal microscope (Zeiss, Oberkochen, Germany).

Statistics

Data were analyzed statistically by Student's *t*-test.

Results

Melanin Synthesis in Cultured Melanocytes

Melanocyte cultures from all three HPS-3 patients expressed endogenous tyrosinase activity levels, as assessed for cell lysates, that were intermediate between melanocytes cultured from light skin Caucasian (NHM_L) and dark skin African-American (NHM_D) individuals (Figure 1a). This is consistent with the intermediate skin pigmentation of individuals of Puerto Rican ancestry.[25] For comparison, cultures of melanocytes from three individuals with HPS-1 were assessed for tyrosinase activity. Interestingly, the two HPS-1 Puerto Rican individuals (see Materials and Methods) expressed endogenous tyrosinase activity similar to that of the three HPS-3 Puerto Rican individuals while the HPS-1 Caucasian expressed tyrosinase activity that was significantly less than that of the HPS-1 and HPS-3 Puerto Rican patients and similar to that of normal Caucasian individuals (NHM_L) (Figure 1a). This is also consistent with the skin coloration of the respective ancestry.

In contrast, endogenous melanin synthesis as assessed for intact melanocytes cultured from the three HPS-3 patients was relatively suppressed and resembled that of melanocytes cultured from normal Caucasian individuals (NHM_L) (Figure 1b). However, the reduction in melanin synthesis exhibited by HPS-3 melanocytes was not as marked as that of HPS-1 melanocytes (Figure 1b). This correlates with the severity of the disease, and specifically, the cutaneous manifestations of HPS-1 and HPS-3. HPS-1 patients exhibit a severe bleeding diathesis and pulmonary fibrosis with marked skin hypopigmentation whereas HPS-3 patients manifest a less severe bleeding diathesis, no pulmonary fibrosis, and minimal skin hypopigmentation.

In contrast to the endogenous tyrosinase activity, the functional tyrosinase activity of intact HPS-3 melanocytes was significantly reduced compared to that of Caucasian

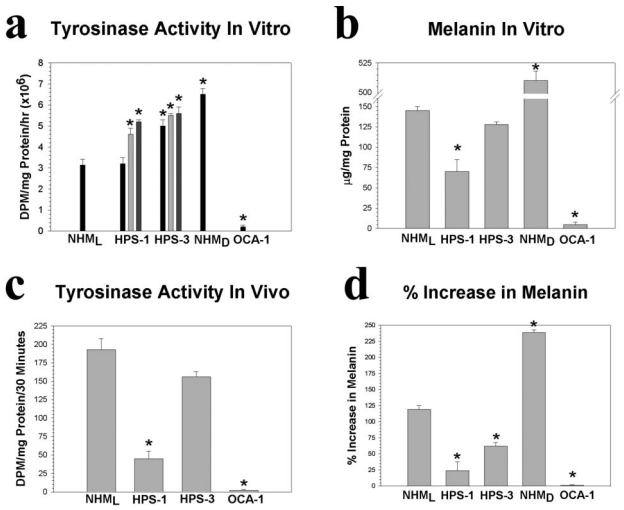

Figure 1. Melanocytes cultured from patients with HPS-3 exhibit suppressed tyrosinase activity and mild hypomelanosis. Cultures of melanocytes derived from a Caucasian individual (NHM_L), an African American individual (NHM_D), an individual with oculocutaneous albinism type 1 (OCA1), and three individuals with either HPS-1 or HPS-3 were tested in triplicate for (**a**) tyrosinase activity in cell lysates [shown separately are each HPS-1 (**bars** from left to right represent patients 3, 1, and 2, respectively) and HPS-3 (**bars** from left to right represent patients 1, 2, and 3, respectively) melanocyte line], (**b**) melanin content in cell lysates (data from each HPS-1 and 3 melanocyte line is grouped by genotype, as for **c** to **d** also), (**c**) tyrosinase activity in intact cells, and (**d**) percent increase in melanin after treatment of cultured melanocytes with isobutyl methylxanthine and cholera toxin for 10 days as described in Materials and Methods. Data are expressed as means ± SEM. *, $P < 0.05$ for each line compared to the NHM_L lines.

melanocytes, but not as reduced as that exhibited by HPS-1 melanocytes (Figure 1c). This demonstrates that HPS-3 melanocytes, unlike and in comparison with normal melanocytes exemplified by the NHM_L line, express a relatively high endogenous amount of tyrosinase activity (ie, significantly greater than NHM_L) that is reduced/suppressed in intact cells (ie, insignificantly different that NHM_L), and results in less melanin synthesis than expected for the level of endogenous tyrosinase that exists (ie, comparable to or significantly lower than NHM_L as assessed in lysates or intact cells, respectively). Melanocytes were also tested for their response to the melanogenic/mitogenic agents isobutyl methylxanthine (IBMX) and cholera toxin (CT) (Figure 1d). Both HPS-3 and HPS-1 melanocytes exhibited a suppressed response for functional tyrosinase activity (not shown) and melanin synthesis (Figure 1d). In contrast, melanocytes from both HPS subtypes exhibited a normal proliferative response to IBMX/CT as indicated by similar increase in protein level as observed for NHM (not shown).

Ultrastructure of HPS-3 Melanocytes

The cell body and dendrites of HPS-3 melanocytes (~30 cells from each of the three HPS-3 individuals) exhibited normal ultrastructural morphology (Figure 2). The only apparent difference between the HPS-3 and control (NHM_L and NHM_D) melanocytes was in the maturation of melanosomes. HPS-3 melanocytes exhibited relatively few mature stage IV melanosomes in both the cell body and the dendrites compared to normal human melanocytes. The structure of stage I premelanosomes and the melanofilament assembly within stage II melanosomes was normal (insets to Figure 2).

DOPA histochemistry was performed on cultured HPS-3 melanocytes to assess cytoplasmic sites of functional tyrosinase. In normal human melanocytes (from both NHM_L and NHM_D), the extra-melanosomal tyrosinase activity identified by DOPA histochemistry was restricted to the trans-most cisternae of the Golgi apparatus and to 50-nm vesicles in the vicinity of the Golgi (Figure 3a) while absent from lateral areas of the cell body (Figure 3a) and the dendrites (Figure 3d). In HPS-3 melanocytes, DOPA treatment created abundant stage IV melanosomes, indicating the presence of sufficient enzyme activity to convert the exogenous supplied substrate into melanin. In addition, numerous DOPA-positive 50-nm vesicles and short tubular elements were present peripheral to the Golgi zone (Figure 3, b and c) as well as within the dendrites (Figure 3e). On occasion, some of the DOPA-positive short tubular elements appeared to be continuous with melanosomes (Figure 3, b and c).

Localization of Melanocyte Proteins in HPS-3

Tyrosinase was immunolocalized in cultured HPS-3 and control (both NHM_L and NHM_D) melanocytes (Figure 4). In control melanocytes, tyrosinase exhibited a granular staining pattern predominant within the cell body as well as throughout the dendrites, indicative of melanosomal localization (Figure 4, a and c). In addition, minimal intergranular fluorescence was detected (Figure 4e). In contrast, tyrosinase in HPS-3 melanocytes exhibited a fine, floccular staining pattern in both the cell body and the dendrites (Figure 4, b and d). With higher magnification, the floccular pattern within HPS-3 melanocytes consisted of a large granular component similar to control melanocytes (Figure 4e) plus fine intergranular staining (Figure 4f). This is consistent with the ultrastructure of the DOPA histochemistry of HPS-3 melanocytes, by which tyrosinase is localized within both melanosome organelles as well as 50-nm vesicles and tubules dispersed throughout the melanocyte. Tyrosinase-related protein-1 (Tyrp1), another regulatory enzyme in melanin synthesis, also exhibited a granular staining pattern in normal human melanocytes and a fine, floccular pattern in HPS-3 melanocytes (Figure 5). In addition, co-localization between tyrosinase and Tyrp1 was not as distinct in all HPS-3 melanocytes (ie, HPS-3-#1 (Figure 5f), -#2 and -#3 (not shown) as in control melanocytes).

DOPAchrome tautomerase (Dct), the enzyme responsible for a later step in melanin synthesis, also exhibited a fine, floccular pattern of distribution within HPS-3 melanocytes (Figure 6, a and b). Two of the three lysosomal-associated membrane proteins, LAMP1 and LAMP3, also exhibited a granular staining pattern in normal human melanocytes and a fine, floccular pattern in HPS-3 melanocytes (Figure 6, c to f). In contrast, the HPS 1 and 2 gene products (HPS1p and β adaptin-3) (Figure 6, g to j), as well as the Silver protein (also known as Pmel17 and

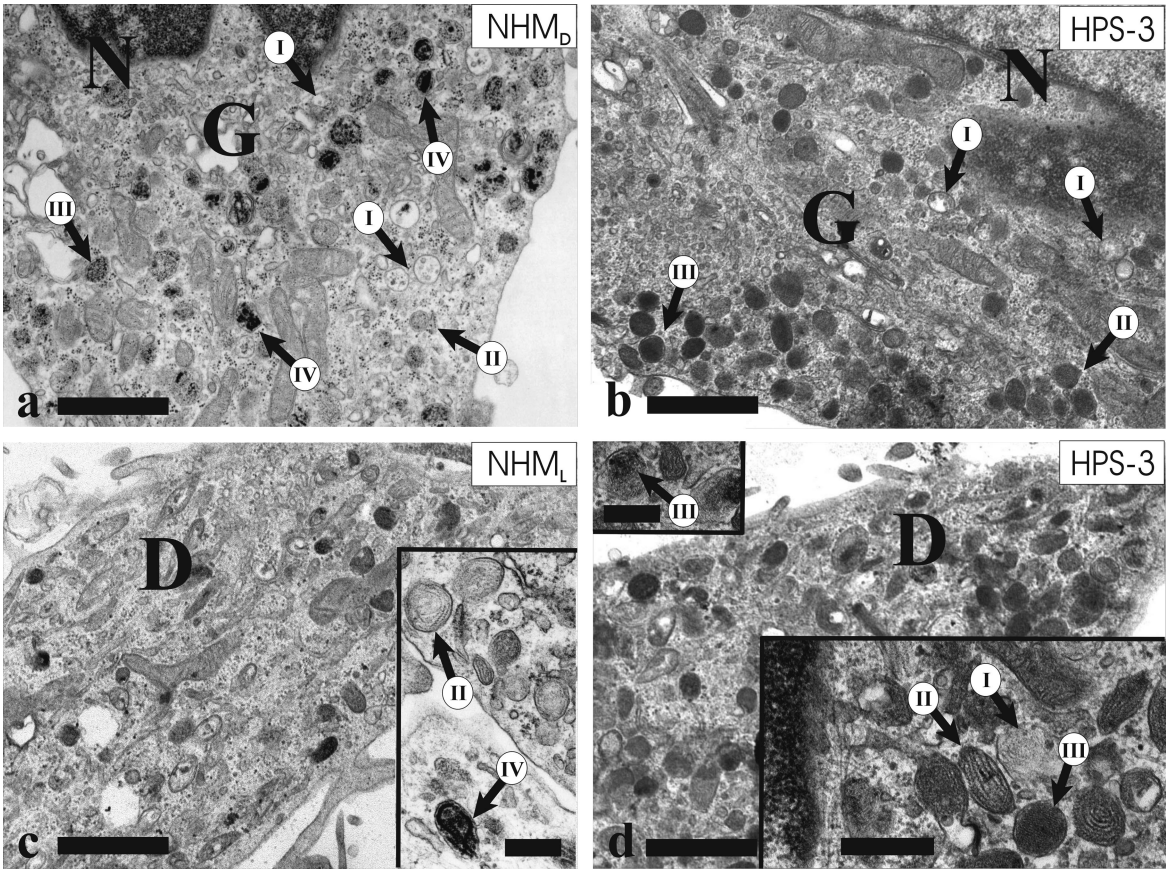

Figure 2. HPS-3 melanocytes exhibit morphologically normal melanosomes with diminished melanin synthesis. As demonstrated by electron microscopy, the Golgi (G) area (**a**) and dendritic (D) area (**c**) of normal human melanocytes (**a** and **c** represent NHM_D and NHM_L, respectively) contain melanosomes ranging from the early (I) to the mature (IV) stage. In contrast, the Golgi (G) area (**b**) and dendritic (D) area (**d**) of melanocytes of patients with HPS-3 (**b** and **d** represent patients 1 and 2, respectively) contain melanosomes representing stages I to III only, with no stage IV melanosomes apparent. **Insets** demonstrate that the melanofilament arrangement within the stage II melanosomes of the HPS-3 melanocytes appears normal. N = nucleus. **Bars**: **a–d**, 2 μm; **insets**, 1 μm.

gp100), Melan-A/MART-1, LAMP2, RAB27, transferrin, and c-kit (data not shown), exhibited normal staining patterns in HPS-3 melanocytes. Normal human melanocytes derived from both light and dark skin individuals were used throughout these immunolocalization studies and demonstrated similar staining pattern between the two complexion coloration types for all protein assessed.

Discussion

Hermansky-Pudlak syndrome is a multi-system disorder with genetic heterogeneity. Patients present with cutaneous and ocular hypopigmentation, a bleeding diathesis, and various organ disorders including pulmonary fibrosis and granulomatous colitis. These physical disorders result from the defective biogenesis of the pigmented melanosome, the platelet dense body, and lysosomes. To date, seven human genes and approximately 15 murine genes have been identified that can result in HPS when expressing a mutation in both alleles. Most of the HPS gene products encode novel proteins that appear to function at specific and varied sites along trafficking pathways commonly used in the biogenesis of the functional melanosome, platelet dense body, and lysosome.[2] Cultured melanocytes have provided an illustrative system for unraveling the multi-step cellular trafficking processes responsible for creating the specific organelles affected by different *HPS* gene mutations.

Reported herein is the identification of the cellular abnormality of the HPS-3 subtype involving trafficking of melanosome-destined proteins out of the Golgi apparatus. Specifically, the major melanocyte-specific enzymes, tyrosinase, Tyrp1, and Dct, in addition to reaching the target melanosome, are uniquely maintained in 50-nm vesicles that are distributed throughout the HPS-3 cell body and dendrites. Of significance is the localization of these tyrosinase-related proteins to the melanosome in some amount. This may suggest that alternate or promiscuous HPS-3-independent pathways may co-exist with the HPS-3-dependent pathway in melanocyte trafficking. The aberrant distribution of tyrosinase appears on electron microscopy as an abundance of DOPA-positive vesicles and tubules beyond the Golgi area and on immunoconfocal microscopy as a fine, floccular pattern of staining. The latter pattern was also observed for Tyrp1, Dct, LAMP1 and LAMP3. We suggest that these aberrant morphological patterns represent the untargeted and undirected delivery of these cargo proteins to the melano-

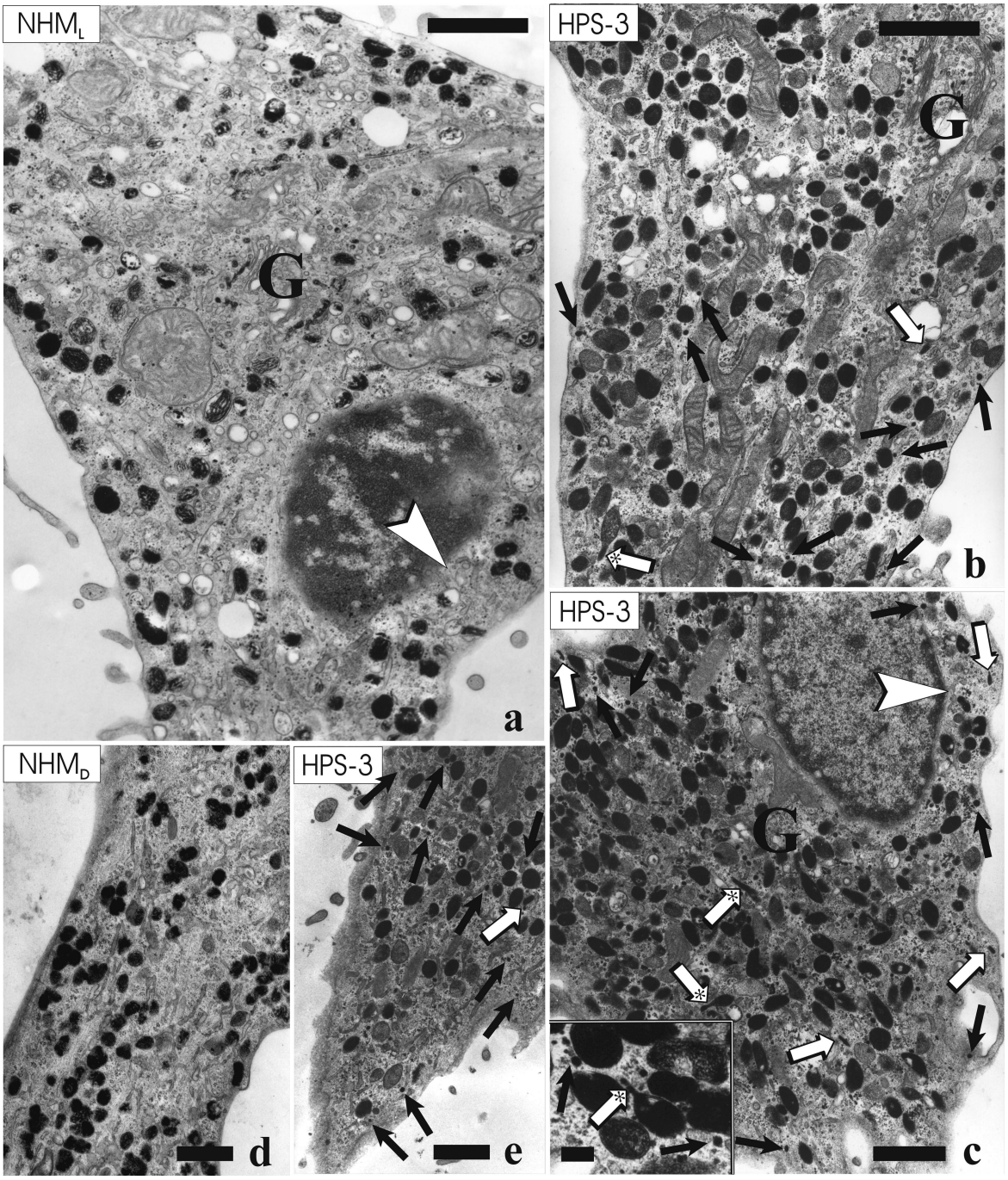

Figure 3. After DOPA histochemistry, HPS-3 melanocytes exhibit an extensive distribution of DOPA-positive vesicles and tubules. As demonstrated by electron microscopy, normal human melanocytes (NHM_L) (**a**) exhibit DOPA-positive vesicles confined to the Golgi (G) area and notably absent from the lateral side of the nucleus (**arrowhead**). In contrast, HPS-3 melanocytes (**b** and **c** represent patients 2 and 1, respectively) exhibit DOPA-positive vesicles (**black arrows**) and tubules (**white arrows**) throughout the cell body peripheral to the Golgi (G) area as well as in the lateral side of the nucleus (**arrowhead**). Some of the DOPA-positive tubular profiles appear to be continuous with melanosomes (**white arrows** with **asterisks** in **c** and **inset** to **c**). In addition, DOPA-positive vesicles (**black arrows**) and tubules (**white arrows**) are absent from the dendrites of (**d**) normal human melanocytes (NHM_D) but (**e**) prevalent in the dendrites of HPS-3 melanocytes (patient 2). **Bars**: **a–e**, 2 μm; **inset** to **c**, 0.3 μm.

some in the immediate vicinity of the trans-Golgi network in the absence of the HPS-3 protein. The 50-nm vesicles represent trafficking vesicles carrying cargo to the melanosome that are unable to recognize, dock, and/or fuse to the premelanosome efficiently and subsequently become waywardly distributed throughout the cell. However, some cargo enzymes do successfully become deposited in melanosomes, as demonstrated by the innate production of moderately pigmented melanosomes (ie, stage III melanosomes) and by the increase in pigmentation resulting from DOPA incubation. The incorporation of these melanosomal proteins may occur either inadvertently (ie,

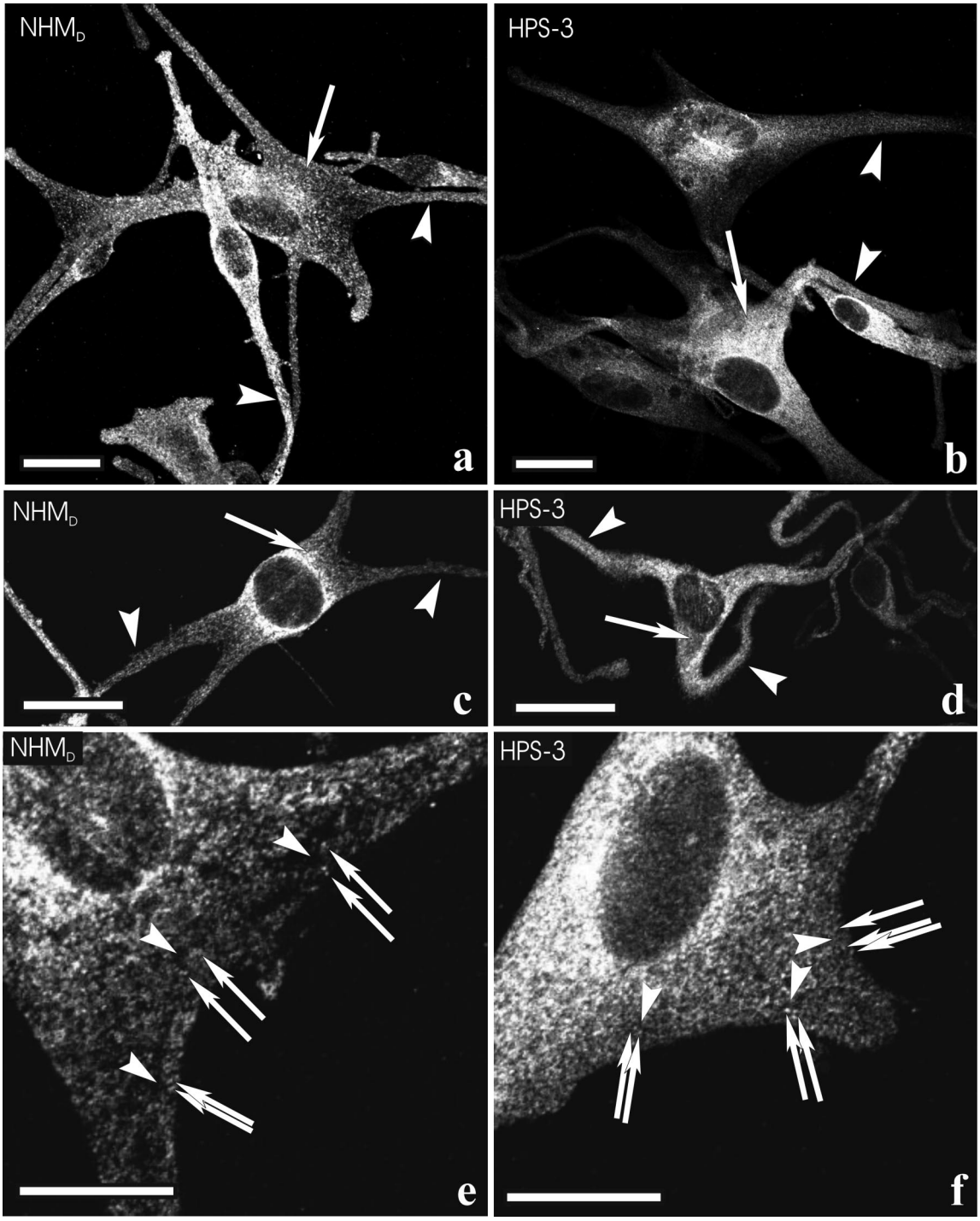

Figure 4. Tyrosinase distribution in HPS-3 melanocytes appears floccular and less granular than in normal human melanocytes (NHM). As demonstrated by immunocytochemical staining of tyrosinase, normal human melanocytes (**a** and **c** represent NHM_D) exhibit a granular staining pattern that is dense in the cell body (**arrow**) and sparse in the dendrites (**arrowheads**). In contrast, HPS-3 melanocytes (**b** and **d** represent patients 1 and 2, respectively) exhibit a finer, more floccular staining pattern, also more intense in the cell body (**arrows**) than in the dendrites (**arrowheads**). Higher magnification of NHM (**e** represents NHM_D) demonstrates that the granular pattern characteristic of NHM represents immunolabeling predominantly of 0.5 to 1.0 μm melanosomes (**arrows**) with lack of immunolabeling between melanosomes (**arrowheads**). In contrast, higher magnification of HPS-3 (**f** represents patient 1) demonstrates that the floccular staining characteristic of HPS-3 melanocytes represents additional staining (**arrowheads**) between the immunolabeled melanosomes (**arrows**). This finer immunolabeling profile represents the numerous DOPA-positive intermelanosomal vesicles and tubules observed with electron microscopy (Figure 3). **Bars**: **a–d**, 20 μm; **e–f**, 8 μm.

non-specific fusion of trafficking vesicles with the melanosome) or by a default mechanism (ie, independent of the HPS3 protein). Regardless, insufficient amounts of enzymes and regulatory proteins are incorporated into the melanosomes so that insufficient melanin synthesis occurs and oculocutaneous albinism subsequently results.

Although the specific function of most HPS gene products is unknown, many of them interact within mod-

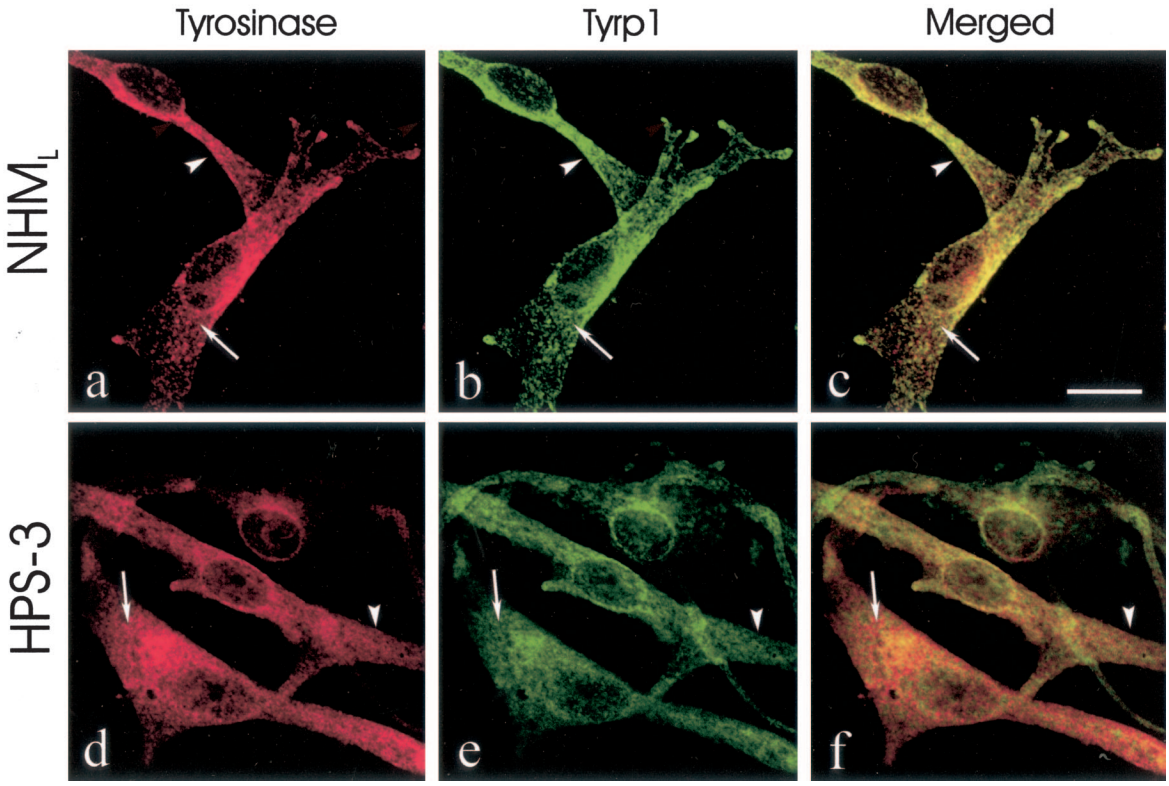

Figure 5. Tyrosinase-related protein-1 (Tyrp1) in HPS-3 melanocytes also appears floccular and less granular and without uniform co-localization with tyrosinase. As demonstrated by dual immunocytochemical staining, normal human melanocytes (NHM) derived from light skin (**a–c**) exhibit a granular staining pattern relatively dense in the cell body (**arrow**) and sparser in the dendrites (**arrowheads**) for both tyrosinase (**a**, red) and Tyrp1 (**b**, green). Tyrosinase and Tyrp1 exhibit marked co-localization in the merged image (**c**). In contrast, HPS-3 melanocytes derived from patient 1 (**d–f**) exhibit a finer, more floccular staining pattern also more intense in the cell body (**arrows**) than the dendrites (**arrowheads**) for both tyrosinase (**d**, red) and Tyrp1 (**e**, green). Merged images demonstrate that tyrosinase and Tyrp1 exhibit less co-localization in HPS-3 melanocytes (**f**) than observed in NHM (**c**). Similar results were obtained using HPS-3-#2 melanocytes (not shown) **Bar**, 15 μm.

ules or complexes thought to facilitate the trafficking of cargo from donor to recipient site. For example, the gene products of pallid (*pa*) and muted (*mu*), two murine models of HPS presently without human homologues, are subunits of a protein complex termed biogenesis of lysosome-related organelle complex-1 (BLOC-1), which interacts *in vitro* with the murine cappuccino (*cno*) gene product,[38] syntaxin-13 (a SNARE family member involved in membrane fusion[39,40]), and the actin cytoskeleton.[41] Yeast two-hybrid studies and co-immunoprecipitation analysis have shown that the gene product of HPS-7, dysbindin, interacts with pallidin suggesting that dys-

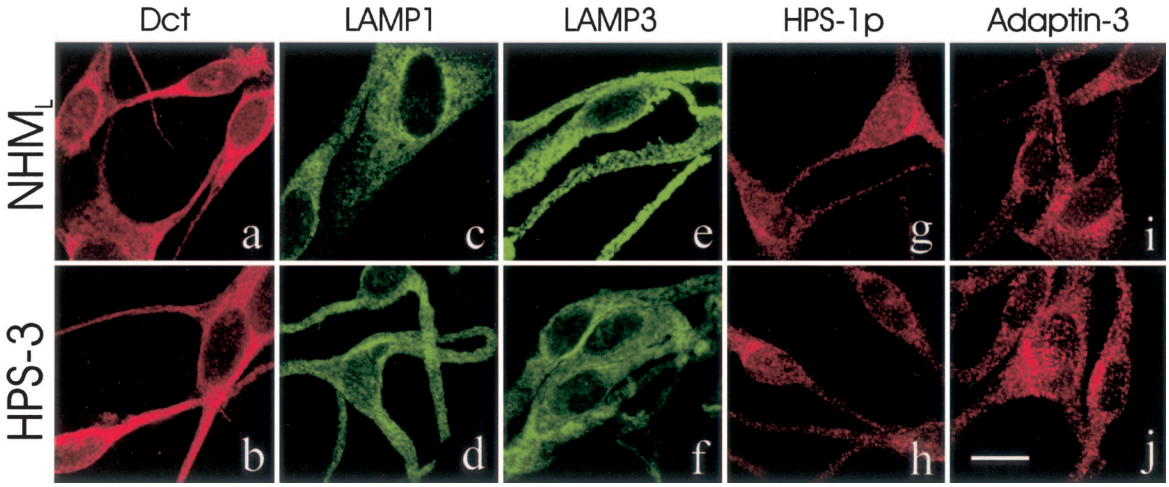

Figure 6. HPS-3 melanocytes exhibit a staining pattern distinct from normal human melanocytes (NHM$_L$) for Dct and LAMP 1 and 3 but not for the HPS1 protein or adaptin-3. Normal and HPS-3 melanocytes were immunolabeled for Dct, LAMP1, LAMP2, HPS1 protein, and adaptin-3 as described in Materials and Methods. Dct (**b** versus **a**), LAMP1 (**d** versus **c**), and LAMP3 (**f** versus **e**) exhibit a finer, more floccular staining pattern in HPS-3 melanocytes, as opposed to the granular pattern in NHM. **Bar**, 10 μm.

bindin co-resides with BLOC-1.[8] Similarly, the HPS1 and HPS4 proteins were demonstrated to interact transiently or indirectly in a BLOC-3 complex.[42] Sedimentation-velocity and co-immunoprecipitation experiments[43,44] have confirmed the interaction between HPS1 and HPS4 in BLOC-3. The HPS5 and HPS6 proteins have been shown to interact and comprise BLOC-2,[13] which may include the HPS3 protein.[45,46] It is postulated that these BLOC modules facilitate formation of cargo vesicles at donor sites, translocation of cargo vesicles through the cytoplasm, and the ultimate recognition/docking/fusing of cargo vesicles with receptor organelles.

Recently, the HPS3 protein was shown to co-immunoprecipitate with the HPS5 and the HPS6 proteins suggesting that it is a component of the 350-kd BLOC-2.[45,46] In addition, the HPS3 protein contains a putative clathrin-binding domain at residues 172–176 consisting of LL-DFE[25,26] that is essential for the accurate localization of LAMP1 in fibroblasts.[47] Therefore, BLOC-2 and its association with clathrin may be necessary for the efficient incorporation of melanogenic cargo targeted to the melanosome. We have recently demonstrated that early endosomes and lysosomes appeared clustered in the perinuclear region of HPS-3 fibroblasts and did not extend into the periphery of the cells as in normal fibroblasts.[47] In addition, we demonstrate herein that melanosome-targeted cargo does not efficiently become incorporated into melanosomes in the perinuclear region but are retained in 50-nm vesicles that extend into the periphery of the HPS-3 melanocyte. Preliminary localization of the HPS3 protein demonstrates that it exists predominantly in the perinuclear area, positioning it at sites that regulate endosome/lysosome translocation and melanosome cargo trafficking (R. Boissy, personal observation).

The morphological aberration in the trafficking of melanocyte-specific proteins in HPS-3 melanocytes is distinct from that observed in HPS-1 and HPS-2 melanocytes. HPS-1 melanocytes are characterized by unique large, membranous complexes containing tyrosinase, Tyrp1, and LAMP3.[29] HPS-2 melanocytes are characterized by the containment of tyrosinase within large vesicles resembling endosome/multi-vesicular-sorting bodies while Tyrp1 is normally trafficked to the melanosome.[48] The distinct morphological manifestations in melanocytes of HPS 1–3 suggest that the respective proteins regulate different sites in the trafficking pathway of cargo from the Golgi to the melanosome.[49] This is consistent with the molecular distinction of these proteins in that HPS1 is a component of BLOC-3, the HPS2 is a component of the adaptin-3 complex, and HPS3 is a component of BLOC-2. The specific function these distinct complexes serve in regulating in cellular trafficking remains to be resolved.

References

1. Hermansky F, Pudlak P: Albinism associated with hemorrhagic diathesis and unusual pigmented reticular cells in the bone marrow: report of two cases with histochemical studies. Blood 1959, 14:162–169
2. Huizing M, Boissy RE, Gahl WA: Hermansky-Pudlak syndrome: vesicle formation from yeast to man. Pigment Cell Res 2002, 15:405–419
3. King RA, Hearing VJ, Creel DJ, Oetting WS: Albinism. The Metabolic and Molecular Bases of Inherited Disease. Edited by Scriver CR, Beaudet AL, Valle DL, Sly WS. New York, McGraw-Hill, 2001, pp 5587–5627
4. Witkop CJJ, Krumwiede M, Sedano H, White JG: Reliability of absent platelet dense bodies as a diagnostic criterion for Hermansky-Pudlak syndrome. Am J Hematol 1987, 26:305–311
5. Gahl WA, Brantly M, Kaiser-Kupfer MI, Iwata F, Hazelwood S, Shotelersuk V, Duffy LF, Kuehl EM, Troendle I, Bernardini I: Genetic defects and clinical characteristics of patients with a form of oculocutaneous albinism (Hermansky-Pudlak syndrome). N Engl J Med 1998, 338:1258–1264
6. Bednar B, Hermansky F, Lojda Z: Vascular pseudohemophilia associated with ceroid pigmentophagia in albinos. Am J Pathol 1964, 45:283–294
7. Schinella RA, Greco MA, Cobert BL, Denmark LW, Cox RP: Hermansky-Pudlak syndrome with granulomatous colitis. Ann Intern Med 1980, 92:20–23
8. Li W, Zhang Q, Oiso N, Novak EK, Gautam R, O'Brien EP, Tinsley CL, Blake DJ, Spritz RA, Copeland NG, Jenkins NA, Amato D, Roe BA, Starcevic M, Dell'Angelica EC, Elliott RW, Mishra V, Kingsmore SF, Paylor RE, Swank RT: Hermansky-Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). Nat Genet 2003, 35: 84–89
9. Oh J, Bailin T, Fukai K, Feng GH, Ho L, Mao J, Frenk E, Tamura N, Spritz RA: Positional cloning of a gene for Hermansky-Pudlak syndrome, a disorder of cytoplasmic organelles. Nat Genet 1996, 14: 300–306
10. Dell'Angelica EC, Aguilar RC, Wolins N, Hazelwood S, Gahl WA, Bonifacino JS: Molecular characterization of the protein encoded by the Hermansky-Pudlak syndrome type 1 gene. J Biol Chem 2000, 275:1300–1306
11. Suzuki T, Li W, Zhang Q, Karim A, Novak EK, Sviderskaya EV, Hill SP, Bennett DC, Levin AV, Nieuwenhius HK, Fong CT, Castellan C, Miterski B, Swank RT, Spritz RA: Hermansky-Pudlak syndrome is caused by mutations in HPS4, the human homolog of the mouse light-ear gene. Nat Genet 2002, 30:321–324
12. Anderson PD, Huizing M, Claassen DA, White J, Gahl WA: Hermansky-Pudlak syndrome type 4 (HPS-4): clinical and molecular characteristics. Hum Genet 2003, 113:10–17
13. Zhang Q, Zhao B, Li W, Oiso N, Novak EK, Rusiniak ME, Gautam R, Chintala S, O'Brien EP, Zhang Y, Roe BA, Elliott RW, Eicher EM, Liang P, Kratz C, Legius E, Spritz RA, O'Sullivan TN, Copeland NG, Jenkins NA, Swank RT: Ru2 and Ru encode mouse orthologs of the genes mutated in human Hermansky-Pudlak syndrome types 5 and 6. Nat Genet 2003, 33:145–153
14. Dell'Angelica EC, Shotelersuk V, Aguilar RC, Gahl WA, Bonifacino JS: Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the β3A subunit of the AP-3 adaptor. Mol Cell 1999, 3:11–21
15. Huizing M, Scher CD, Strovel E, Fitzpatrick DL, Hartnell L, Anikster Y, Gahl WA: Nonsense mutations in ADTB3A cause complete deficiency of the β3A subunit of adaptor complex-3 and severe Hermansky-Pudlak syndrome type 2. Pediatr Res 2001, 51:150–158
16. Robinson MS, Bonifacino JS: Adaptor-related proteins. Curr Opin Cell Biol 2001, 13:444–453
17. Swank RT, Novak EK, McGarry MP, Rusiniak ME, Feng L: Mouse models of Hermansky-Pudlak syndrome: a review. Pigment Cell Res 1998, 11:60–80
18. Kantheti P, Qiao X, Diaz M, Peden AA, Meyer GE, Carskadon SL, Kapfhamer D, Sufalko D, Robinson MS, Noebels JL, Burmeister M: Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. Neuron 1998, 21:111–122
19. Feng L, Seymour AB, Jiang S, To A, Peden AA, Novak EK, Zhen L, Rusiniak ME, Eicher EM, Robinson MS, Gorin MB, Swank RT: The β3A subunit gene (Ap3b1) of the AP-3 adaptor complex is altered in the mouse hypopigmentation mutant pearl, a model for Hermansky Pudlak syndrome and night blindness. Hum Mol Genet 1999, 8:323–330
20. Wilson S, Yip R, Swing DA, O'Sullivan N, Zhang Y, Novak ED, Swank RT, Russell LB, Copeland NG, Jenkins NA: A mutation in Rab27a

causes the vesicle transport defects observed in ashen mice. Proc Natl Acad Sci USA 2000, 97:7933–7938
21. Suzuki T, Oiso N, Gautam R, Novak EK, Panthier JJ, Suprabha PG, Vida T, Swank RT, Spritz RA: The mouse organellar biogenesis mutant buff results from a mutation in Vps33a, a homologue of yeast vps33 and Drosophila carnation. Proc Natl Acad Sci USA 2003, 100:1146–1150
22. Detter JC, Zhang Q, Mules EH, Novak EK, Mishra VS, Li W, McMurtrie EB, Tchernev VT, Wallace MR, Seabra MC, Swank RT, Kingsmore SF: Rab geranylgeranyl transferase alpha mutation in the gunmetal mouse reduces Rab prenylation and platelet synthesis. Proc Natl Acad Sci USA 2000, 97:4144–4149
23. Wildenberg SC, Oetting WS, Almod¢var C, Krumwiede M, White JG, King RA: A gene causing Hermansky-Pudlak syndrome in a Puerto Rican population maps to chromosome 10q2. Am J Hum Genet 1995, 57:755–765
24. Fukai K, Oh J, Frenk E, Almodovar C, Spritz RA: Linkage disequilibrium mapping of the gene for Hermansky-Pudlak syndrome to chromosome 10q23.1-q23.3. Hum Mol Genet 1995, 4:1665–1669
25. Anikster Y, Huizing M, White J, Bale S, Gahl WA, Toro J: Mutation of a new gene causes a unique form of Hermansky-Pudlak syndrome in genetic isolate of central Puerto Rico. Nat Genet 2001, 28:376–380
26. Suzuki T, Li W, Zhang Q, Novak EK, Sviderskaya EV, Wilson A, Bennett DC, Roe BA, Swank RT, Spritz RA: The gene mutated in cocoa mice, carrying a defect of organelle biogenesis, is a homologue of the human Hermansky-Pudlak syndrome-3 gene. Genomics 2001, 78:30–37
27. Huizing M, Anikster Y, Fitzpatrick DL, Jeong AB, D'Souza M, Rausche M, Kaiser-Kupfer MI, White JG, Gahl WA: Hermansky-Pudlak syndrome type 3 in Ashkenazi Jews and other non-Puerto Rican patients with hypopigmentation and platelet storage pool deficiency. Am J Hum Genet 2001, 69:1022–1032
28. Shotelersuk V, Hazelwood S, Larson D, Iwata F, Kaiser-Kupfer MI, Kuehl E, Bernardini I, Gahl WA: Three new mutations in a gene causing Hermansky-Pudlak syndrome: clinical correlations. Mol Genet Metab 1998, 64:99–107
29. Boissy RE, Zhao Y, Gahl WA: Altered protein localization in melanocytes from Hermansky-Pudlak syndrome: support for the role of the HPS gene product in intracellular trafficking. Lab Invest 1998, 78: 1037–1048
30. Medrano EE, Nordlund JJ: Successful culture of adult human melanocytes from normal and vitiligo donors. J Invest Dermatol 1990, 95:441–445
31. Halaban R, Alfano FD: Selective elimination of fibroblasts from cultures of normal human melanocytes. In Vitro Cell Dev Biol 1984, 20:447–450
32. Abdel-Malek Z, Swope VB, Pallas J, Krug K, Nordlund JJ: Mitogenic, melanogenic, and cAMP responses of cultured neonatal human melanocytes to commonly used mitogens. J Cell Physiol 1992, 150:416–425
33. Zhao H, Boissy Y, Abdel-Malek Z, King RA, Nordlund JJ, Boissy RE: On the analysis of the pathophysiology of Chediak-Higashi syndrome: defects expressed by cultured melanocytes. Lab Invest 1994, 71:25–34
34. Zhao H, Boissy RE: Distinguishing between the catalytic potential and apparent expression of tyrosinase activities. Am J Med Sci 1994, 308:322–330
35. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 1965, 27:137 (Abstract)
36. Karnovsky MJ: Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. J Cell Biol 1971, 51:146 (Abstract)
37. Virador VM, Muller J, Wu X, Abdel-Malek ZA, Yu Z-X, Ferrans VJ, Kobayashi N, Wakamatsu K, Ito S, Hammer JA, Hearing VJ: Influence of α-melanocyte-stimulating hormone and of ultraviolet radiation on the transfer of melanosomes to keratinocytes. FASEB J 2001, 16:105–107
38. Ciciotte SL, Gwynn B, Moriyama K, Huizing M, Gahl WA, Bonifacino JS, Peters LL: Cappuccino, a mouse model of Hermansky-Pudlak syndrome, encodes a novel protein that is part of the pallidin-muted complex (BLOC-1). Blood 2003, 101:4402–4407
39. Huang L, Kuo YM, Gitschier J: The pallid gene encodes a novel, syntaxin 13-interacting protein involved in platelet storage pool deficiency. Nat Genet 1999, 23:329–332
40. Moriyama K, Bonifacino JS: Pallidin is a component of a multi-protein complex involved in the biogenesis of lysosome-related organelles. Traffic 2002, 3:666–677
41. Falcon-Perez JM, Starcevic M, Gautam R, Dell'Angelica EC: BLOC-1, a novel complex containing the pallidin and muted proteins involved in the biogenesis of melanosomes and platelet-dense granules. J Biol Chem 2002, 277:28191–28199
42. Chiang PW, Oiso N, Gautam R, Suzuki T, Swank RT, Spritz RA: The Hermansky-Pudlak syndrome 1 (HPS1) and HPS4 proteins are components of two complexes, BLOC-3 and BLOC-4, involved in the biogenesis of lysosome-related organelles. J Biol Chem 2003, 278: 20332–20337
43. Martina JA, Moriyama K, Bonifacino JS: BLOC-3, a protein complex containing the Hermansky-Pudlak syndrome gene products HPS1 and HPS4. J Biol Chem 2003, 278:29376–29384
44. Nazarian R, Falcon-Perez JM, Dell'Angelica EC: Biogenesis of lysosome-related organelles complex 3 (BLOC-3): a complex containing the Hermansky-Pudlak syndrome (HPS) proteins HPS1 and HPS4. Proc Natl Acad Sci USA 2003, 100:8770–8775
45. Gautam R, Chintala S, Li W, Zhang Q, Tan J, Novak EK, Di Pietro SM, Dell'Angelica EC, Swank RT: The Hermansky-Pudlak syndrome 3 (cocoa) protein is a component of the biogenesis of lysosome-related organelles complex-2 (BLOC-2). J Biol Chem 2004, 279:12935–12942
46. Di Pietro SM, Falcon-Perez JM, Dell'Angelica EC: Characterization of BLOC-2, a complex containing the Hermansky-Pudlak syndrome proteins HPS3, HPS5, and HPS6. Traffic 2004, 5:276–283
47. Helip-Wooley A, Westbroek W, Dorward H, Mommaas M, Boissy R, Gahl WA, Huizing M: Hermansky Pudlak syndrome type 3 protein interacts with clathrin and functions in trafficking of lysosome-related organelles. Traffic, in press
48. Huizing M, Sarangarajan R, Strovel E, Zhao Y, Gahl WA, Boissy RE: AP-3 mediates tyrosinase but not TRP-1 trafficking in human melanocytes. Mol Biol Cell 2001, 12:2075–2085
49. Richmond B, Huizing M, Knapp J, Koshoffer A, Zhao Y, Morris R, Gahl WA, Boissy RE: Melanocytes from Hermansky-Pudlak syndrome types 1–3 express distinct defects in cargo trafficking. J Invest Dermatol 2004, DOI: 10.111/j.0022-202x, 2004. 23585.x