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Essential role of the molecular chaperone gp96 in regulating melanogenesis

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Summary

Through a process known as melanogenesis, melanocyte produces melanin in specialized organelles termed melanosomes, which regulates pigmentation of the skin, eyes and hair. Gp96 is a constitutively expressed heat shock protein in the endoplasmic reticulum whose expression is further up-regulated upon ultraviolet irradiation. However, the roles and mechanisms of this chaperone in pigmentation biology are unknown. In this study, we found that knockdown of gp96 by RNA interference significantly perturbed melanin synthesis and blocked late melanosome maturation. Gp96 knockdown did not impair the expression of tyrosinase, an essential enzyme in melanin synthesis, but compromised its catalytic activity and melanosome translocation. Further, mice with melanocyte-specific deletion of gp96 displayed decreased pigmentation. A mechanistic study revealed that the defect in melanogenesis can be rescued by activation of the canonical Wnt pathway, consistent with the critical roles of gp96 in chaperoning Wnt-co-receptor LRP6. Thus, this work uncovered the essential role of gp96 in regulating melanogenesis.

Keywords

Gp96; Melanogenesis; Tyrosinase; Melanosome

Introduction

Melanin is a heterogeneous biopolymer produced only by melanocytes, which synthesize and deposit the melanin pigment in specialized membrane-bound organelles known as melanosomes. Ultraviolet (UV) radiation stimulates melanin production which acts as a

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direct shield against UV-induced damage, via its optical and chemical filtering properties, thus playing an important protective role in the skin against UV-induced DNA damage and cancer (Agar and Young, 2005, Abdel-Malek et al., 2010). Understanding the mechanisms of melanin synthesis and melanosome formation is important for effective prevention and treatment for diseases of pigmented cells such as vitiligo.

Melanogenesis has been an area of intense research for many decades, and significant advances have been made in understanding the mechanisms of melanogenesis through studies of various pigmentary diseases, such as Oculocutaneous Albinism (OCA) (Gronskov et al., 2007) and Hermansky-Pudlak syndrome (HPS) (Boissy et al., 2005). Genes that regulate pigmentation in humans include Tyrosinase, P-locus, Tyrosinase-related protein 1 (TYRP1) and membrane-associated transporter protein (MATP), mutations of which cause OCA1, OCA2, OCA3 and OCA4 respectively (Gronskov et al., 2007). A large number of genes have been linked to the various forms of HPS in humans, and so far 10 subtypes of HPS have been defined, all being associated with the trafficking of proteins to lysosome-related organelles (LROs), including melanosomes. Patients with HPS have hypopigmentation but also have disrupted functions in other tissues, especially those related to LRO function. It is clear that a number of factors involved in the post-translational modification and trafficking of melanosome (and other LROs) constituents are critical to the functions of those organelles and disruptions of those cause hypopigmentary diseases such as OCA and HPS. Thus, although much is now known about factors involved in the assembly of melanosomes, many aspects of that process remain poorly understood.

As a member of the HSP90 family, gp96 (also known as HSP90b1 or grp94) is a molecular chaperone localized in the lumen of the endoplasmic reticulum (ER) and is important for protein maturation and homeostasis (Randow and Seed, 2001, Yang and Li, 2005, Liu et al., 2010). Gp96 is ubiquitously expressed, and its expression can be further induced upon a variety of stress conditions such as glucose starvation, ER-calcium store depletion, glycosylation blockade, reducing environment, proteasome inhibition and overexpression of misfolded proteins in the ER (Kozutsumi et al., 1988, Kim et al., 1987, Bush et al., 1997, Ramakrishnan et al., 1995, Christianson et al., 2008). A previous study suggested that gp96 is a constituent of melanosomes (Basrur et al., 2003); yet, the role of gp96 in melanogenesis is unknown. Herein, we addressed the essential role of gp96 in melanogenesis using a short-hairpin RNA (shRNA) strategy and a melanocyte-specific gp96 knockout mouse model. We demonstrate that gp96 is essential for melanin synthesis and that its function is critical to the normal distribution of tyrosinase to late melanosomes

Results

Knockdown of gp96 reduces melanin production

Using the well-characterized and well-pigmented B16F1 murine melanoma cell line, we found that gp96 can be significantly induced both at the mRNA and the protein levels upon UVB exposure (Fig.S 1A, 1B). To study the role of gp96 in melanogenesis, we knocked down gp96 in B16 cells using a lentivirus-based shRNA strategy as previously described (Liu et al., 2010). An immunoblot assay demonstrated that gp96 was efficiently depleted in gp96 shRNA transduced knocked down (KD) cells compared with a control virus

transduced wild type (WT) cells (Fig. 1A). Remarkably, efficient depletion of gp96 in B16 cells led to significant hypopigmentation (Fig. 1B). As melanin levels are responsible for the pigmentation of melanocytes, we quantified melanin level both in B16-KD and in B16-WT cells. A significant reduction of melanin content was observed in B16-KD cells compared to control B16-WT cells (Fig. 1C). These data indicate that gp96 is essential for melanin homeostasis.

Melanocyte-specific deletion of gp96 leads to depigmentation

To address the role of gp96 in melanogenesis *in vivo*, we crossed *Tyr-cre/ERT2* mice with *Hsp90b1^{flox/flox}* mice to generate a Tamoxifen-inducible melanocyte-specific gp96 knockout (KO) mouse model. We topically applied 4-hydroxytamoxifen (4-OHT) to shaved skin on the right flank of *Tyr-cre/ERT2-Hsp90b1^{flox/flox}* (KO) mice to induce specific gp96 deletion from skin melanocytes and left flank were treated with vehicle. The color of the re-grown coat hair was then monitored. The vehicle-treated left flank of KO mice regrew black hair, whereas hairs that regrown in the 4-OHT-treated area were grey/white in color (Fig. 2A). For the control, *Tyr-cre/ERT2-Hsp90b1^{flox/wt}* mice with topical treatment of either 4-OHT or vehicle did not result in a change of hair color. Further histological study demonstrated decreased levels of melanin in hair bulbs and hair shafts in the 4-OHT treated areas in KO mice compared to WT mice (Fig. 2B, 2C). Taken together, we conclude that gp96 is essential for melanin synthesis.

Knockdown of gp96 impairs melanosome maturation

Melanin is synthesized and stored in melanosomes (Orlow, 1995). Melanosome biogenesis occurs through distinct sequential morphological steps. Stage I early melanosomes are round, membrane-bound structures that are generally found in the perinuclear area. As they mature into stage II melanosomes (premelanosomes), they develop a characteristic array of fibrous luminal striations which serve as sites for melanin deposition. In subsequent stages of maturation, melanogenic enzymes are trafficked to premelanosomes and initiate melanin biosynthesis, leading to the development of stage III partially pigmented melanosomes and stage IV fully pigmented melanosomes. We examined B16-KD and B16-WT cells using transmission electron microscopy. Consistent with the decreased level of melanin, we observed a significant reduction of stage III and IV melanosomes in B16-KD cells compared with B16-WT cells (Fig. 3), further indicating that gp96 is required for full melanosome maturation.

Knockdown of gp96 disrupts the enzymatic function and trafficking of tyrosinase

To address the underlying mechanism by which gp96 is involved in melanin synthesis, we examined the expression levels of Tyrosinase, Tyrosinase-related protein-1 (Tyrp1) and Tyrosinase-related protein-2 (DCT), all of which are important for melanin synthesis. We observed no change in the expression levels of these melanogenic enzymes in the presence or absence of gp96 (Fig. 4A). Furthermore, no difference was observed in the Endoglycosidase H sensitivity of these proteins in the presence or absence of gp96, suggesting that they are properly transported through the ER to the post-ER compartments (Fig. 4B-4D). As expression levels of those melanogenic enzymes were not changed and

thus not reflect the reduced pigmentation, we further analyzed the catalytic activity of tyrosinase, the first and rate-limiting enzyme in the biochemical synthesis of melanin (Raposo and Marks, 2007). The enzymatic activity was measured based on the conversion of L-DOPA into DOPAchrome via DOPAquinone using whole cell lysates of WT and gp96 KD cells. We found that the catalytic activity of tyrosinase from B16-KD melanocytes was significantly reduced when compared with control B16-WT cells (Fig. 5A). The compromised enzymatic activity was also confirmed by an in-gel activity assay when the solubilized cell lysates were resolved first by non-reducing SDS-PAGE followed by incubation with L-DOPA and staining with 3-methyl-2-benzothiazolinone hydrazone (Fig. 5B). With the B16-WT lysate, the DOPA oxidase activity was resolved around the ~70 kDa region, corresponding to the molecular weight of tyrosinase (Figure 5B). Taken together, our results indicate that gp96 plays an important role in melanogenesis by controlling tyrosinase function but not its expression.

We then examined the subcellular localization of tyrosinase in melanocytic cells in the presence or absence of gp96 function using confocal microscopy (Fig. 5D). First, we confirmed the localization of gp96 in the ER of B16-WT cells using Bip (also known as Grp78) as a specific marker of the ER. Most gp96 colocalized with Bip and a minor amount colocalized in the Golgi (GM130); none colocalized with lysosomal markers LAMP1 or LAMP2 (Figure 5D). HMB45 is a specific marker of early (stage II) melanosomes whereas α -PEP7 stains tyrosinase which is localized in stage III and stage IV late melanosomes (Figure 5C). Interestingly, in B16-WT cells, there is a minor colocalization of tyrosinase with gp96 but no colocalization of gp96 and HMB45. The distribution of early melanosomes (HMB45-positive) is in the perinuclear area and the more mature melanosomes (α PEP7-positive) are distributed more in the dendritic peripheries of the cells (indicated by white arrows). However, in the B16-KD cells, no gp96 was detectable (as expected) and the distribution of HMB45 was unaffected. However, tyrosinase distribution in B16-KD cells was homogenous throughout the cytoplasm and was not localized peripherally in late melanosomes. This is consistent with the disruption of the enzymatic function of tyrosinase in melanosomes and the decreased pigmentation that results in the absence of gp96 function.

gp96 modulates tyrosinase activity by controlling the WNT/LRP6/ β -catenin signaling pathway

Previous studies have reported that activation of the Wnt canonical pathway is essential for melanogenesis and melanocyte differentiation by upregulating the expression of melanogenesis-associated enzymes (Bellei et al., 2008, Chien et al., 2009). We demonstrated recently that gp96 is a critical chaperone for the Wnt co-receptor LRP6 (Liu et al., 2013). Gp96 is required for full maturation and surface expression of LRP6. Furthermore, without gp96, LRP6 does not interact efficiently with MesD, and there is a total loss of canonical Wnt signaling. Thus, we asked if the hypopigmentation in gp96 KO B16 cells was due to a deficiency of the canonical Wnt/ β -catenin pathway. To address this possibility, we activated the Wnt/ β -catenin signaling pathway in B16-KD cells using the GSK3 β inhibitor TWS119. We found that treatment with TWS119 can rescue the pigmentation of B16-KD cells (Figure 6A), which corresponds to the restoration of tyrosinase enzymatic activity (Figure 6B and C). Consistent with a previous report (Bellei et al., 2008), the GSK3 β inhibitor TWS119 can

dramatically up-regulate tyrosinase expression both in B16-WT and in B16-KD cells (Figure 6D). Given the lack of expression of Wnt co-receptor by gp96 KO cells, treatment of cells with Wnt3a however can only induce more pigmentation from WT cells but not from gp96 KD cells (Figure 6E).

Discussion

Melanogenesis is a complex metabolic pathway controlled by a family of enzymes known as tyrosinase-related proteins, which are synthesized and glycosylated in the ER and Golgi and then further trafficked to melanosomes where they synthesize and deposit melanin. A previous proteomics study showed that the ER resident chaperone gp96 is also present within melanosomes (Basrur et al., 2003) and our results in this study demonstrate that knockdown of gp96 dramatically reduced pigmentation. Furthermore, we revealed that gp96 positively regulates melanogenesis and melanosome development by facilitating tyrosinase trafficking and enzymatic function via chaperoning the Wnt canonical pathway.

ER has a quality-control system for ‘proof-reading’ newly synthesized proteins, so that membrane-bound and secretory proteins leave the ER pool and are transported to the cell surface only if they are correctly folded, modified and assembled in the ER (Yang and Li, 2005, Ellgaard and Helenius, 2003). A number of chaperone proteins, including Bip, calnexin, calreticulin, gp96, thiol-disulphide oxidoreductase, protein disulphide isomerase (PDI) and ERp57, within the ER act in concert to guide protein maturation through trafficking, folding, assembling and sorting, and preventing the export of improperly folded protein from the ER (Yang and Li, 2005, Ellgaard and Helenius, 2003). Tyrosinase is a type I membrane glycoprotein trafficked to and localized in the melanosomal membrane which requires proper N-glycosylation for its enzymatic activity (Petrescu et al., 1997, Takahashi and Parsons, 1992, Negroiu et al., 1999). During these glycosylation and maturation processes, multiple ER lectin chaperones, such as calnexin/calreticulin and Bip, have been reported to interact with tyrosinase (Halaban et al., 2000, Toyofuku et al., 2001), and play an essential role in folding and endowing the enzymatic activity of tyrosinase. In this study, we found that gp96 is required for tyrosinase function, but not its expression level, proper glycosylation and acquisition of Endo H resistance. Immunoprecipitation studies do not show a direct interaction between gp96 and tyrosinase, yet knockdown of gp96 function impairs tyrosinase trafficking to late melanosomes. These results suggest that gp96 may not chaperone tyrosinase *per se* or that the role of gp96 in folding tyrosinase is functionally important but is biochemically and subtly mediated by a very low affinity interaction, which is consistent with the general lack of colocalization of gp96 and tyrosinase by immunohistochemical analysis. Alternatively, since gp96 is physically localized in melanosomes, it remains possible that gp96 is required for regulating tyrosinase trafficking via a glycosylation-independent fashion, directly or indirectly, to early melanosomes for exerting its optimal catalytic activity. Fully elucidating the role of gp96 as a chaperone to promote trafficking of tyrosinase to late melanosomes will be the focus in future study. Intriguingly, we found that the defect in melanogenesis in the absence of gp96 can be rescued by activating the canonical Wnt pathway. The Wnt canonical signaling pathway is essential in regulating melanocyte differentiation and pigmentation (Bellei et al., 2008, Chien et al., 2009, Yamaguchi et al., 2008, Yamaguchi et al., 2004, Rabbani et al., 2011).

DKK1, an antagonist of the canonical Wnt signaling pathway, can bind to LRP6 with high affinity and prevents the Frizzled-Wnt-LRP6 complex formation. Yamaguchi *et al.* found that DKK1, which is secreted at a high level by fibroblasts in the skin on the palms and soles, impairs skin pigmentation (Yamaguchi et al., 2008, Yamaguchi et al., 2004). We demonstrated recently that the Wnt co-receptor LRP6 is a *bona fide* client of gp96, in that deletion of gp96 abrogates LRP6 surface expression, resulting in the loss of canonical Wnt signaling (Liu et al., 2013). In this study, we observed that activation of the Wnt downstream signaling pathway with the GSK3 β inhibitor TWS119 rescued the hypopigmentation of B16-KD cells, which correlated with the tyrosinase expression level as well as its enzymatic activity. How melanogenesis is positively regulated by the Wnt pathway is not entirely clear. Possibilities include the roles of Wnt signaling in directly upregulating melanogenic enzymes, in facilitating melanosome assembly, in controlling trafficking of OCA gene products to the proper subcellular compartments and/or in regulating possible yet unidentified co-factors for melanogenesis.

Human skin is repeatedly exposed and damaged by various environmental insults, especially by solar UV radiation. UVB is particularly cytotoxic and mutagenic. It can cause sunburn, cataract formation, and has been associated with the development of skin cancers (Brenner and Hearing, 2008). Epidermal melanin has important physiological protection implications because of its optical and chemical filtering properties (Brenner and Hearing, 2008). Indeed, higher skin pigment levels are closely associated with lower latitude region and higher UV radiation levels, indicating that epidermal pigmentation is a critical protective mechanism against UV radiation-induced damage. In this study, we have conclusively linked the function of gp96 with an essential role in regulating melanogenesis. Further studies into the precise mechanism of gp96 in this process during normal and UVB-stressed conditions may uncover the clinical implications of our findings in a variety of skin pigmentation disorders, including vitiligo and solar lentigines as well as melanoma.

Materials and Methods

Mice

Hsp90b1^{flox/flox} mice were described previously (Yang et al., 2007, Staron et al., 2011) and have been backcrossed onto C57BL/6 for more than 10 generations. *Tyr-cre/ERT2* mice (stock No. 012338) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed and treated in accordance with protocols approved by the Institutional Care and Use Committee for animal research at the Medical University of South Carolina.

Cell culture and gp96 knockdown

B16 melanocytes were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The gp96 knockdown in B16 cell line was generated by transduction with lentivirus-based pLL3-gp96 shRNA system as previously reported (Liu et al., 2010).

Measurement of melanin

For determination of melanin content, B16 cells were washed with phosphate-buffered saline and boiled in 1 N NaOH for 15 min. The absorbance at 400 nm was measured with a multi-well plate reader (Bio-Rad, Hercules, CA).

Assay of tyrosinase activity

Tyrosinase activity was assayed as described by Yang et al. (Yang et al., 2006). Briefly, cells were washed twice with PBS, and lysed with 20 mM Tris-0.1% Triton X-100 (pH 7.5) in PBS buffer. After centrifugation to remove the nuclei, tyrosinase activity in the supernatant was analyzed spectrophotometrically by following the oxidation of DOPA to DOPACHrome at 475 nm. The reaction mixture containing 100 μ L of freshly prepared substrate solution [0.1% L-DOPA in 0.1 mol/L sodium phosphate (pH 6.0)] and 50 μ L of enzyme solution was incubated at 37°C. The absorbance change was measured during the first 10 min of the reaction while the increase of the absorbance was linear, and corrections for auto-oxidation of L-DOPA in the controls were made. The specific activity of Tyrosinase in gp96 KD B16 cells was normalized by the control cells. For in gel colorimetric staining, cell lysates were subjected to 12% acrylamide gels under non-reducing conditions. Electrophoresis was performed at 4°C, and at a constant current of 25 mA. Colorimetric staining in the presence of 3-methyl-2-benzothiazolinone hydrazone was carried out by incubating the gels, previously equilibrated at pH 6.0, at 37°C from 15 min to 30 min in a solution of 1.5 mM L-DOPA supplemented with 4 mM 3-methyl-2-benzothiazolinone hydrazone (Jimenez-Cervantes et al., 1993).

Western blot and antibody

Western blotting was performed on cell lysates in RIPA buffer with a cocktail of protease inhibitors P2714 (Pierce Chem Co, Rockford, IL location) (Morales et al., 2009, Liu et al., 2012). The protein concentration of each sample was determined by the Bradford method according to manufacturer's protocol (Bio-Rad, Hercules, CA). Samples were applied to polyacrylamide SDS gels and subjected to electrophoresis, followed by protein transfer to a nylon membranes and immunoblotting with the specified antibodies. The Tyrosinase (EP1577Y) rabbit monoclonal antibody was purchased from Epitomic Co. (Burlingame, CA). Polyclonal antibodies for Tyrosinase (α -PEP7) (Jimenez et al., 1991), Tyrp1 (α -PEP1) (Jimenez et al., 1988) and Dct (α -PEP8') (Tsukamoto et al., 1992) were previously described. The gp96 antibody (9G10) was obtained from Stressgen (Victoria, BC, Canada) and the β -actin antibody (AC-74) was purchased from Sigma (St. Louis, MO).

RT-PCR

After 50 mJ/cm² ultraviolet irradiation with wavelength 405 nm (UVP, LLC), total RNA was extracted from cultured B16 cells at indicated times using TRIZOL (Invitrogen Life Technologies, Grand Island, NY). Total mRNA was then reverse-transcribed using oligo (dT)12–18 primer (Promega, Madison, WI) according to the manufacturer's protocol. Complementary DNAs of interests were quantified by quantitative-polymerase chain reaction (Q-PCR) with a Bio-Rad iCycler, in triplicate. Q-PCR data were analyzed with the corresponding software, and the number of PCR cycles to reach the threshold of detection

was calculated. β -Actin (used as an internal control) and gp96 were amplified using the following primers: β -Actin: forward, 5'-GGTCATCACTATTGGCAACG-3', reverse 5'-ACGGATGTCAACGTCACACT-3'; gp96: forward, 5'-TCGTCAGAGCTGATGATGAAGT-3'; reverse, 5'-GCGTTTAACCCATCCAACCTGAAT-3'.

Electronic microscopy

Cells grown in T25 flasks were fixed with 2% Cacodylate Buffered Glutaraldehyde for 30 min and were then rinsed with Cacodylate Buffer. After fixation, samples were incubated with 2% Aqueous Osmium Tetroxide for 30 min. Samples were then dehydrated, followed by embedding and sectioning. Images were acquired using a JEOL JEM 1010 Transmission Electron Microscope.

Immunofluorescence and confocal microscopy

The expression of proteins of interest was detected by indirect dual immunofluorescence as described previously (Valencia et al., 2006). Briefly, cells were seeded at a density of 5×10^3 cells/well into 2 well plastic chamber-slides (Fisher, Pittsburgh, PA). Cells were cultured for at least 48 hr prior to fixation with 4% paraformaldehyde for 20 min at 4°C. Then, cells were permeabilized with cold methanol or Triton X100 (for Bip only) for 20 min at 4°C, were air dried and rehydrated with PBS. Cells were then blocked with 5% normal goat serum (NGS) and normal horse serum (NHS) (Vector Labs, Burlingame, CA, USA), and then incubated with primary antibodies overnight at 4°C. The murine anti-tyrosinase antibody, α PEP7 is a rabbit polyclonal antibody (Jimenez et al., 1991). Antibodies against cytoplasmic markers were all mouse monoclonal antibodies and were from different sources: a) Cell Signaling (Danvers, MA): BIP, endoplasmic reticulum, GM130 (Golgi), LAMP1 and LAMP2 (lysosomes); b) Santa Cruz (Santa Cruz, CA): HMB-45 (pre-melanosomes). The next day, cells were washed 3 times with PBS and were then incubated with a biotinylated anti-rabbit secondary antibody (Vector) for 30 min and then were washed 3 times with PBS. Cells were incubated for 45 min in a solution containing: 2% NHS, FITC-streptavidin and Texas-red anti-mouse antibody (Vector, Burlingame, CA). Slides were mounted with mounting medium containing DAPI (Vector) for nuclei counterstain. Confocal images were acquired using a Zeiss LSM 780 (Jena, Germany).

Statistical analysis

Results are presented as mean $\pm$ standard deviation (SD). All experiments were repeated at least 2 times, with documented reproducibility. Data were analyzed for statistical significance using Student's *t*-Test. P values of <0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of significance

Using the combination of genetic and biochemical tools, this work uncovered for the time the critical roles of a molecular chaperone gp96 in melanogenesis. In particular, gp96 is shown to be functionally required for melanin synthesis and for controlling the enzymatic activity of tyrosinase. This study suggests that stress induction of gp96 is a novel protective mechanism against UV-induced skin damage. Targeted inhibitor against gp96 may prove useful, cosmetically and medically, for the treatment of a variety of skin hyperpigmentation diseases.

Fig. 1.

Knockdown of gp96 in B16 cells inhibits melanin synthesis. (A) Knockdown of gp96 in B16 cells was achieved using a lentiviral shRNA system. gp96 silencing was confirmed by Western blot. (B) Gp96 knockdown B16 cell (B16-KD) pellets show decreased pigmentation compared with control vector transduced B16 cell pellets (B16-WT). (C) Quantification of melanin content in B16-KD and B16-WT cells (Mean $\pm$ SD). Shown is a representative data from three independent experiments. *

P<0.05, ** P<0.01

Fig. 2.

Deficiency of gp96 in melanocytes results in decreased hair pigmentation. (A) Hair on the backs of *Tyr-cre/ERT2-Hsp90b1^{flox/flox}* (Knockout, KO) and *Tyr-cre-Hsp90b1^{flox/wt}* (Wild type, WT) mice was shaved, followed by topical application of 4-OHT (10 mg/ml) or vehicle control (ethanol) on the right and left side, respectively, once a day for 4 weeks. The color of regrown hair is shown. Two independent experiments were performed with 4 mice for each experiment. (B) Epidermal melanocyte pigmentation with ethanol (left) and 4-OHT (right) treatment in KO (top) and WT (bottom) mice (400×, bars = 50 μm). (C) Lack of melanin in plucked hair shafts of KO mice compared to WT mice, 400×. Two experiments were done with similar findings.

Fig. 3.

Disrupted melanosome development in B16-KD cells. Ultrastructure of B16-KD and B16-WT cells was analyzed by transmission electronic microscopy. Decreased pigmentation in melanosomes is observed in B16-KD cells. Magnification: Left, 10000×, bar=10 μm; Right, 300000×, bar=500 nm. Arrowheads indicate mature (stage IV) melanosomes. Two experiments were done with similar findings.

Fig. 4.

Lack of gp96 does not interrupt expression or N-linked glycosylation of melanogenesis-associated enzymes. (A) Protein expression of Tyr, Tyrp1 and Dct in B16-WT and B16-KD cells by immunoblotting with an anti-tyrosinase monoclonal antibody (EP1577Y, EPITOMICS), an anti-Tyrp1 antibody (α -PEP1) and an anti-Dct antibody (α -PEP8'). (B,C,D) Western blotting of indicated proteins in non-digested cell lysates (-), or lysates treated with Endoglycosidase H (Endo H) or PNGase F, respectively. Data represent similar results from 2-3 experiments.

Fig. 5.

Tyrosinase catalytic activity and its distribution in the late melanosome is altered in the absence of gp96. Tyrosinase activity in whole cell lysates from B16-WT and B16-KD cells was measured by spectrophotometric assay (Mean $\pm$ SD) (A) and by an in-gel colorimetric assay after electrophoretic separation (B). ** $P < 0.01$. Equal amounts of cell lysates were used, as indicated by the β -actin loading control. Three independent experiments were performed with similar findings. (C) A schematic diagram of melanocytic markers during melanosome maturation. (D) Confocal immunofluorescence analysis of subcellular localization of gp96, tyrosinase (α -PEP7) and HMB45 along with ER (Bip), Golgi (GM130) and lysosome (LAMP1/LAMP2) markers, as noted, in B16-WT and in B16-KD cells. Pictures shown are representative images from two independent experiments.

Fig. 6.

Defect in melanogenesis in the absence of gp96 can be rescued by activating the Wnt/LRP6/ β -catenin signaling pathway. (A) Pigmentation of cell pellets of B16-KD and B16-WT cells cultured without (UT) or with the GSK3 β inhibitor TWS119 (1 μ M) for 2 weeks. (B) Whole cell lysates from B16-WT and B16-KD cells treated with or without TWS119 (1 μ M) were prepared, and Tyrosinase activity were measured by spectrophotometric assay (Mean $\pm$ SD). Results from three independent experiments were presented. **P<0.01. (C) In-gel colorimetric assay of tyrosinase after electrophoretic separation from the indicated cell lysates. (D) Tyrosinase expression level by immunoblot in B16-WT and B16-KD cells with or without TWS119 treatment. (E) Pigmentation of cell pellets of B16-KD and B16-WT cells cultured without (UT) or with the Wnt ligand Wnt 3a (200 ng/ml) for 2 weeks. Two experiments were done with similar findings.