Dual Loss of ER Export and Endocytic Signals with Altered Melanosome Morphology in the *silver* Mutation of Pmel17[□]

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Pmel17 is a pigment cell-specific integral membrane protein that participates in the formation of the intraluminal fibrils upon which melanins are deposited in melanosomes. The Pmel17 cytoplasmic domain is truncated by the mouse *silver* mutation, which is associated with coat hypopigmentation in certain strain backgrounds. Here, we show that the truncation interferes with at least two steps in Pmel17 intracellular transport, resulting in defects in melanosome biogenesis. Human Pmel17 engineered with the truncation found in the mouse *silver* mutant (hPmel17si) is inefficiently exported from the endoplasmic reticulum (ER). Localization and metabolic pulse-chase analyses with site-directed mutants and chimeric proteins show that this effect is due to the loss of a conserved C-terminal valine that serves as an ER exit signal. hPmel17si that exits the ER accumulates abnormally at the plasma membrane due to the loss of a di-leucine-based endocytic signal. The combined effects of reduced ER export and endocytosis significantly deplete Pmel17 within endocytic compartments and delay proteolytic maturation required for premelanosome-like fibrillogenesis. The ER export delay and cell surface retention are also observed for endogenous Pmel17si in melanocytes from *silver* mice, within which Pmel17 accumulation in premelanosomes is dramatically reduced. Mature melanosomes in these cells are larger, rounder, more highly pigmented, and less striated than in control melanocytes. These data reveal a dual sorting defect in a natural mutant of Pmel17 and support a requirement of endocytic trafficking in Pmel17 fibril formation.

INTRODUCTION

Melanins are complex pigments synthesized by melanocytes and eye pigment epithelia in mammals. Because of the potentially toxic nature of melanin intermediates, melanin biosynthesis and storage are sequestered within membrane-bound organelles called melanosomes (Marks and Seabra, 2001). The synthesis of melanins and the formation of melanosomes are exquisitely controlled by a host of factors, many of which have been identified as targets of genetic mutations in mouse strains with coat color dilution due to altered pigmentation (Hearing, 2000; Bennett and Lamoreux, 2003). Some of these strains have pleiotropic phenotypes in which

multiple tissue types are affected because of malformation of other lysosome-related organelles (Bonifacino, 2004; Li *et al.*, 2004; Di Pietro and Dell'Angelica, 2005), whereas others are due to defects in pigment cell-specific components thought to be involved only in melanin synthesis or melanosome architecture (Oetting and King, 1999). Analysis of the products encoded by the normal and mutant genes from these loci and the specific defects observed in mutant pigment cells provide important clues to the function of the respective genes or the mechanisms by which their activity or localization are regulated.

silver was characterized as a recessive mouse mutant with progressive coat color dilution on certain backgrounds (Dunn and Thigpen, 1930). Hair follicle melanocytes in affected *silver* mice are depleted with age (Quevedo *et al.*, 1981), suggesting a role for the corresponding gene product in melanocyte viability. An effect of the *silver* mutation on melanocyte health and viability is consistent with the prolonged doubling times of immortalized melanocytes from *silver* mice (Spanakis *et al.*, 1992). The *Si* locus, defective in *silver* mice, encodes Pmel17 (Pmel; also known as gp100, ME20, gp85 and Silver), a pigment cell-specific matrix protein present in melanosomes (Theos *et al.*, 2005). Pmel is a major structural and biogenetic component of the fibrillar structures within melanosome precursors upon which melanins are deposited as they are synthesized (Berson *et al.*,

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Abbreviations used: endoH, endoglycosidase H; ER, endoplasmic reticulum; hPmel, human Pmel17; IEM, immunoelectron microscopy; IFM, immunofluorescence microscopy; mPmel, mouse Pmel17; PE, phycoerythrin; Pmel, Pmel17; WT, wild type.

2001, 2003; Raposo *et al.*, 2001) and may interact directly with melanin intermediates (Donatien and Orlow, 1995; Chakraborty *et al.*, 1996; Lee *et al.*, 1996; Fowler *et al.*, 2006). Pmel is synthesized as a type I transmembrane protein with a ~600-residue luminal domain, a single membrane-spanning domain, and a 45-residue cytoplasmic domain (Kwon *et al.*, 1987; Adema *et al.*, 1994). Proteolytic cleavage by a proprotein convertase within the luminal domain liberates a large fibrillogenic fragment within melanosome precursors that is incorporated into and required for the formation of the striated fibrils (Berson *et al.*, 2003). Subsequent cleavage events may occur within maturing melanosomes (Kushimoto *et al.*, 2001; Yasumoto *et al.*, 2004). Although Pmel has been proposed to be targeted directly to premelanosomes from smooth endoplasmic reticulum (ER; Kushimoto *et al.*, 2001; Basrur *et al.*, 2003; Yasumoto *et al.*, 2004), abundant evidence indicates that Pmel accesses premelanosomes via endocytic compartments (Berson *et al.*, 2001, 2003; Raposo *et al.*, 2001; Salas-Cortes *et al.*, 2005; Theos *et al.*, 2006; reviewed in Theos *et al.*, 2005). Interestingly, Pmel expressed in nonpigment cells induces the formation of melanosome-like fibrils within late endocytic structures (Berson *et al.*, 2001), requiring transport through multivesicular intermediates (Theos *et al.*, 2006), and the fibrillogenic fragment *in vitro* forms fibrillar structures with features of amyloid (Fowler *et al.*, 2006). These data suggest that Pmel may be the sole melanocyte-specific structural component of the fibrils. Consistent with a critical role in pigment regulation, mutations in the gene encoding Pmel in chicken (Kerje *et al.*, 2004), zebrafish (Schonthaler *et al.*, 2005), and dog (Clark *et al.*, 2006) result in hypopigmentation in the skin and eyes. It has not yet been determined whether the hypopigmentation in these animal models results from an intrinsic failure to generate pigment in melanocytes, melanocyte loss as observed in *silver* mice, or to other defects such as in transfer of melanosomes to keratinocytes.

The mutant *Si* gene of *silver* mice (*Si^{si}*) encodes a truncated Pmel product with a deletion of the C-terminal 25 residues of the cytoplasmic domain (Martínez-Esparza *et al.*, 1999; Solano *et al.*, 2000). It is not known how this mutation affects Pmel activity or localization; indeed, we have shown that post-endocytic sorting of Pmel to multivesicular endosomes is independent of the cytoplasmic domain (Theos *et al.*, 2006). Here we show that the *silver* mutation impedes two sorting steps in the intracellular itinerary of Pmel: early biosynthetic transport from the ER to the Golgi and internalization from the plasma membrane. As a consequence, Pmel accumulation within premelanosomes is significantly decreased, with concomitant alterations in melanosome morphology including reduced striations and loss of shape. Nevertheless, pigment continues to deposit within melanosomes in *silver* melanocytes, such that the melanocytes themselves are fully or even hyperpigmented. The data demonstrate how a natural mutation can alter multiple transport steps of an integral membrane protein and illuminate an important role for endosomes and ER exit in Pmel function and for the premelanosome fibrils in melanocyte survival.

MATERIALS AND METHODS

Cell Culture and Transfection

Melan-si-1 (Spanakis *et al.*, 1992) and melan-a (Bennett *et al.*, 1987) cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 0.1 mM 2-mercaptoethanol, 200 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) and antibiotics. Melan-si-1 cells (genotype *Si^{si}/Si^{si}*, *Tyrp1^b/+*, C57Bl/6J) were occasionally passaged on feeder layers of mitomycin C-treated XB2 keratinocyte cells as described (Spanakis *et al.*, 1992), but all experiments shown were done on cells passaged at least once in the absence of feeders. Melanocyte

lines melan-si-3, -4, and -5 were grown from skins of separate neonatal *Si^{si}/Si^{si}*, Ink4a-Arf $-/-$ C57Bl/6J mice (*Tyrp1* $+/+$). The Ink4a-Arf null phenotype yields primary melanocytes that are immediately immortal (Sviderskaya *et al.*, 2002) (and <http://www.sgul.ac.uk/depts/anatomy/pages/protocols/msmelpri.html>). HeLa cells were cultured as described (Berson *et al.*, 2001) and transfected using FuGene6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions with 2 μ g of total plasmid DNA. Expression levels were varied by modifying the amount of specific DNA (50 ng to 1 μ g) relative to "carrier" (empty vector). Cells were analyzed 1–2 d after transfection.

Antibodies

Anti-Pmel mAbs HMB-45, HMB-50, and NKI-beteb (Esclamado *et al.*, 1986) were obtained from Lab Vision (Fremont, CA). Rabbit antibody α Pep13h to the human Pmel cytoplasmic domain has been described (Berson *et al.*, 2001). Rabbit antiserum α mPmel-N was generated by Genemed Synthesis (South San Francisco, CA) to a synthetic peptide (CEGSRNQDWLGVPRLVTK-CO2H) corresponding to the predicted mouse Pmel17 NH2 terminus (residues 25–42) with an appended N-terminal cysteine residue for conjugation to keyhole limpet hemocyanin and affinity-purified on peptide coupled to SulfoLink beads (Pierce Chemical). mAbs H4A3 to human LAMP-1 was from Developmental Studies Hybridoma Bank (Iowa City, IA), 7G7.B6 to Tac and TA99 (Mel-5) to *Tyrp1* were from American Type Culture Collection (Manassas, VA), C8.B6 to calnexin was from Chemicon (Temecula, CA), and YL1/2 to tubulin was from Serotec (Raleigh, NC). Fluorochrome-conjugated species- and isotype-specific secondary antibodies were obtained from Molecular Probes (Eugene, OR), Southern Biotechnology (Birmingham, AL) or Jackson ImmunoResearch (West Grove, PA).

Plasmids

The plasmid encoding hPmel in the pCI vector has been previously described (Berson *et al.*, 2001). hPmel^{si} was generated by one-step PCR amplification using a 3' primer antiparallel to the coding region for amino acids H⁶³⁹–H⁶⁴³ of hPmel with a stop codon and XbaI site appended, a 5' primer upstream of a unique BglII site, and subcloning of the BglII-XbaI fragment into the corresponding site of pCDM8.1-hPmel17 (Berson *et al.*, 2001). The insert was subsequently subcloned into pCI using unique SalI-XbaI sites. hPmel(V668D) and hPmel^{si}(H643V) were similarly generated by one-step PCR mutagenesis in which the C-terminal codon of the relevant construct was altered (V668D and H643V), and inserted into pCI-hPmel17 using unique BstXI and NotI sites. hPmel[LL>AA] was generated by site-directed mutagenesis using the GeneEditor *in vitro* Mutagenesis System (Promega, Madison, WI) according to the manufacturer's instructions. TTP was generated by two-step PCR using pCDM8.1-TTMB (Marks *et al.*, 1995) and pCI-hPmel17 as templates, such that a fragment encoding the Tac transmembrane and Pmel cytoplasmic domains was subcloned into the BglII-XbaI sites of pCDM8.1-TTMB as described (Marks *et al.*, 1995). TTP^{si} was similarly generated using the same 3' primer as for hPmel^{si}. TTP and TTP^{si} inserts were subcloned into pCI using EcoRI and XbaI sites. Sequences of all PCR-generated fragments and of junctions of all subcloned fragments were verified by automated sequencing using the University of Pennsylvania Cell Center core facility. Details of the sequence and PCR reactions will be provided upon request.

Immunofluorescence Microscopy and Antibody Uptake

Cells were fixed with 2% formaldehyde/phosphate-buffered saline (PBS) for 15–30 min at RT, washed twice with PBS, and then permeabilized with 0.2% saponin and labeled with primary and fluorochrome-conjugated secondary antibodies as described (Marks *et al.*, 1995). In some experiments, cells were labeled with primary antibodies on ice and washed twice with ice-cold PBS before fixation and processing with secondary antibodies. For antibody uptake, cells were incubated for 40 min at 4°C with primary antibody, warmed to 37°C for 10 min, washed in medium, and then chased for 15 min at 37°C before fixation and processing with secondary antibodies. Cells were analyzed on a Leica DM IRBE microscope (Leica Microsystems, Bannockburn, IL) equipped with a Hamamatsu (Hamamatsu, Hamamatsu City, Japan) Orca digital camera, and images captured and manipulated using Improvision (Lexington, MA) OpenLab software. Most images shown were generated from sequential Z-series images captured at 0.2- μ m intervals and deconvolved using either the OpenLab Volume Deconvolution module or the Iterative Deconvolution module from Improvision Velocity software.

Metabolic Labeling, Immunoprecipitation, and Immunoblotting

Cells were metabolically pulse-labeled with [³⁵S]methionine/cysteine and chased as described (Marks *et al.*, 1995), using 15–30-min pulses and chase times as indicated. Fresh or frozen cell pellets were lysed in 1% (wt/vol) Triton X-100 (TX-100) for 15–30 min as described (Berson *et al.*, 2000), and lysates were clarified by centrifugation for 15–20 min at 20,000 $\times$ g. Immunoprecipitations, treatments with endoglycosidase H (endoH; New England Biolabs, Beverly, MA), fractionation of eluted proteins by SDS-PAGE on 10% polyacrylamide gels, and phosphorimaging analysis were performed as de-

Figure 1. Cytoplasmic truncation of Pmel17 is inefficiently exported from the ER. (A) Schematic diagram of the luminal, transmembrane (tm) and cytoplasmic (cyt) domains of hPmel. Primary sequences of cytoplasmic domains of WT hPmel (WT), hPmel17si (si), hPmel[LL>AA] (LL>AA), hPmel17(H643V), and hPmel17(V668D) are shown. Di-leucine and C-terminal valine residues are underlined and substitutions are highlighted in red. (B–K) IFM analysis of HeLa cells expressing WT hPmel and hPmel17si at 24 h (B–G) and 48 h (H–K) after transfection and colabeled for Pmel (with HMB-50; B, E, and H) and for LAMP-1 (C and J), or calnexin (F). (D, G, and K) Merged images. Insets in D and K, 2.5× magnification of the indicated regions. Structures colabeled for LAMP-1 and WT Pmel (D, arrowheads) or hPmel17si (K, arrows) are indicated. Bar, 20 μm.

scribed (Berson *et al.*, 2000). Immunoblotting using whole cell lysates prepared with 1% SDS were as described (Berson *et al.*, 2000) using 10% PAGE/5% methanol transfer buffer. TX-100-insoluble pellets were resolubilized in 0.5% SDS, 1% 2-mercaptoethanol before loading. Immobilon-P membranes (Millipore, Billerica, MA) with transferred proteins were probed with indicated antibodies, and bands were detected with alkaline-phosphatase-conjugated goat anti-rabbit, anti-mouse, or anti-rat immunoglobulin (Ig), ECF and phosphorimaging analysis using a Molecular Dynamics STORM 860 (Sunnyvale, CA) and Imagequest software (Amersham Biosciences, Piscataway, NJ).

Electron Microscopy

For immunoelectron microscopy (IEM), cells were fixed with 2% (wt/vol) paraformaldehyde/0.1% (wt/vol) glutaraldehyde in 0.1M phosphate buffer, and immunogold labeling of ultrathin cryosections was performed as described (Raposo *et al.*, 1997, 2001) using protein A conjugated to 10- or 15-nm gold particles (PAG-10 or -15). Sections were observed and photographed under a Philips CM120 Electron Microscope (FEI Company, Eindhoven, The Netherlands). For conventional electron microscopy, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 90 min, post-fixed with 2% OsO₄, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were counterstained with uranyl acetate before observation.

Flow Cytometry

Flow cytometry was performed essentially as described (Marks *et al.*, 1996). Briefly, mouse melanocytes or transfected HeLa cells were harvested by treatment with PBS/5 mM EDTA for 5–10 min at 37°C and then washed in ice-cold medium. Cells were then resuspended in 100 μl of primary antibody diluted in FACS buffer (PBS/5 mM EDTA/0.1% BSA) for 30–60 min on ice.

Cells were then washed twice with ice-cold FACS buffer and incubated with phycoerythrin (PE)-conjugated anti-rabbit or anti-mouse IgG in FACS buffer for 30 min on ice. Cells were then washed twice and analyzed immediately on a FACScan using CellQuest software (BD Biosciences Immunocytometry Systems, San Jose, CA). For transfected HeLa cells, analyses focused on cells gated for EGFP expression at comparable levels; cells lacking EGFP expression served as negative controls.

RESULTS

Mislocalization of Pmel with the silver Mutation to the ER and Plasma Membrane

We have extensively characterized the biosynthesis and intracellular trafficking of human Pmel in human melanocytic cells and transfected HeLa cells (Berson *et al.*, 2001, 2003; Nichols *et al.*, 2003). To take advantage of reagents specific for human Pmel (hPmel), we reproduced the *silver* mutation in the context of hPmel and studied its biosynthetic transport in transfected HeLa cells. To this end, a stop codon was engineered following the codon for His⁶⁴³ in hPmel (Figure 1A), analogous to His⁶⁰¹ in mouse Pmel (mPmel) after which a stop codon is inserted by the *silver* mutation, to generate hPmel17si. HeLa cells transduced with wild-type (WT) hPmel or hPmel17si were first analyzed by indirect immunofluorescence microscopy (IFM; Figure 1, B–K) using antibody

Figure 2. hPmel17-si accumulates on the cell surface and can access MVBs in transfected HeLa cells. Ultrathin cryosections of HeLa cells expressing (A and B) WT hPmel or (C–E) hPmelsi were immunogold labeled with HMB-50 (PAG-10). Cells were analyzed 48 h (A–C, and E) or 24 h (D) after transfection. WT hPmel is detected nearly exclusively on internal membranes of multivesicular bodies (MVB; A and B) and dense fibrillar structures within them (arrows, B). Labeling for hPmelsi is found occasionally on internal membranes of multivesicular bodies (MVB; arrows, C) and more consistently on ER membranes (D) or at the plasma membrane (PM; arrowheads, E). Bars, 200 nm.

HMB-50, which recognizes an epitope within amino acid residues 236 and 297 in the Pmel luminal domain (DCM, ACT, and MSM, unpublished results). As previously shown (Berson *et al.*, 2001), WT hPmel localized to discrete puncta throughout the cell that were completely coincident with a subset of LAMP-1-containing compartments (Figure 1, B–D); only in cells with massively high overexpression was cell surface labeling also observed (unpublished data). The same pattern was observed regardless of the time after transfection. By contrast, 24 h after transfection, hPmelsi localized predominantly to a reticular network and the nuclear enve-

lope (Figure 1, E–G). The reticular pattern overlapped with labeling for calnexin, a resident ER protein, indicating that hPmelsi was predominantly localized to the ER. Additional labeling was observed in discrete puncta and at the cell surface (faint cell surface labeling is more evident by IFM of cells labeled without saponin permeabilization and by IEM; see Figure 2 below); the punctate and cell surface labeling became more apparent by 48 h after transfection, and the ER labeling often became relatively more muted (Figure 1, H–K). The puncta largely colocalized with a fraction of LAMP-1-containing late endosomes (Figure 1K), indicating

that the defect in localization imparted by the *silver* mutation is not absolute. Together, these data show that hPmelsi is largely confined to the ER at early time points, suggesting inefficient ER exit, with a secondary aberrant localization to the plasma membrane.

To confirm these data, we analyzed the cells by IEM. Ultrathin cryosections of transfected HeLa cells were labeled with HMB-50 and 10-nm gold-labeled protein A. In cells expressing WT hPmel, labeling was observed primarily over the intraluminal vesicles of multivesicular late endosomes and over fibrillar structures that formed within these organelles (Berson *et al.*, 2001, 2003; Figure 2, A and B). By contrast, in cells expressing hPmelsi, labeling was detected in the ER (Figure 2D) and additionally at the plasma membrane (Figure 2E), with only a small fraction of gold particles localizing to multivesicular endosomes (Figure 2C). Fibrils were not observed. Consistent with IFM results, ER labeling was predominant early after transfection and in cells expressing low levels of hPmelsi (Figure 2D), whereas plasma membrane labeling was particularly evident in cells expressing high levels of hPmelsi and in all cells at later times after transfection (Figure 2E). These results are consistent with a primary defect in ER exit and a secondary defect in transport from the plasma membrane. Interestingly, the low level of labeling observed over the intraluminal vesicles of multivesicular structures suggests that the fraction of hPmelsi that accesses the endocytic pathway localizes appropriately in HeLa cells to multivesicular endosomes. This is consistent with a function for the luminal domain in facilitating the partitioning of Pmel to multivesicular endosomal domains (Theos *et al.*, 2006).

Inefficient Maturation of Pmel17 with the *silver* Mutation

To determine if the ER localization of hPmelsi reflected a defect in maturation, we analyzed the biosynthetic processing of hPmel and hPmelsi expressed in HeLa cells by metabolic pulse-chase and immunoprecipitation. Cells were labeled for 30 min with [³⁵S]methionine/cysteine and chased for various periods of time, and then cell lysates were immunoprecipitated with the anti-Pmel antibody HMB-50. Immunoprecipitates were left untreated or treated with endoH, which releases only high-mannose N-linked oligosaccharides that have not been modified by medial Golgi oligosaccharide transferases, and then analyzed by SDS-PAGE and phosphorimaging. As observed previously (Berson *et al.*, 2001), WT hPmel after the pulse appeared as a single P1 precursor that was fully susceptible to digestion by endoH (Figure 3A, lanes 1 and 2). After 1 h of chase, P1 is partially converted to a slower migrating, endoH-resistant P2, representing the full-length, Golgi-modified precursor and to the faster migrating M α and M β , which represent the products of proprotein convertase cleavage of P2 (Berson *et al.*, 2001, 2003; Figure 3A, lanes 3 and 4). By 4 h of chase, most of the material had disappeared from the detergent lysates primarily because of insolubility of the M α fragment upon fibril formation (Berson *et al.*, 2001, 2003; Figure 3A, lanes 7 and 8). Parallel analysis of hPmelsi revealed a significant delay in maturation from endoH-sensitive P1 to endoH-resistant P2 (Figure 3B). This led to an overall stabilization of hPmelsi relative to WT Pmel. The extent of the delay in maturation and of the stabilization of hPmelsi relative to WT hPmel17 (measured as the increase in the time at which half of the starting material became resistant to endoH) varied with each experiment from a minimum of twofold to a maximum in which essentially no maturation was observed (2 experiments of at least 10 are shown representative of an extreme phenotype in panel B and an intermediate phenotype in

Figure 3. Maturation of hPmel17-si is delayed compared with WT. (A and B) HeLa cells expressing WT hPmel (A) and hPmelsi (B) were metabolically pulse-labeled and chased as indicated. Pmel isoforms were immunoprecipitated from cell lysates with luminal-directed HMB-50 antibody, fractionated by SDS-PAGE with (+) or without (–) prior digestion with endoH, and analyzed by PhosphorImager. Top panels, regions surrounding P1, P2, and M α (arrows; see text); bottom panels, regions surrounding M β ; right, migration of molecular-weight markers. Note the appearance of M β after 1 h of chase for WT and its relative absence for hPmelsi. In this experiment, very little maturation of P1 to the Golgi-processed P2 isoform was observed for hPmelsi (representative of 6 independent experiments). (C and D) Similar to the experiment shown in A and B, except without EndoH treatment and using HeLa cells transfected with lower levels of WT hPmel and hPmelsi (low) as well as higher levels of hPmelsi (high). In this experiment, significant maturation of hPmelsi to the P2 isoform was observed at both low and high levels of expression, but was delayed compared with WT hPmel (representative of 5 independent experiments). (D) Overexposure of lanes in panel C (high*) containing hPmelsi expressed at high levels. The presence of products of proteolytic maturation, M β ^{si} and M α (arrowhead), are evident at later chase time points.

panel C). The variation was at least partially dependent on expression level (at high levels of expression, maturation of both WT Pmel and hPmelsi was slowed; Figure 3C) and minor variations in incubation temperature (maturation was slowed at 35°C relative to 37°C; unpublished data). These data support the localization of hPmelsi to the ER and indicate that the *silver* mutation results in a defect in ER exit.

Notably, the P2 form of hPmelsi that was observed during the chase persisted for long periods of time (Figure 3C). Moreover, generation of M α and M β was severely reduced in cells expressing hPmelsi relative to WT hPmel, indicating impaired proteolytic maturation (although prolonged exposure of the gels revealed faint bands corresponding to M α and the expected truncated M β ^{si}, indicating that some proteolytic processing occurred; Figure 3D, arrowhead). This will be discussed later.

The *silver* Mutation Deletes an ER Exit Signal from Pmel17

What is the basis for the defect in ER exit? The slowing of ER export at reduced temperatures relative to higher temperatures is less consistent with a folding defect and more consistent with a kinetic effect on ER-to-Golgi transport. One potential explanation is loss of an ER exit signal that can efficiently interact with the COPII coat complex, required for sorting cargo in the ER to Golgi-bound vesicles. To determine whether the *silver* mutation interfered with a cytoplasmic ER export signal, we tested whether the cytoplasmic deletion conferred delayed ER exit to a heterologous integral membrane protein. We generated chimeric proteins in which the luminal and transmembrane domains of a monomeric cell surface protein, Tac, were appended to the cytoplasmic domain of WT hPmel (to generate TTP) or hPmelsi (to generate TTPsi; Figure 4A). IFM analysis of HeLa cells expressing these chimeric proteins at low levels showed a modest accumulation of TTPsi in the ER, whereas TTP localized predominantly to the plasma membrane and endosomes (unpublished data).

Metabolic pulse-chase analyses of HeLa cells expressing these two chimeric proteins demonstrated a clear decrease in the rate of Golgi maturation of TTPsi relative to TTP, reflected by the increase in M_r of the Tac luminal domain upon processing of its N- and O-linked oligosaccharides

(Leonard *et al.*, 1984; Figure 4B). By these criteria, the half-time for maturation of TTP was 23 min compared with 60 min for TTPsi, representing a 2.6-fold decrease in the rate of ER exit imparted by the *silver* mutation. This delay in maturation is comparable to that observed in some experiments for hPmelsi and strongly supports the notion that the *silver* mutation eliminates an efficient ER exit signal. Consistent with this, appendage of the well-characterized di-acidic signal from the Vesicular Stomatitis Virus glycoprotein (Nishimura and Balch, 1997) to hPmelsi resulted in a modest—albeit heterogeneous—increase in vesicular localization at the expense of ER localization by IFM analysis (unpublished data).

What might the ER exit signal be? At the C-terminus of all sequenced Pmel orthologues is a valine residue (see Figure 1A); a C-terminal valine has been implicated in binding to COPII coats and facilitating ER exit of several cargo proteins (Briley *et al.*, 1997; Nakamura *et al.*, 1998; Urena *et al.*, 1999; Nufer *et al.*, 2002; Crambert *et al.*, 2004; Paulhe *et al.*, 2004). To determine whether loss of the C-terminal valine was responsible for the inefficient ER exit of hPmelsi, we generated two additional mutants (Figure 1A). In hPmelsi(H643V), the C-terminal histidine residue of hPmelsi was converted to a valine residue. In hPmel(V668D), the C-terminal valine residue of WT hPmel was converted to an aspartic acid residue,

Figure 5. Loss of the di-leucine motif is responsible for cell surface localization of hPmelsi. (A–J) IFM analysis of HeLa cells expressing hPmel WT (A–C), hPmel[LL>AA] (LLAA, D–F), and hPmelsi(H643V) (G–J). Cells were fixed and colabeled for Pmel (with HMB-50; A, D, G) and LAMP-1 (B, E, and H). Merged images are shown in C, F and J; insets, 2.5× magnification of indicated regions. Note that although hPmel[LL>AA] and hPmelsi(H643V) are observed at the cell surface, intracellular material localizes to LAMP-1-positive puncta like the WT protein (insets, arrows and arrowheads). Bar, 20 μ m.

shown to be unfavorable for efficient ER exit (Nufer *et al.*, 2002). HeLa cells expressing these variants were analyzed by IFM. As shown in Figure 4, C–E, the ER localization observed for hPmelsi was abolished in the H643V variant, suggesting that the C-terminal histidine was indeed responsible for ER retention of hPmelsi. By contrast, although essentially no WT hPmel localized to the ER at steady state, the V668D variant was ER localized to a similar degree as hPmelsi (Figure 4, F–H), suggesting that the C-terminal valine was required for efficient ER export of hPmel. In support of this conclusion, metabolic pulse-chase analyses indicate that the H643V mutation restores rapid ER export to hPmelsi, whereas the V668D mutation slows ER export of full-length hPmel to a similar degree as the *silver* mutation (Figure 4J). Taken together, these data indicate that the *silver* mutation abolishes a C-terminal valine-dependent ER exit signal.

The *silver* Mutation Deletes an Additional Internalization Signal

As noted in Figures 1 and 2, the *silver* mutation not only retards ER exit for hPmel, but also results in increased labeling at the plasma membrane. This plasma membrane labeling could potentially be due to loss of an endocytic signal. Indeed, a canonical acidic di-leucine-based E/DxxxLL endocytic motif (Bonifacino and Traub, 2003) is present within the region of the cytoplasmic domain eliminated by *silver* (Figure 1A). To determine if this motif was responsible for clearance of Pmel from the plasma membrane, we used site-directed mutagenesis to alter the two leucine residues to alanines to generate hPmel[LL>AA] (see Figure 1A). When

expressed in HeLa cells, hPmel[LL>AA] localized predominantly to vesicles that also contained LAMP-1, indicating proper localization to late endosomes like WT hPmel (Figure 5, A–F); this is consistent with the predominant role of a luminal determinant in localization to multivesicular endosomes (Theos *et al.*, 2006). However, unlike WT hPmel and like hPmelsi, significant labeling was also observed at the plasma membrane in most cells (Figure 5, D–F), even after permeabilization with saponin. This pattern of localization to both LAMP-1-positive compartments and the plasma membrane is identical to that observed for the H643V mutant, which rescued the ER exit phenotype of hPmelsi (Figure 5, G–J).

To quantitate the effect of the *silver* mutation and assess the influence of the ER exit and di-leucine-based endocytic signals on cell surface expression, the relative degree of cell surface labeling between the different constructs was quantitated by flow cytometry (Figure 6). HeLa cells coexpressing Pmel constructs and EGFP from a bicistronic plasmid vector were labeled 24 h after transfection with the anti-Pmel antibody NK1-beteb and PE-conjugated secondary antibody at 4°C and then analyzed for EGFP and PE fluorescence. The degree of EGFP fluorescence showed a roughly linear relationship with that of surface Pmel expression in cells expressing hPmelsi, hPmel[LL>AA], and hPmelsi(H643V) over the entire range of EGFP expression (Figure 6A). By contrast, in cells expressing WT hPmel, a much lower level of surface labeling was observed in cells expressing low levels of EGFP, with a sharp rise in hPmel surface expression at higher levels of EGFP (Figure 6A, WT). We interpret the latter as a reflection of overexpression of WT hPmel and

Figure 6. Surface accumulation of hPmel is due to an endocytosis defect. (A) HeLa cells were transfected with bicistronic plasmids encoding EGFP and hPmelwt (WT), hPmel si (Si), hPmel si(H643V) (H643V), or hPmel[LL>AA] (LLAA) or with a comparable empty vector (–). Cells were labeled on ice with NK1-beteb anti-Pmel and then PE-conjugated anti-mouse Ig. PE signal intensity corresponding to surface Pmel labeling is plotted against EGFP fluorescence intensity. (B) Cells gated for low but positive levels of EGFP fluorescence (see gate, panel A; chosen based on nonsaturating levels of WT Pmel, panel A) were analyzed for surface Pmel labeling, mean fluorescence intensity values were obtained, and all values were calculated relative to the mean value for WT Pmel within each experiment. Results from three independent experiments performed in triplicate are presented; error bars, SD. (C–M) HeLa cells expressing hPmel WT (WT; C and H), hPmel si (si; D and J), hPmel[LL>AA] (LLAA; E and K), hPmel(V668D) (F and L) or hPmel si(H643V) (G and M) were incubated on ice with HMB-50 antibody to label protein exposed on the cell surface. Cells were then washed and fixed either immediately (C–G) or after a 15-min incubation at 37°C (H–M), and analyzed by IFM with anti-mouse secondary antibodies. Note labeling for all constructs at the cell surface at 4°C (C–G) and only intracellular labeling for WT and V668D after 15 min at 37°C (H and L). Bar, 20 μ m.

consequent saturation of the sorting machinery that acts on the di-leucine-based sorting signal, resulting in default retention at the plasma membrane (Marks *et al.*, 1996). To quantitatively compare surface expression at nonsaturating levels, Pmel surface expression was analyzed in cells expressing similar low levels of EGFP above background (Figure 6A, –), as indicated by the gate (Figure 6A, shaded box), and represented relative to WT Pmel in Figure 6B. hPmel si-expressing HeLa cells display a 15-fold increase in cell surface labeling compared with WT. Interestingly, even greater steady state cell surface accumulation (~30-fold over WT) was observed with cells expressing di-leucine-deficient hPmel si(H643V) and hPmel[LL>AA], likely reflecting the increase in kinetics of delivery of cell surface delivery of these mutants compared with hPmel si; both mutants

showed a similar degree of cell surface accumulation. These data are consistent with the presence of a di-leucine-based endocytosis signal that is eliminated by the *silver* mutation. Moreover, the relative accumulation of hPmel si, hPmel si(H643V), and hPmel[LL>AA] suggests that 1) the ER exit block imposes a twofold difference in Pmel cell surface delivery and 2) no other active endocytic sorting signal besides the di-leucine-based motif is removed by the *silver* mutation.

To determine whether the increased surface expression of the hPmel mutants was due to impaired endocytosis, we monitored intracellular accumulation of anti-Pmel antibody exposed to living cells at 37°C. Transfected HeLa cells were first incubated with anti-Pmel antibody at 4°C to label the cell surface. As expected, although cell surface expression

Figure 7. mPmel17si in *silver* mouse melanocytes is inefficiently sorted and expressed at low levels in melanosomes. (A and B) Immortalized melanocyte lines derived from WT (melan-a) and *silver* mice (melan-si-1; referred to here as melan-si) were metabolically pulse-labeled and chased as indicated. Endogenous protein was immunoprecipitated using α mPmel-N to the N-terminus of mPmel, subjected to mock (–) or endoH (+) digestion, and then fractionated by SDS-PAGE and analyzed by PhosphorImager. Top panels, regions surrounding P1, endoH-processed P1 (P1^H), and potential P2 bands (P2?); bottom panels, regions surrounding Mβ and Mβ' (representing small Mβ fragment derived from mPmelsi); right, migration of molecular-weight markers. Note that detection of processing intermediates for mPmel is not as consistent as for hPmel, and assignment of a band to P2 is more difficult. (C) Quantitation of mean P1 band intensity over time. Error bars, mean $\pm$ SD for 7 experiments for melan-a (○) and 5 experiments for melan-si (■). (D) Immunoblot analyses of melan-a and melan-si TX-100-soluble (Sol), TX-100-insoluble (Insol) fractions, and total cell lysates probed with anti-Pmel antibodies α Pep13h (to the C-terminus of hPmel) or anti-tubulin as indicated. Migration of molecular-weight standards are shown on the right. The reduced intensity of the HMB-45-reactive bands in total cell lysates relative to SDS-solubilized TX-100-insoluble pellets is consistent and likely reflects inefficient recapture of the epitope in SDS from dilute whole cell lysates. Phosphorimaging analysis of both total cell lysates and Insol fractions show fivefold less HMB-45-reactive Pmel, relative to tubulin, in melan-si than in melan-a. (E–K). IFM analysis of melan-a (E–G) and melan-si-1 (H–K) cells. Cells were fixed, permeabilized, and costained with HMB-45 (E and H) and α mPmel-N (F and J). Images were all taken at the same exposure to illustrate the significant difference in fluorescence intensity of HMB-45 labeling in melan-a versus melan-si-1 (compare E with H). (E and H) The summation of a z-series; (F and J) single raw images from within this series. Corresponding bright field images are shown in G and K. Bar, 20 μ m.

Figure 8. Surface accumulation of mPmel in *silver* melanocytes. (A–D) IFM analysis of unpermeabilized melan-a (A and B) and melan-si-1 (C and D) melanocytes labeled with α mPmel-N (A and C) to detect cell surface-exposed Pmel. Corresponding bright field images are shown in B and D. Bar, 20 μ m. (E–H) melan-a (E and F) and melan-si-1 (G and H) cells were labeled with either polyclonal α Pep13h as a negative control (thin line, –control) or α mPmel-N (thick line, Pmel) on ice and then anti-rabbit IgG-PE and analyzed by flow cytometry. (E and G) Forward scatter is plotted against side scatter for melan-a (E) and melan-si-1 (G) cells. The melan-si-1 cells are more homogeneous and display less forward scatter and greater granularity than melan-a cells because of their smaller size and greater degree of melanization. (F and H) PE fluorescence intensity is plotted against cell counts. Mean fluorescence intensity values are given for each sample. The increase in α mPmel-N staining in melan-si-1 compared with melan-a is thus $\sim$ 15–20-fold. Representative of three experiments.

was detectable by IFM for all Pmel variants, labeling was more frequently observed and was apparent upon shorter exposures for hPmel[LL>AA] and hPmelsi(H643V) (Figure 6, C–G). Cells were then incubated for an additional 10-min pulse at 37°C with anti-Pmel antibody and then washed and chased for 15 min at 37°C in the absence of antibody to allow for internalization before analysis by IFM. Those cells expressing WT hPmel internalized essentially all bound antibody (Figure 6H) to punctate endocytic structures, whereas the majority of antibody remained at the plasma membrane in cells expressing either hPmelsi, hPmel[LL>AA], or the efficient ER-exit variant H643V (panels J, K, and M, respectively); in most cells expressing these constructs, labeling remained at the plasma membrane even after 30–60 min of chase (unpublished data). Consistent with the separation of the ER exit and endocytosis defect, what little anti-Pmel antibody was bound to the surface by the V668D variant (Figure 6F) was efficiently internalized by 15 min of chase (Figure 6L). Taken together, these data indicate that the *silver* mutation eliminates a di-leucine-based internalization signal responsible for clearing Pmel from the cell surface.

Interestingly, unlike hPmelsi, steady state analysis of permeabilized cells expressing hPmel[LL>AA] and H643V show efficient localization to LAMP-1-containing structures (Figure 5F) despite the block in clearance from the plasma membrane (Figure 6, compare K with H). This suggests that the di-leucine-based signal is not absolutely necessary for the localization of Pmel to late endosomes and functions primarily as an endocytic signal at the plasma membrane, consistent with the role of the luminal domain in regulating postendocytic sorting (Theos *et al.*, 2006). Furthermore, metabolic pulse-chase analysis of hPmel[LL>AA] shows efficient proteolytic processing of the Golgi-modified P2 form to the mature M α and M β forms (Figure 4J, LLAA) with only a short delay, contrasting with the inefficient proteolytic processing of hPmelsi (Figure 4J, si). Given that proteolytic

processing is mediated by a post-Golgi resident proprotein convertase (Berson *et al.*, 2003) and in HeLa cells requires delivery to endosomal compartments (Theos *et al.*, 2006), these data suggest that in addition to the endocytosis defect, the *silver* mutation is unlikely to eliminate any additional signal required for endosomal delivery of Pmel.

Defects in ER Exit and Endocytosis Abrogate Melanosome Accumulation of Pmel17 in Melanocytes from *silver* Mice

The processing intermediates detected for hPmel in human melanocytic cells and transfected HeLa cells are difficult to detect in mouse melanocytes (Kobayashi *et al.*, 1994), likely because of different kinetics of processing and fibril formation by the M α cleavage product. Nevertheless, processing likely occurs by the same mechanism because the M β cleavage product can be detected by Western blotting (Berson *et al.*, 2001) and the kinetics of disappearance of the P1 precursor band from detergent lysates are identical to those of the human protein (Kobayashi *et al.*, 1994). As shown above, ER retention of hPmelsi was accompanied by stabilization of the protein as assessed by metabolic pulse chase. To determine if Pmelsi was ER-retained in mouse melanocytes, we used metabolic pulse chase and immunoprecipitation to compare the stability of Pmel in melan-a cells (Bennett *et al.*, 1987), derived from control C57Bl/6 mice, and in melan-si-1 cells (Spanakis *et al.*, 1992), derived from *silver* mice. Detergent lysates of both lines at all time points were immunoprecipitated with an antibody raised to the N-terminus of mature mPmel, immunoprecipitates were left untreated or treated with endoH, and the products were analyzed by SDS-PAGE and phosphorimaging. As observed previously (Kobayashi *et al.*, 1994), only the endoH-sensitive P1 form of mPmel is detected throughout the chase in melan-a cells, but the half-life of this fragment is rather short; in some experiments, a small amount of P2 and M β could be detected (Figure 7A). By contrast, in melan-si-1 cells, endoH-sensitive P1 had a

Figure 9. Silver melanocytes are depleted of organized stage II and III melanosomes. (A) Ultrathin cryosections of melan-si-1 mouse melanocytes were labeled with antibodies to LAMP-1 (PAG-15) and the mature melanosome protein Tyrp1 (PAG-10). Note labeling for LAMP-1 within late endosomal membranes (arrows) and multilamellar lysosomes (Lys) and labeling for Tyrp1 on large, spherical mature melanosomes (arrowheads). (B and C) Ultrathin cryosections of melan-si-1 (B) and WT melan-a (C) mouse melanocytes were single-labeled for Tyrp1 (PAG-10). Note the presence of aberrant enlarged spherical densely melanized mature Tyrp1-positive melanosomes in melan-si-1 (B) compared with the smaller more ellipsoidal compartments in melan-a (C). Bars, 200 nm.

longer half-life (Figure 7B), confirming our observations of hPmelsi and the Tac chimeras in HeLa cells. Although the degree of maturation varies between experiments, P1 is consistently lost from melan-a lysates efficiently by 4-h chase compared with stabilization out to 6 h in melan-si-1 (Figure 7C). These data confirm that endogenous mPmelsi in *silver*-derived melanocytes has an ER exit defect similar to that of the “humanized” hPmelsi in HeLa cells. Consistent with this finding, labeling of saponin-permeabilized melanocytes with the antibody to the Pmel N-terminus, which detects wild-type Pmel primarily in the ER and Golgi, shows a slightly larger steady state pool of immature mPmelsi in melan-si-1 cells compared with mPmel in wild-type melan-a melanocytes (Figure 7, F and I). The mean difference in half-life between mPmel and mPmelsi in melan-a and melan-si-1 is less dramatic than that between hPmel and hPmelsi in transfected HeLa cells, likely reflecting a cell-type difference in efficiency of Pmelsi export.

The HMB-50 antibody used to recognize hPmel does not react with mPmel, and our antibodies to the N-terminus of mPmel do not recognize the mature forms of Pmel by IFM (likely because of post-Golgi oligomerization and masking of the epitope; Figure 7, F and J) or by immunoblotting (likely because of rapid proteolytic processing; see below). However, the HMB-45 antibody recognizes a neuraminidase-sensitive determinant within the luminal domain of Pmel that is generated in most immortal melanocytic cell lines upon glycoprotein processing in the Golgi or *trans*-Golgi network (Kapur *et al.*, 1992; Chiamanti *et al.*, 1996; Raposo *et al.*, 2001). A reduction in ER export would therefore be expected to result in a reduced level of labeling by HMB-45. Consistent with this, immunoblotting of melan-a and melan-si-1 cell lysates with HMB-45 shows at least a fivefold reduction in the total expression level of HMB-45-reactive bands (Figure 7D), which represent cleavage products of Pmel formed by proteolytic digestion of the M α

Figure 10. The aberrant melanosome morphology of *silver* is not due to the *Tyrp1*^{+/+} genotype. (A and B) Paraffin-embedded section of melan-a (WT control, A) and melan-si-4 (B) mouse melanocytes. Bars, 2 μ m. Inset in A, magnification of melanosome clusters from melan-a containing organelles of different stages of melanosome development and inset in B, magnification of melan-si-4 melanosome cluster containing relatively fewer immature melanosomes. Note the larger size and more spherical geometry of the melanosomes from melan-si-4 compared with melan-a. Inset bars, 0.5 μ m.

fragment (Chiamenti *et al.*, 1996; Yasumoto *et al.*, 2004). These data confirm that Pmel in *silver* melanocytes is defective in post-ER transport and accumulates to a fivefold lower degree in melanosomes. Consistent with this finding, IFM labeling of melan-si-1 cells with HMB-45 yields a significantly reduced signal intensity when compared with melan-a in images taken at the same exposure (Figure 7, compare E and H), contrasting with the slightly increased signal intensity upon labeling with the antibody to the N-terminus (Figure 7, F and J). The weak HMB-45 labeling that is observed in melan-si-1 cells displays similar low/partial coincidence with markers of mature melanosomes and late endocytic compartments as observed in wild-type cells (Supplementary Figure S1), suggesting that residual mature mPmelsi localizes similarly to wild-type mPmel.

Because expression of hPmelsi in HeLa fibroblasts results in an approximate 15-fold increase in cell surface expression, we hypothesized that cell surface accumulation of endogenous mPmelsi in *silver* melanocytes might account for this substantial reduction in HMB-45-reactive material in melanosomes. In agreement with this, a substantial accumulation of Pmel at the cell surface was observed at steady state in unpermeabilized melan-si-1 cells by IFM compared with melan-a controls (Figure 8). This difference in cell surface Pmel was quantitated by flow cytometric analysis of unfixed melanocytes at 4°C (Figure 8, E–H). The data suggest an endocytic defect for mPmelsi within the melanocyte comparable to that observed in transfected HeLa cells (compare Figure 8 with Figure 6), with an ~15-fold increase in cell surface expression of Pmel in melan-si-1 cells relative to melan-a cells. These data confirm and extend the critical role for cytoplasmic sorting signals in the delivery of Pmel to the endocytic system before fibril formation in stage II melanosomes.

Altered Melanosome Morphology in Melanocytes from *silver* Mice

Despite the dramatic depletion of Pmel from stage II melanosomes of melan-si-1 cells, the cells are highly pigmented relative to WT melan-a cells (Figure 7, compare G and K, and Figure 8, compare B and D). Nevertheless, because Pmel is a major biogenetic component of the fibrous striations of stage II and III melanosomes, we anticipated that the morphology of melanosomes in melan-si-1 cells would be aberrant. Indeed, IEM analysis of melan-si-1 cells shows a marked depletion of stage II and III melanosomes and the appearance of aberrantly large and round melanosomes (Figure 9, A and B). These melanosomes were devoid of HMB-45 labeling for Pmel (unpublished data) and displayed a more uniform electron density than the striated melanosomes in melan-a cells (Figure 9C). A few stage II and III melanosomes were observed within the population of melan-si-1 cells (our unpublished observations), but they were much less numerous than in melan-a cells. The melan-si-1 line is derived from mice that are homozygous for the mutant *silver* allele and also heterozygous for the *brown* mutation at the *Tyrp1* locus (*Tyrp1^b*), which also affects coat color. To confirm that the morphological defects observed in melan-si-1 were not due to the *Tyrp1* mutation, we analyzed by thin section EM another melanocyte line derived from mice carrying the *silver* mutation with no *Tyrp1* mutation—in which the *silver* mutation imparts no coat color dilution (M. L. Lamoreux, unpublished observations; Figure 10). Like melan-si-1, these cells harbored enlarged, uniform melanosomes lacking internal striations, indicating that aberrant melanosomes are a consequence of the *silver* mutation and not associated with the *Tyrp1^b*/+ genotype. These data confirm our prediction

that a genetic defect in Pmel that depletes the accumulation of mature Pmel in melanosomes would affect fibril formation and show that Pmel trafficking both out of the ER and into the endocytic system is required for the efficient morphogenesis of organized melanosomes.

DISCUSSION

Here we have uncovered the molecular mechanism underlying the cellular defects of the *silver* mouse, a natural mutant with impaired coat pigmentation on black backgrounds that was described more than 75 years ago (Dunn and Thigpen, 1930). The genetic mutation in the *Pmel17/Si* gene in *silver* mice is a nucleotide substitution within the coding region, introducing a stop codon predicted to result in premature truncation of the cytoplasmic domain (Martínez-Esparza *et al.*, 1999). Consistent with the prediction that the mutation would affect melanosomal sorting (Kwon *et al.*, 1995; Martínez-Esparza *et al.*, 1999), we show here that the truncation removes two signals required for the efficient intracellular transport of Pmel and accumulation in endosomal/melanosomal compartments: a C-terminal valine-dependent ER exit signal and a di-leucine-based endocytic signal. Consistent with a requirement for ER exit and endocytic routing in premelanosome targeting of Pmel, melanosomes in melanocytes from *silver* mice are depleted of Pmel and morphologically deformed. Although the mice from which these lines were derived show coat color dilution, we show that the deformation of the melanosome does not impair pigment accumulation within the melanocyte. Our data provide a novel example of a natural mutation in an integral membrane protein that deletes at least two sequential sorting signals, lend further support to the requirement for endocytic trafficking in Pmel access to melanosomes, and indicate that melanosome fibrils inhibit hair pigmentation in a manner independent of melanogenesis within the melanosome.

Numerous genetic disorders are caused by mutations that render integral membrane proteins incapable of escaping the ER toward their ultimate destination elsewhere in the cell. For example, common forms of oculocutaneous albinism type I and ocular albinism are caused by missense mutations in the genes encoding tyrosinase (Berson *et al.*, 2000; Halaban *et al.*, 2000; Toyofuku *et al.*, 2001) and OA1 (d'Addio *et al.*, 2000; Shen *et al.*, 2001), respectively, which result in ER retention and depletion of the gene products from melanosomes or associated compartments. However, most of these mutations result in protein misfolding and recognition by the ER quality control machinery (Berson *et al.*, 2000; Halaban *et al.*, 2000). To our knowledge, the only other verified case in which disease is caused by loss of an ER export signal is in a variant of the cystic fibrosis transporter that is associated with a common form of cystic fibrosis (Wang *et al.*, 2004). We provide a second example of such a mechanism with the *silver* mutation in Pmel, which eliminates a critical C-terminal valine residue implicated in COPII binding and ER export of numerous cargo proteins (Nakamura *et al.*, 1998; Nufer *et al.*, 2002; Crambert *et al.*, 2004; Paulhe *et al.*, 2004). These recent examples suggest that loss of ER export signals may be a more common mechanism than once thought for loss-of-function mutations within integral membrane proteins. Interestingly, appendage of the exit signal of Vesicular Stomatitis Virus glycoprotein (Nishimura and Balch, 1997) was inefficient in rescuing ER export of hPmelsi compared with the addition of a C-terminal valine (unpublished data). This suggests that the context of an ER exit signal is crucial for its function.

The second defect conferred by the *silver* mutation is a loss of endocytosis, resulting in increased expression of ER-exported protein at the plasma membrane. This defect was more predictable, given the presence of a canonical di-leucine-based internalization signal within the region of the cytoplasmic domain deleted by the *silver* mutation; similar signals are known to facilitate clathrin-dependent endocytosis, presumably by interaction with the AP-2 adaptor (Bonifacino and Traub, 2003). As predicted from the sequence of this signal, Pmel is internalized in wild-type mouse melanocytes with kinetics consistent with clathrin-dependent endocytosis (initial rate of ~50% per min; unpublished data), and site-directed mutagenesis of the di-leucine signal enhanced cell surface appearance of Pmel and decreased uptake of Pmel antibody into transfected HeLa cells, suggesting a major defect in internalization. Loss of this signal through the *silver* mutation thus explains the increased cell surface expression of Pmelsi in HeLa cells and *silver* melanocytes. Our data do not rule out that the di-leucine signal facilitates, in addition to endocytosis, a direct sorting pathway from the Golgi to early endosomes that bypasses the plasma membrane. Its absence would thus lead to increased delivery of Pmel to the cell surface, concomitant with decreased endocytosis. The relevance of such a route for many constituents of the late endosomal pathway has been a matter of controversy for nearly 20 years and is largely still unresolved (e.g., for a recent contribution to the controversy see McCormick *et al.*, 2005).

We have shown previously that the localization of Pmel to late endosomes, in which WT Pmel efficiently accumulates, is dependent on a luminal determinant (Theos *et al.*, 2006). What function, then, does the di-leucine-based internalization signal serve? We propose that sorting from the limiting membrane to internal membranes of early endosomes, mediated by the luminal determinant, occurs subsequent to delivery of Pmel to early endosomes. Efficient delivery of Pmel to early endosomes would be facilitated by the cytoplasmic di-leucine signal. Consistent with earlier results (Theos *et al.*, 2006), the loss of the di-leucine signal slowed but did not prevent access of Pmel to late endocytic compartments of HeLa cells or to stage II melanosomes of melan-si-1 cells, in which WT Pmel efficiently accumulates. Consistently, in HeLa cells, the efficient ER exit and delayed internalization at the cell surface of hPmel[LL>AA] and hPmelsi(H643V) had only a minor effect on the kinetics of proteolytic processing. These data indicate that although endocytosis mediated by the di-leucine signal may enhance the subsequent postendocytic sorting step, it is not absolutely required and may be partially compensated by clathrin-independent endocytic mechanisms. Such mechanisms may be more prevalent in HeLa cells than in melanocytes, perhaps explaining the dramatic loss of mature Pmel from melanosomes of melan-si cells. Importantly, the data indicate that the di-leucine motif is not an *a priori* signal for sorting to melanosomes as previously predicted (Vijayasaradhi *et al.*, 1995; Calvo *et al.*, 1999; Simmen *et al.*, 1999).

The combined effects of the defects in ER export and endocytosis in *silver* melanocytes was sufficient to cause a fivefold decrease in the accumulation of Pmel in premelanosomes. Moreover, cleavage of the Golgi processed precursor of Pmel to the mature M α and M β forms, which is required for the maturation of premelanosome fibrils (Berson *et al.*, 2003), was dramatically reduced in *silver* Pmel and somewhat delayed in hPmelsi(H643V) and hPmel[LL>AA]. This supports previous data that cleavage occurs in endocytic compartments (Theos *et al.*, 2006) and requires acidification (Berson *et al.*, 2003). Taken together, the data support the

requirement for endocytic routing of Pmel in transport to premelanosomes and are inconsistent with a model for direct transport of Pmel to premelanosomes from the ER (Yasumoto *et al.*, 2004). Interestingly, although rescue of the ER exit defect in hPmelsi(H643V) and hPmel[LL>AA] restored proteolytic processing to nearly wild-type levels (albeit delayed), a defect in ER exit in hPmelsi and hPmel(V668D) resulted in a dramatic decrease in the accumulation of processed products. This may reflect either a kinetic effect (i.e., processing and disappearance of processed products may be more rapid than ER exit) or a mass action effect (i.e., sorting to internal membranes and consequent processing may require concentrations of endosomal Pmel that are not sustained by the ER exit defect). In vitro studies will likely be required to distinguish these possibilities.

The most striking effect of the *silver* mutation is the dramatic change in mature melanosome morphology from the characteristic ellipsoidal shape with longitudinal internal fibers to the larger, more spherical shape without detectable internal organization. These aberrant melanosomes were nevertheless highly pigmented, more so than those in melan-a cells, as judged both by light microscopy and by side scatter values by flow cytometry. The aberrant size and morphology of these mature melanosomes must contribute to the pigment dilution of *silver* mice in a manner that is not reflected by cell autonomous pigmentation. Analogous mutants in humans have yet to be detected, but are likely to exist. Indeed, mutations in the chicken (Kerje *et al.*, 2004), zebrafish (Schonthaler *et al.*, 2005), and dog (Clark *et al.*, 2006) *SILV* genes also result in hypopigmentation, likely as a result of altered melanosome morphology and consequent melanocyte or melanosome instability or poor transfer to keratinocytes. A modest mutation that causes a short truncation such as *silver* may result in subtle pigment dilution that would be far less dramatic than a full deletion and perhaps more difficult to distinguish in an outbred population; indeed, the *silver* mutation has little gross effect on coat pigmentation in certain backgrounds (Dunn and Thigpen, 1930). Despite the partial loss of function due to the multiple sorting defects of this short truncation, the *silver* mutant phenotype confirms a role for Pmel in the formation of intraluminal amyloid-like fibrils consistent with our previous studies (Berson *et al.*, 2003; Fowler *et al.*, 2006; Theos *et al.*, 2006).

The pigment dilution phenotype of *silver* in mice with certain genetic backgrounds implies a novel role for melanosomal Pmel in the proliferation in vitro and survival in vivo of melanocytes in these mice (Quevedo *et al.*, 1981; Spanakis *et al.*, 1992). In addition to forming a morphological template for melanosomes, Pmel is also capable of binding to melanin intermediates such as dihydroxyindole and dihydroxyindole carboxylic acid (Chakraborty *et al.*, 1996; Lee *et al.*, 1996; Fowler *et al.*, 2006), and has been suggested to serve as a sink within melanosomes for the detoxification of cytotoxic intermediates. It will be of interest to determine whether it is the loss of this binding capacity or of the ability to form amyloid-like fibrils that results in the loss of melanocyte viability observed in affected strains of *silver* mice. The latter might contribute in some way to the transfer of melanin from melanocyte to keratinocyte, such that melanin in *silver* mice accumulates within the melanocyte, with consequent effects on viability. It will also be important to dissect why the pigment dilution is penetrant only on certain backgrounds.

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