Melanocytic Galectin-3 Is Associated with Tyrosinase-Related Protein-1 and Pigment Biosynthesis

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Galectin-3 is a family member of the carbohydrate-binding proteins widely expressed by many cell types and exhibits multiple cellular functions. We demonstrate that melanocytes express galectin-3, which is predominantly localized to the cell body peripherally along the Golgi zone. Downregulation of galectin-3 in human melanocytes using short hairpin RNA technology resulted in the reduction of both melanin synthesis and expression/activity of tyrosinase-related protein-1 (Tyrp-1). In the cell body, galectin-3 colocalizes with melanosome-destined cargo, specifically tyrosinase and Tyrp-1. We studied melanocytes cultured from patients with forms of Hermansky–Pudlak syndrome (HPS) containing defects in trafficking steps governed by biogenesis of lysosome-related organelle complex-2 (BLOC-2) (HPS-5), BLOC-3 (HPS-1), and adaptin-3 (HPS-2). We found that galectin-3 expression mimicked the defective expression of the tyrosinase cargo in dendrites of HPS-5 melanocytes, but it was not altered in HPS-1 or HPS-2 melanocytes. In addition, galectin-3 colocalized predominantly with the HPS-5 component of BLOC-2 in normal human melanocytes. These data indicate that galectin-3 is a regulatory component in melanin synthesis affecting the expression of Tyrp-1.

Journal of Investigative Dermatology (2015) **135,** 202–211; doi:10.1038/jid.2014.315; published online 21 August 2014

INTRODUCTION

The melanocyte synthesizes a premelanosome organelle derived from the endosomal system within the cell (Marks and Seabra, 2001). Subsequently, several enzymes (specifically tyrosinase, tyrosinase-related protein-1 (Tyrp-1), and Tyrp-2) and regulatory proteins that are responsible for converting tyrosine to melanins are trafficked from the Golgi apparatus, through the endosomal system, and targeted for incorporation to the premelanosome (Boissy *et al.*, 2006; Raposo and Marks, 2007). Predominate chaperones that facilitate this trafficking process are the family of protein complexes called BLOCs (biogenesis of lysosome-related organelles complex) (Dell'Angelica 2004; Di Pietro *et al.*, 2006). Many of the subunits of these BLOCs are products of genes that when mutated result in the Hermansky–Pudlak syndrome (HPS) (Bonifacino, 2004; Di Pietro and Dell'Angelica 2005; Wei, 2006). BLOC-2 is composed of at least the HPS-3, -5, and -6 proteins (Di Pietro *et al.*, 2004) and BLOC-3 is composed of at least the HPS-1 and -4 proteins (Nazarian *et al.*, 2003; Kloer *et al.*, 2010). When mutated, each of these HPS proteins compromise the integrity of their respective BLOCs and impair efficient trafficking of the requisite enzyme to the melanosome in a distinctive manner, resulting in reduced melanin synthesis and cutaneous and ocular hypopigmentation (Richmond *et al.*, 2005; Boissy *et al.*, 2006). The molecular mechanisms used by these BLOCs to recruit and shuttle melanosome-destined cargo remains unclear. However, two small GTPase, Rab32 and Rab38, have recently been shown to cooperate with BLOC-2 and/or BLOC-3 in trafficking of melanogenic enzymes (Marks, 2012; Bultema and DiPietro, 2013). Also unknown are additional protein and regulatory components that may be part of or participate with BLOCs.

Galectin-3 is a member of carbohydrate-binding proteins that interact primarily with β-galactoside residues of cell surface and extracellular matrix glycoprotein molecules (Wang *et al.*, 2004; Dumic *et al.*, 2006). By virtue of this property, galectin-3 has been implicated in cell–cell and cell–substrate recognition. In addition, it has been proposed that galectin-3 may function as a chaperone involved in intracellular trafficking of cytosolic glycoproteins in various cell types (Liu *et al.*, 2002; Delacour *et al.*, 2006, 2009; Vagin *et al.*, 2009). Expression of galectin-3 by the melanocytes has not been reported. However, brief mention of galectin-3 being a possible component of the melanosome was demonstrated in melanosomes purified using sucrose density gradient centrifugation and subsequently analyzed by protein digestion and mass spectrometry (Basrur *et al.*, 2003).

In this report, we demonstrate that galectin-3 is expressed by melanocytes, regulates melanogenesis, maintains the expression of Tyrp-1, and colocalizes to chaperones upstream of the melanosome, particularly HPS-5, in the melanocyte cell

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Abbreviations: BLOC, biogenesis of lysosome-related organelle complex; HPS, Hermansky–Pudlak syndrome; NHM, normal human melanocyte; shRNA, short hairpin RNA; Tyrp-1, tyrosinase-related protein-1

Received 23 August 2013; revised 13 March 2014; accepted 8 April 2014; accepted article preview online 23 July 2014; published online 21 August 2014

body. These data implicate galectin-3 as a regulator of melanization by maintaining Tyrp-1.

RESULTS

Expression of galectin-3 by epidermal cells

Galectin-3 was identified at the expected molecular weight of 30 kDa in cultured melanocytes from both light- and dark-skinned individuals (Figure 1aA_1). Galectin-3 was also identified in an established line of human melanoma cells, cultured human keratinocytes, and fibroblasts, as well as in epidermal lysates from dark and light skin. The 30-kDa galectin-3 molecule expressed by cultured melanocytes was not found to be secreted into the medium (Figure 1aA_2).

Silencing of galectin-3 results in reduced melanin synthesis

Cultures of normal human melanocytes (NHMs) derived from dark, medium, and light skins were silenced for galectin-3 expression by transfection with several short hairpin RNAs (shRNAs), as described in Materials and Methods section. The dark line (1800 ng melanin per ug protein) and the medium line (800 ng melanin per ug protein) were silenced using the Santa Cruz shRNA (Supplementary Figure 1 online) and the light line (25 ng melanin per ug protein) was silenced using the SigmaTRCN clones (Figure 1b). Galectin-3 was markedly reduced in all cell lines by 25–40%. Concurrently, melanin amount was significantly reduced in all melanocyte lines by 20–50% compared with the control shRNA transfectants (Figure 1c).

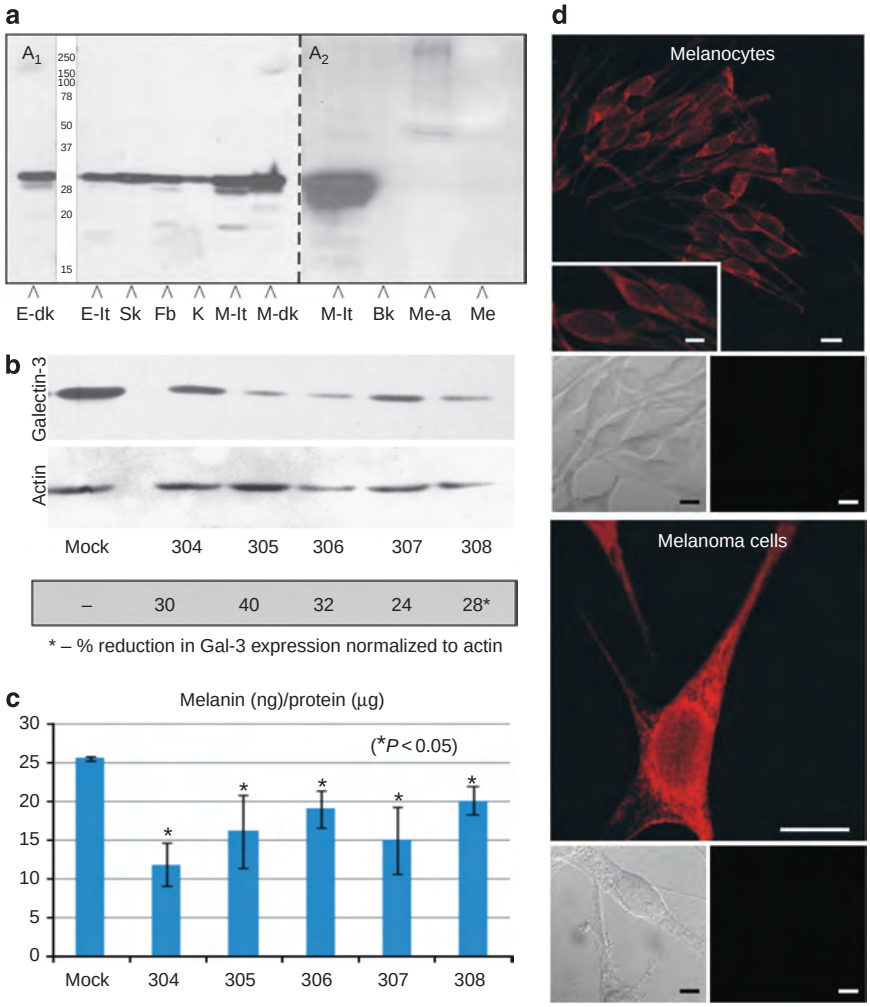

Figure 1. (a) Expression and secretion of galectin-3 by cutaneous cells and tissues. (A_1) Galectin-3 of 30 kDa was identified by immunocytochemistry in lysates of dark (E-dk) and light (E-lt) neonatal foreskins and in cultured cells of skMel melanoma (Sk), dermal fibroblasts (Fb), keratinocytes (K), and melanocytes derived from light (M-lt) and dark (M-dk) skin. (A_2) Medium collected from light skin–derived melanocytes before (Me) or after (Me-a) acetone precipitation did not exhibit the 30-kDa galectin-3. However, the latter did exhibit a 45- to 50-kDa triplet and a 250-kDa doublet. (Bk, blank lane). (**b**, **c**) Silencing of galectin-3 results in reduced melanin synthesis. Cultured melanocytes derived from one light skin was transfected with control shRNA (Mock) or one of six galectin-3 shRNAs (304-8). The amount of (**b**) galectin-3 protein and (**c**) melanin content was reduced in the galectin-3-silenced melanocyte lines by 24–40% and 20–50%, respectively, compared with the mock transfectants in all melanocyte lines. (**d**) Cellular profile of galectin-3. Cultured normal human melanocytes and skMEL melanoma cells demonstrated a prominent perinuclear localization of galectin-3 and minimal dendritic localization. Doublet insets below the immunofluorescence micrographs represent phase (left) and corresponding fluorescence (right) images of cells stained with the galectin-3 primary antibody omitted and secondary used to demonstrate lack of nonspecific staining. C, cytoplasm; N, nucleus. Bars = 10 µm; insets = 5 µm.

Cellular profile of galectin-3

In NHMs and melanoma cells, galectin-3 is expressed in a granular pattern that is prominent within the cell body and moderate along the dendrites, while being relatively devoid in the nucleus (Figure 1d). There is a distinctive lattice-like and vesicular profile in the cell body that resembles the location of the endoplasmic reticulum and endosomes, suggesting that galectin-3 could participate in the trafficking of glycoproteins in this significant area.

Tyrp-1 expression and activity is reduced in galectin-3-silenced NHMs

Cultures of NHMs derived from light skin were silenced for galectin-3 expression by shRNA transfection, as described in Materials and Methods section and presented in Figure 1. Control shRNA and Gal3 shRNA transfectants exhibited and lacked galectin-3 expression, respectively (Figure 2aB and E). In addition, control shRNA and Gal3 shRNA transfectants demonstrate abundant and reduced expression of Tyrp-1 throughout the cell, respectively (Figure 2aC and F). In contrast, expression of tyrosinase was not altered in control shRNA and Gal3 shRNA transfectants (Figure 2aG–L). Signal intensity per cell representing the expression of Tyrp-1 in control shRNA and Gal3 shRNA transfectants was $17 \times 10^4 \pm 15$ and $1.9 \times 10^4 \pm 0.4$, respectively ($P = 0.021$). For tyrosinase expression, signal intensity values were $116 \times 10^4 \pm 19$ and $157 \times 10^4 \pm 20$, respectively ($P = 0.111$).

A fourth round of silencing on a light NHM line was performed using three of the SigmaTRCN clones and assessed for expression of Tyrp-1 and tyrosinase. Expression of galectin-3 was reduced by 20–30% (Figure 2b), Tyrp-1 by 20–42% (Figure 2b), and tyrosinase (data not shown) was unchanged. Concurrently, the tyrosine hydroxylase activity specific for human Tyrp-1 was significantly reduced in the galectin-3-silenced NHMs (Figure 2c). These results show that the expression and/or maintenance of Tyrp-1, as opposed to tyrosinase, was dependent on galectin-3.

Colocalization of galectin-3 with melanosome-destined cargo (i.e., tyrosinase and Tyrp-1)

Galectin-3 was found to colocalize with both tyrosinase and Tyrp-1 in NHMs (Figure 3). Both tyrosinase and Tyrp-1 had

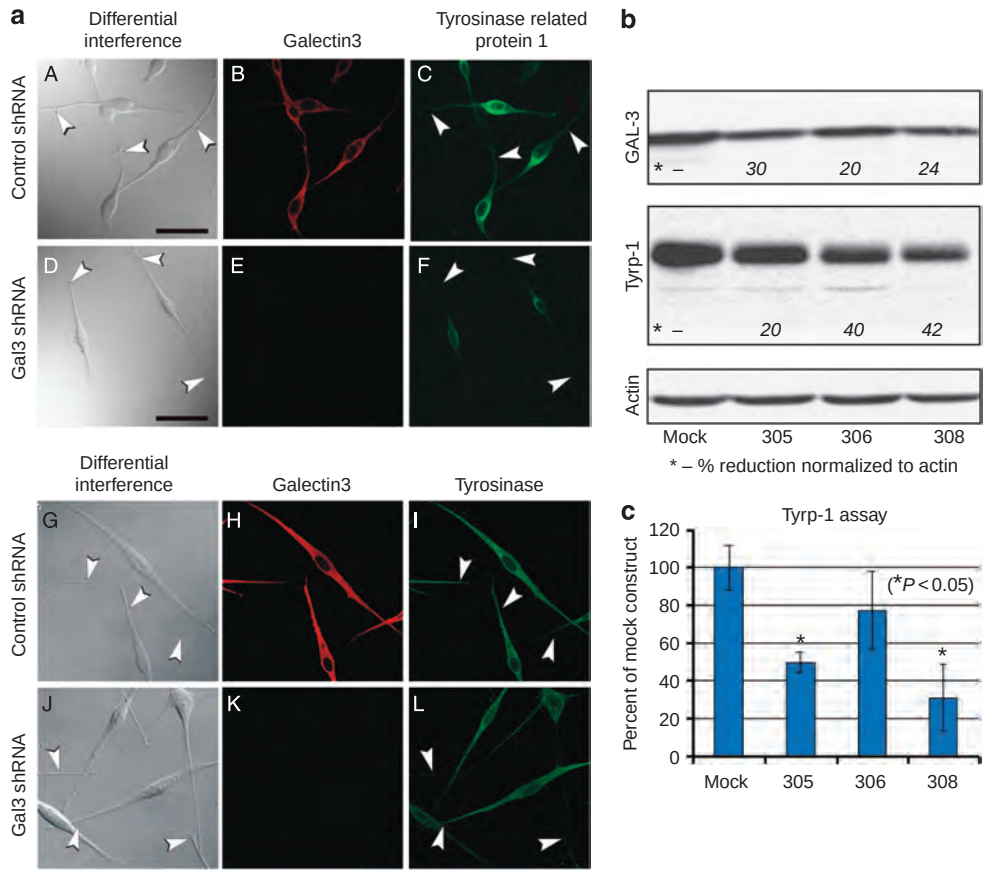

Figure 2. Galectin-3 influences tyrosinase related protein-1 (Tyrp-1) expression but not that of tyrosinase. (**a**) Cultured melanocytes derived from light skins were transfected with control short hairpin RNA (shRNA) (Control shRNA) or galectin-3 shRNA (Gal3 shRNA) and colabeled by immunofluorescence for galectin-3 and Tyrp-1 or tyrosinase. The amount of Tyrp-1 expression in shRNA transfectants (A, C) was reduced in the Gal3 shRNA tranfectants (D, F). In contrast, tyrosinase expression was similar in both the shRNA transfectants (G, I) and the Gal3 shRNA transfectants (J, L). Bars = 40 μm. (**b**) Cultured melanocytes transfected with control (Mock) or three galectin-3 (305, 306, and 308) shRNAs demonstrating a reduction in expression of galectin-3 (ranging between 20 and 30%) and Tyrp-1 (ranging between 20 and 42%) by silencing. (**c**) Cell lines represented in (**b**) demonstrating a concurrent reduction in the catalytic activity of human Tyrp-1.

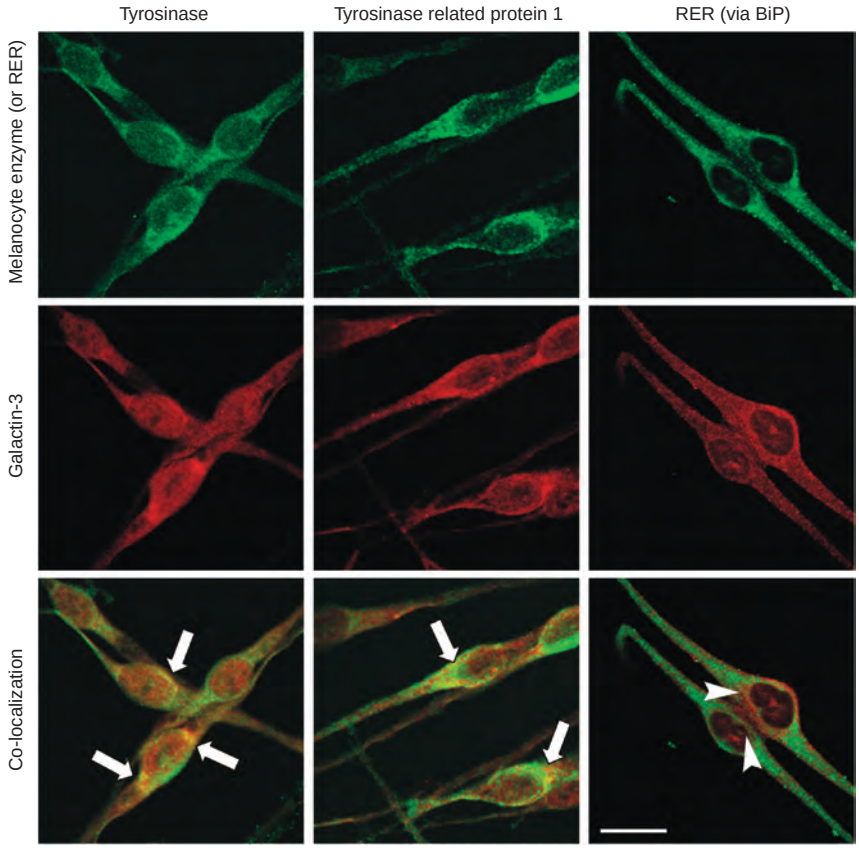

Figure 3. Galectin-3 colocalizes with melanosome-destined cargo. Galectin-3 colocalizes with both tyrosinase (left panel) and tyrosinase-related protein-1 (middle panel). This colocalization is prominent in the peri-Golgi area (arrows) and does not occur in the dendrites. In contrast, the colocalization of galectin-3 with the rough endoplasmic reticulum (RER; right panel) appeared minimal. Bars = 30 μm.

Table 1. Quantification of galectin-3 coexpression with tyrosinase and Tyrp-1

Cell type	Cell area	Colocalized proteins	Mander's coefficient[1]
NHM	Cell body	Galectin-3 and Tyrp-1	0.945 ± 0.04[2],
NHM	Cell body	Galectin-3 and tyrosinase	0.977 ± 0.01
NHM	Cell body	Galectin-3 and RER	0.732 ± 0.02[3]
NHM	Cell body	Galectin-3 and nucleus	0.277 + 0.01[3]
NHM	Entire cell	Galectin-3 and tyrosinase	0.856 ± 0.038
HPS-1 (BLOC-3)	Entire cell	—	0.647 ± 0.116[4]
HPS-2 (adaptin-3)	Entire cell	—	0.693 ± 0.062[4]
HPS-5 (BLOC-2)	Entire cell	—	0.864 ± 0.101

Abbreviations: BLOC, biogenesis of lysosome-related organelle complex; HPS, Hermansky–Pudlak syndrome; NHM, normal human melanocyte; RER, rough endoplasmic reticulum; Tyrp-1, tyrosinase related protein-1.

[1]Mander's overlap coefficient ranges between 1 and 0, with 1 being high colocalization and 0 being low.

[2]Average plus SD.

[3]$P<0.05$ compared with galectin-3 and Tyrp-1 in cell body of NHMs.

[4]$P<0.05$ compared with galectin-3 and tyrosinase in entire cell of NHMs.

similar colocalization patterns with galectin-3. The Mander's coefficient quantifying these colocalizations within the cell body is presented in Table 1. In contrast, colocalization of galectin-3 with the rough endoplasmic reticulum and the nucleus was relatively minimal. The colocalization of galectin-3 with Tyrp-1 and tyrosinase occurs lateral to the Golgi,

where Golgi-derived cargo vesicles containing tyrosinase and Tyrp-1 fuse with either endosomes or stage II melanosomes. Galectin-3 also seemed to be somewhat restricted from the perinuclear Golgi zone. In addition, colocalization between galectin-3 and both tyrosinase and Tyrp-1 does not appear to occur in the dendrites of the melanocytes. This pattern of colocalization between galectin-3 and the two melanocyte-specific enzymes suggests that galectin-3 may be involved in the trafficking of these enzymes to melanosomes.

Colocalization of galectin-3 with melanosome-destined proteins in BLOC-2, BLOC-3, and adaptin-3-deficient HPS melanocytes

HPS-1 melanocytes contain genetic mutations that prevent the normal functioning of the BLOC-3 chaperone. These mutations cause the HPS-1 melanocytes to express macroautophagosomes to which tyrosinase and Tyrp-1 are targeted to because of their ineffective trafficking to melanosomes (Smith *et al.*, 2005). In HPS-1 melanocytes, galectin-3 demonstrated varied amounts of colocalization with melanocyte-specific proteins in HPS-1 macroautophagosomes (Figure 4a). There was either marked, moderate, or no colocalization observed in various cells. This suggests that galectin-3 probably does not uniquely function in the BLOC-3 regulatory step in trafficking, but it may be incorporated into macroautophagosomes by default. In support of this subjective evaluation, the Mander's coefficient demonstrates that the colocalization of galectin-3 with tyrosinase throughout the entire cell is lower in the HPS-1 cells as opposed to control NHMs (Table 1).

HPS-2 melanocytes contain genetic mutations that affect the functioning of the adaptin-3 chaperone. These mutations cause HPS-2 melanocytes to exhibit normal expression of Tyrp-1 and a drastically reduced expression of tyrosinase, which is mainly localized to multivesicular endosomes (Huizing *et al.*, 2001). In HPS-2 melanocytes, galectin-3 exhibited a normal colocalization pattern with Tyrp-1 in the peri-Golgi zone (Figure 4b). Galectin-3 did not colocalize with tyrosinase outside of the Golgi zone in multivesicular endosomes. This suggests that galectin-3 is probably not involved in the adaptin-3 step of cargo trafficking. In support of this subjective evaluation, the Mander's coefficient demonstrates that the colocalization of galectin-3 with tyrosinase is lower in the HPS-2 cells as opposed to control NHMs (Table 1).

HPS-5 melanocytes contain genetic mutations that affect the normal function of the BLOC-2 chaperone. These mutations cause HPS-5 melanocytes to retain tyrosinase and Tyrp-1 in many cargo vesicles that become distributed through the dendrites and impart a fine granular pattern rather than the normal course granular fluorescence pattern in the dendrites (Boissy *et al.*, 2005; Helip-Wooley *et al.*, 2007). In HPS-5 melanocytes, galectin-3 also demonstrated the abnormal fine granular fluorescent pattern resembling cargo vesicles and colocalization with tyrosinase in the dendrites (Figure 4c). In support of this subjective evaluation, the Mander's coefficient demonstrates that the colocalization of galectin-3 with tyrosinase did not differ between the HPS-5 and the control NHMs, in contrast to the difference in HPS-1 and HPS-2 (Table 1). This suggests that galectin-3 may participate in BLOC-2-regulated trafficking step that is thought to function in the recognition, docking, and fusion of cargo vesicles with premelanosomes.

Colocalization of galectin-3 with BLOC-2 and BLOC-3

With immunocytochemistry, galectin-3 appears to colocalize to a greater extent, but not exclusively, to the BLOC-2 component HPS-5 compared with the BLOC-3 component HPS-1. This is seen by a greater overall area of yellow regions in the HPS-5 compared with the HPS-1 melanocytes (Figure 5). This was confirmed by the quantitative analysis of the images using MetaMorph (Supplementary Table 2 online; Supplementary material online). In the latter study, the analysis of the colocalization was restricted to the Golgi and peri-Golgi areas of the melanocytes. The percentages determined by MetaMorph analysis were used in a *t*-test to confirm a significant difference in the colocalization between galectin-3 and HPS-1 versus HPS-5. MetaMorph analysis confirmed the increase in colocalization between galectin-3 and HPS-5 compared with the colocalization between galectin-3 and HPS-1 (Supplementary Table 2 online; Supplementary material online). As HPS-5 is a component of BLOC-2 and HPS-1 of BLOC-3, this suggests that galectin-3 may participate in BLOC-2-regulated trafficking, which functions in the recognition, docking, and fusion of cargo vesicles with premelanosomes, rather than BLOC-3-regulated trafficking.

DISCUSSION

Galectin-3 is a member of a family of carbohydrate-binding proteins that is widely expressed by cells and with multiple functions depending on the cell type (Wang *et al.*, 2004). Within the cell, galectin-3 can regulate cell growth, cell cycle, and apoptosis via its relation to known intracellular pathways associated with these processes (Liu *et al.*, 2002). An interesting and less explored cellular function of galectin-3 is the trafficking of glycoprotein cargo through the cell. In Madin-Darby canine kidney epithelial cells, galectin-3 contributes to delivering non-raft-dependent glycoproteins to the lumen of lactase-phlorizen hydrolase-associated vesicles in a carbohydrate-dependent manner, and depletion of galectin-3 results in missorting of the glycoprotein cargo (Delacour *et al.*, 2006). In the trafficking of non-raft-independent glycoproteins, galectin-3 interacts directly with beta-galactoside moieties of the cargo resulting in cross-linked clusters to facilitate trafficking and targeting (Delacour *et al.*, 2007).

It has previously been demonstrated that galectin-3 exists in the melanocytes (Basrur *et al.*, 2003), as well as in benign and malignant melanocytic lesions (Abdou *et al.*, 2010; Braeuer *et al.*, 2012). In the former report, galectin-3 was identified using melanosomes purified by sucrose density gradient centrifugation and subsequently analyzed by protein digestion and mass spectrometry (Basrur *et al.*, 2003). In our present report, we confirmed that melanocytes express galectin-3 and exhibit a distinct localization pattern prevalent in the cell body peripheral to the Golgi zone. This positions galectin-3 to be a participant or chaperone in the trafficking of melanosome-destined cargo from the Golgi

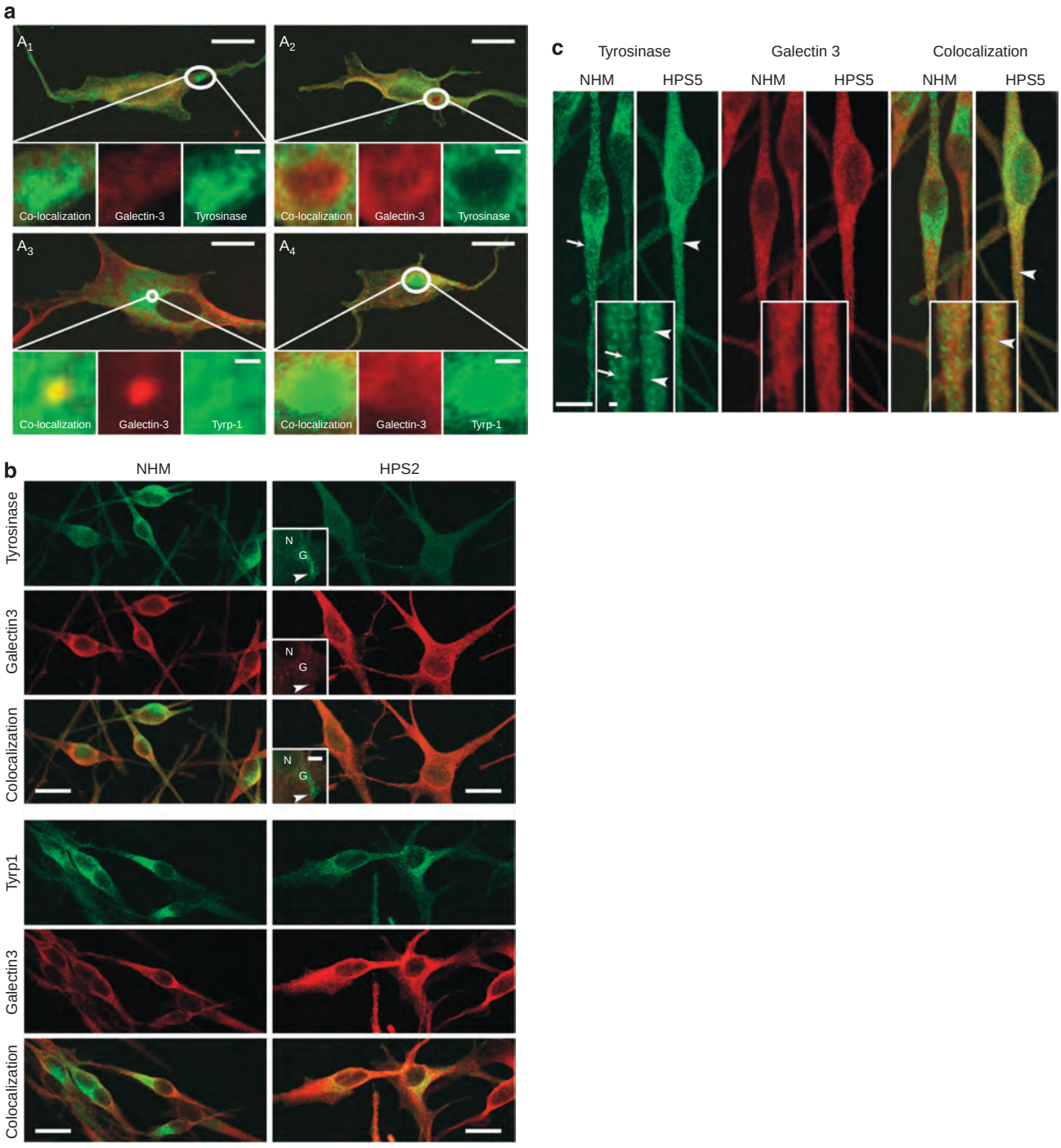

Figure 4. (a) Galectin-3 expression in Hermansky–Pudlak syndrome-1 (HPS-1) melanocytes. In cultured human melanocytes from a patient with HPS-1, galectin-3 exhibits a variation in its localization to macroautophagosomes consisting of (A_1) no, (A_2, A_4) minimal, or (A_3) prominent localization, respectively. In addition, the colocalization of galectin-3 with either tyrosinase or tyrosinase-related protein-1 (TYRP-1) in the macroautophagosomes also varied consisting of (A_1, A_2) no, (A_4) minimal, or (A_3) marked colocalization, respectively. Bars = 10 μm; insets = 3 μm. (**b**) Galectin-3 expression in HPS-2 melanocytes. In cultured human melanocytes from a patient with HPS-2, galectin-3 expression is restricted from the HPS-2-specific macroautophagosomes that accumulate tyrosinase (arrowhead). In contrast, colocalization of galectin-3 with TYRP-1 is normal, i.e., prevalent around the perinuclear area as depicted in Figure 2. G, Golgi zone; N, nucleus. Bar = 10 μm; insets = 5 μm. (**c**) Galectin-3 expression in HPS-5 melanocytes. (Left doublet) In cultured human melanocytes from a patient with HPS-5, tyrosinase exhibits the characteristic fine (arrowheads), as opposed to granular (arrows), staining pattern present in normal human melanocytes (NHMs), particularly noticeable in the dendrites. (Center doublet) Minimal expression of galectin-3 in the dendrites of both cultured NHMs and HPS-5 melanocytes was intensified for observation by increasing the gain of the laser during image capture. (Right doublet) In NHMs, there is minimal colocalization of galectin-3 and tyrosinase. In contrast, in HPS-5 melanocytes, there is colocalization between galectin-3 and tyrosinase in a fine pattern (arrowheads) containing 50-nm vesicles, as opposed to a granular pattern in NHMs. Bars = 10 μm; insets = 2 μm.

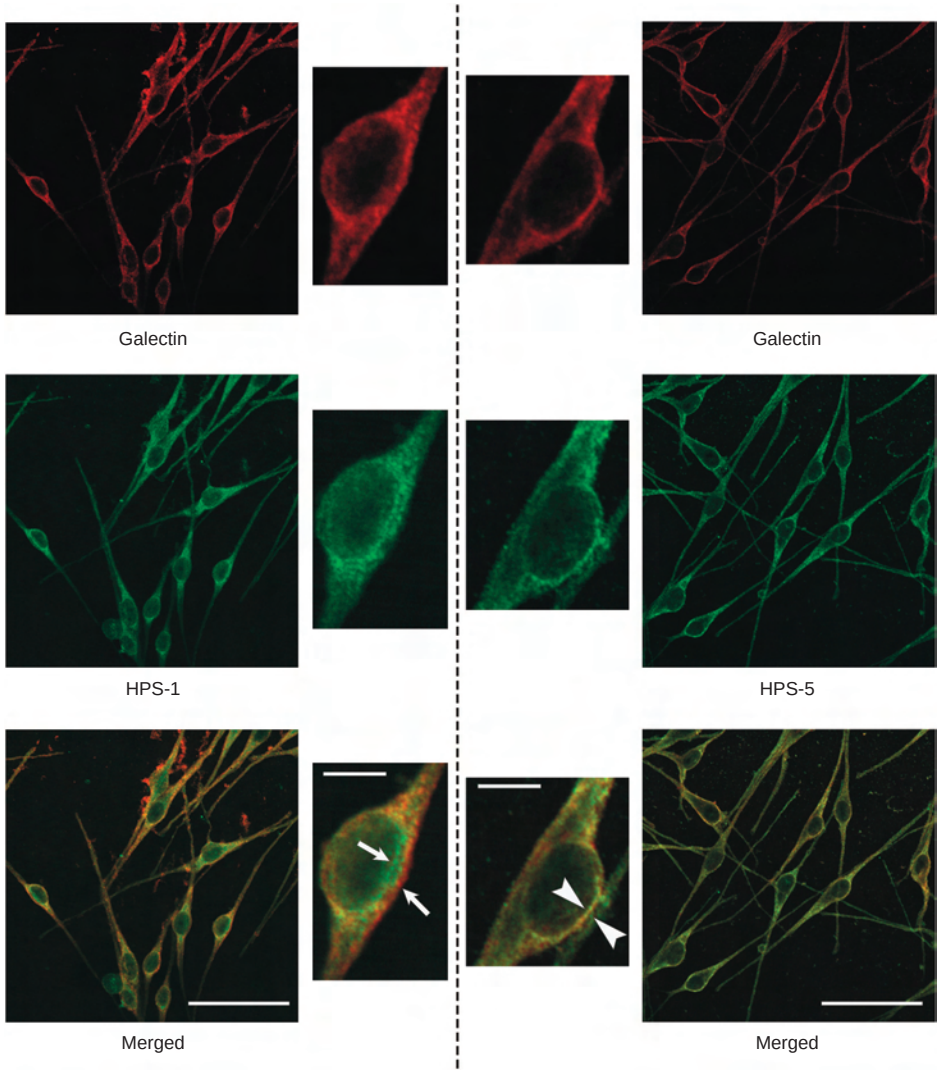

Figure 5. Galectin-3 appears to colocalize more prominently with the biogenesis of lysosome-related organelle complex-2 (BLOC-2) component Hermansky–Pudlak syndrome-5 (HPS-5) than the BLOC-3 component HPS-1. Immunocytochemical staining of normal human melanocytes for galectin-3 with either HPS-1 (left panel) or HPS-5 (right panel) demonstrated relatively minimal colocalization in the former (arrows) and abundant colocalization in the latter (arrowheads). Bars = 50 μm; insets = 10 μm.

to the premelanosome. Colocalization of galectin-3 with tyrosinase and Tyrp-1 at the periphery of the Golgi zone strengthens the hypothesis of this function.

BLOCs have recently been identified as trafficking components in the biogenesis of lysosome-related organelles including the melanosome (Dell'Angelica 2004; Di Pietro *et al.*, 2006). Mutations affecting the functions of BLOCs, as well as the chaperone adaptin-3, result in distinct forms of HPS (Richmond *et al.*, 2005; Boissy *et al.*, 2006). To further explore the role of galectin-3 in trafficking during the biogenesis of the melanosome, we analyzed the expression pattern in melanocytes cultured from patients with HPS. As described in the Materials and Methods section, cultured melanocytes from each form of HPS exhibit distinct defects in the trafficking profile of melanosome-destined cargo. Specifically, mutations affecting BLOC-3 (i.e., HPS-1 and -4) result in the accumulation of macroautophagosomes in the melanocytes where tyrosinase and Tyrp-1 are targeted (Smith *et al.*, 2005). Mutations affecting BLOC-2 (i.e., HPS-3, -5, and -6) result in the accumulation of 50-nm vesicles containing tyrosinase and Tyrp-1 cargo throughout the cell body and dendrites, with the inefficient incorporation of these enzymes into the melanosome (Boissy *et al.*, 2005; Helip-Wooley *et al.*, 2007). Mutations affecting adaptin-3 (i.e., HPS-2) result in the accumulation of tyrosinase, but not Tyrp-1, in mulivescicular bodies of the perinuclear area (Huizing *et al.*, 2001). We rationalized that if galectin-3 participated in the trafficking step governed by either BLOC-3, BLOC-2, or adaptin-3, an aberrant expression pattern for galactin-3 would result as well and mimic that of the associated cargo in melanocytes from the respective HPS form. The data demonstrated that only in BLOC-2-defective HPS-5 melanocytes does the colocalization of galectin-3 uniformly accompany that of the mistrafficked cargo. A recent report demonstrated that galectin-3 expression was increased in HPS pulmonary fibrosis (Cullinane *et al.*, 2014).

To confirm that galectin-3 is predominantly associated with the BLOC-2 pathway, we performed quantitative analysis of the colocalization of galectin-3 with either BLOC-2 (via association with the HPS-5 component) or BLOC-3 (via association with the HPS-1 component). This analysis supported the hypothesis that galectin-3 participates primarily, but not exclusively, in the BLOC-2 trafficking step. The inter-related roles of BLOCs and adaptins in shuttling cargo from the trans-Golgi cisterna, through various endosomal compartments and onto the pre-melanosome, has yet to be clearly delineated. However, it is predicted that these chaperone modules work in tandem and/or concurrent pathway. It is not surprising that galectin-3 had some association with BLOC-3 in the universal mechanism of relaying cargo to the melanosome. Regardless, we demonstrate in preliminary studies that downregulation of galectin-3 results in diminished melanin synthesis and a putative mechanism consists of the inefficient maintenance and/or targeting of at least Tyrp-1 but not tyrosinase to melanosomes that are ultimately translocated down the dendrites.

Galectin-3 binds galactose and lactose residues on glycoproteins. Tyrosinase and Tyrp-1 are replete with these sugar residues to which galectin-3 could conceivably interact (García-Borrón and Solano, 2002). However, this is improbable, as the sugar residues of Tyrp-1 and other cargo are sequestered in the lumen of the transport vesicles and melanosomes, making them inaccessible to cytosolic galectin-3. Of interest is a report of an alternatively spliced form of galectin-3 that contains a single transmembrane-spanning region and a leucine zipper-like stalk domain that positions galectin-3 across the plasma membrane (Gorski *et al.*, 2002). The doublet observed for galectin-3 on western blot analysis (Figure 1aA_1) may represent galectin-3 with and without the transmembrane domain.

Galectin-3 can also participate in protein–protein interactions (Liu *et al.*, 2002) and contains proline/glycine-rich domain (PGAYPGXXX) known to be involved in protein binding (Pawson and Scott, 1997). Galectin-3 directly interacts with the carboxyl terminal 50 amino acids of Gemin4, a member of the SMN (survival of motor neuron) complex (Park *et al.*, 2001). Galectin-3 has been shown to bind Alix (Chen *et al.*, 2009), a component of the endosomal protein sorting complex pathway required for the biogenesis of multivesicular bodies (Odorizzi, 2006). In addition, endosomal protein sorting complex is required for Tyrp-1 transport to the melanosomal membranes (Truschel *et al.*, 2009), similar to BLOC-2. In conclusion, we demonstrate that galectin-3 in the melanocyte is a candidate chaperone molecule during melanosome biogenesis. Where and how galectin-3 molecularly interfaces with the cargo and known chaperones (i.e., endosomal protein sorting complex, BLOCs, adaptins, RABs, and so on) in this pathway needs to be explored.

MATERIALS AND METHODS

Cell culturing

Human melanocyte cultures were established from individual neonatal foreskins obtained from the University Hospital in Cincinnati or from the Christ Hospital in Cincinnati (IRB #03-1-23-1). Patient consent for experiments was not required because USA laws consider human tissue left over from surgery as discarded material. HPS cell cultures were established from skin biopsies provided by W Gahl (NIH, Bethesda, MD) from patients enrolled in a protocol approved by the National Human Research Institute Review Board with written informed consent. This study was conducted according to the declaration of Helsinki principles. Tissues were transported to the laboratory in DMEM with 2% antibiotic/antimycotic. Using routine sterile techniques, the melanocytes were isolated from the tissues as follows. Tissues were incubated in 0.25% trypsin at 4 °C overnight. The epidermis was separated from the dermis by vortexing in phosphate-buffered saline (PBS). The tissue was pelleted at 1,500 r.p.m. for 5 minutes at 4 °C, and the PBS supernatant was aspirated off. The epidermis and cell suspension were resuspended in either melanocyte or keratinocyte medium, and the dermis was resuspended in fibroblast growth medium and plated in a T25 corning tissue culture flask. The melanocyte growth medium consisted of MCDB-153 medium (Sigma-Aldrich, St Louis, MO) supplemented with 4% fetal bovine serum (Fisher Scientific, Pittsburgh, PA), 5 μg ml^{-1} insulin, 0.6 ng ml^{-1} basic fibroblast growth factor (PeproTech, Rocky Hill, NJ), 13 μg ml^{-1} bovine pituitary extract (Hammond Cell Tech, Windsor, CA), 8 nM 12-O-tetradecanoylphorbol-13-acetate, and 1% antibiotic/antimycotic (Fisher Scientific). The keratinocyte growth medium consisted of KC Epilife supplemented with HKGS (Cascade Biologics, Portland, OR) and 1% antibiotic/antimycotic. The fibroblast growth medium consisted of DMEM medium (Sigma, St Louis, MO) supplemented with 5% fetal bovine serum, 2 mM glutamine, 0.5 mM sodium pyruvate, and 1% penicillin–streptomycin (10,000 units ml^{-1} and 10,000 mg ml^{-1}, respectively) (Gibco, Carlsbad, CA). SKMEL-188 (a gift from R Srinivasan, Sloan-Kettering Institute for Cancer Research, New York, NY) was maintained in the DMEM medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine (Fisher Scientific), 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA), and 1% antibiotic/antimycotic. All the above reagents were purchased from Sigma-Aldrich unless otherwise stated. Cultures were maintained in a humidified incubator with 95% air and 5% CO_2 at 37 °C. Subconfluent melanocytes and fibroblasts at passages 2–7 and keratinocytes at passages 1–2 were used for all experiments.

Western blotting

Cells were lysed in RIPA extraction buffer and analyzed for their protein content. Each lane on a 10% acrylaminde gel was loaded with 30 ug of protein. After electrophoresis and transfer, the blot was blocked in 5% milk in TNET (Tris-buffered saline-Tween) overnight, stained with amido black and destained, and immunoblotted with rabbit anti-galectin-3 (1:2,000) (Novus Biologicals, Littleton, CO), mouse anti-Tyrp-1 (Mel 5, Covance, Princeton, NJ), or rabbit anti-actin horseradish peroxidase-conjugated (Santa Cruz, Dallas, TX) antibodies for 2 hours. The blot was washed three times in TNET buffer, incubated in goat anti-rabbit horseradish peroxidase in 5% milk in TNET (1:5,000 Santa Cruz), and washed twice with a final rinse in Tris-buffered saline. The blot was then developed using a Pierce ECL kit in a dark room (Pierce, Rockford, IL).

To assess for the secretion of galectin-3, the medium from NHM cultures was extracted and saved, whereas the cells were trypsinized and pelleted. One milliliter of medium was put into a centrifuge tube along with 4 ml of −20 °C acetone. The tube was vortexed and stored for 1 hour in a −20 °C freezer. The tube was centrifuged for 10 minutes at 13,000 r.p.m. at 4 °C. The supernatant was removed without dislodging the protein pellet, and the extra acetone was

allowed to evaporate from the uncapped tube in a fume hood at room temperature for 30 minutes. A volume of 100 ul of RIPA + PI buffer was added to the pellet and it was vortexed to bring it to solution. This acetone-precipitated sample was then used in a western blot as described above.

Silencing methodology

Normal human melanocytes were plated in six-well plates at a concentration of 2×10^5 per well in 1 ml of melanocyte growth medium. The cells were incubated overnight at 37 °C, 5% CO_2 to allow them to attach. The cells were then infected with 2.5 ug ml^{-1} polybrene and 1.25×10^5 IFU shRNA. Each plate contained a well that was not infected, plus a well that contained either control shRNA (sh-), green fluorescent protein shRNA, or one of six different galectin-3 shRNA. A dark line (dk) and one medium line (med) were infected with Santa Cruz cat # sc-108080, sc-108084, and sc-155994-V in the green fluorescent protein–infected well. A light line was infected with one of five shRNA galectin 3 lentivirus plus a green fluorescent protein clone and a mock shRNA clone obtained through Cincinnati Children's Hospital Medical Center Heart Institute's Lenti-shRNA Library Core. The viruses were generated using Sigma TRCN clones TRCN0000029304, TRCN0000029305, TRCN0000029306, TNCR0000029307, and TRCN0000029308 (Sigma). For the dk and the med lines, the medium containing lentiviral particles was aspirated and replaced with fresh melanocyte growth medium. For the light line, 40 ul of virus was added and the cells were left for 5 days. A second round of infection was done with 40 ul more plus 0.8 ug ml^{-1} hexadimethrine bromide (Sigma H9268). After infection of all groups, puromycin (Sigma P8833) was added at a concentration of 2 ug ml^{-1}. The cells were treated with several rounds of puromycin selection. After sufficient puromycin selection, the cells were used for immunofluorescence analysis, western blot analysis, and for the determination of melanin content.

Quantification of melanin content

Total melanin content was determined as follows. Cells were pelleted after trypsinization and lysed (50 mM Tris pH7.54, 2 mM EDTA pH 7.87, 150 mM sodium chloride, 1% Triton-X 100) in the presence of protease inhibitors (Complete, Roche, Indianapolis, IN). Cell extracts were spun at 14,000 r.p.m. for 5 minutes at 4 °C. The supernatant was removed and protein content was determined (Pierce). The pellet was washed twice in ethanol: ether (1:1) and dissolved in 0.2 N sodium hydroxide at 60 °C. A 75-µl aliquot was measured for absorbance at 490 nm in a plate reader (Bio-Rad, Hercules, CA).

Tyrp-1 assay

To assess the tyrosine hydroxylase activity of Tyrp-1, we used the method developed by Zhao *et al.* (2004). Specifically, melanocyte lysates (10–25 µg protein) were incubated in 250 µl of reaction mixture (0.1 µCi ml^{-1} tyrosine plus 0.08 mM unlabeled tyrosine) for 19 hours at 37 °C. Subsequently, tritium H_2O produced was assessed with a Packard 1900 CA scintillation analyzer (PerkinElmer, Shelton, CT).

Immunofluorescent methodology

Cells were plated on chamber slides at 2×10^4 cells per well and fixed after 24 hours with 3% paraformaldehyde. Cells were washed with PBS and blocked with a 5% BSA solution. Primary antibodies at optimized concentrations in a 1% BSA/PBS solution were added to the slide and left at room temperature for 1 hour. Cells were washed and Alexa Fluor secondary antibodies were added at a 1:250 ratio in a 1% BSA/PBS solution at room temperature for 45 minutes in the dark. The slide was mounted and allowed to dry overnight before viewing on a Zeiss LSM510 META confocal microscope (Zeiss, Thornwood, NY). The primary antibodies used consisted of goat polyclonal antiserum to galectin-3 provided by F-T Liu (University of California at Davis, Sacramento, CA); rabbit polyclonal antiserum to tyrosinase provided by R King and W Oetting (hPEP1; University of Minnesota, Minneapolis, MN); mouse monoclonal antiserum to Tyrp-1 (Mel-5; Signet Laboratories, Dedham, MA); rabbit polyclonal antiserum to BiP (Santa Cruz); rabbit polyclonal serum to the HPS-1 and HPS-5 proteins provided by B Gahl (NIH); rabbit polyclonal antiserum to adaptin-3 provided by MS Robinson (β3A; Cambridge Institute for Medical Research, Cambridge, UK).

Metamorph methodology

For quantification of the expression of tyrosinase and Tyrp-1 in control and galectin-3-silenced NHMs, captured photographs were cropped to include the entire cell ($n = 25$ per group), and fluorescence intensity per cell was quantified using MetaMorph (Molecular Devices, Sunnyvale, CA) and expressed as signal intensity per cell. For quantifying the expression of galectin-3 with HPS-1 or HPS-5, captured photographs were cropped to include only individual cell's nuclei and surrounding cell body ($n = 25$). Dendrites were not included in the cropped images. In the former group, the fluorescence intensity per cell was quantified using the quantitative software MetaMorph and expressed as signal intensity in the former group. In the latter group, the color channels were separated, each of which represented a different primary antibody: either galectin-3, HPS-1, or HPS-5. Each color channel from each photograph was set on a threshold value, and then the colocalization of each color was measured. A *t*-test was done on the colocalization values comparing galectin-3 with HPS-1 and galectin-3 with HPS-5 to test for a significant difference between the primary antibodies.

Analysis of Mander's coefficient

To quantify the Mander's coefficient for colocalization of (a) galectin-3 with Tyrp-1 or tyrosinase in cell bodies (20 cells per group) or (b) galectin-3 with tyrosinase in HPS cells (25 cells per group), the following was performed. Digital images were obtained using the Zeiss LSM510 META confocal microscope. Using the quantitative software Image J (NIH, Bethesda, MD), images were analyzed with the Intensity Correlation Analysis plugin that generated Mander's coefficients. Mander's overlap coefficient ranges between 1 and 0, with 1 being high colocalization and 0 being low. Appropriate thresholds were set for each image to eliminate noise.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was funded by a National Institutes of Health grant R21 AR056059 (REB). The authors appreciate the recommendations of the reviewers.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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