Introduction

More than 95 genetic loci have been reported to regulate mammalian melanogenesis [1]. Tyrosinase is a critical enzyme for melanin synthesis and is transported from *trans*-Golgi network (TGN) to early-stage melanosomes via endosomal compartments [2, 3]. Tyrosinase-related protein 1 (Tyrp1), functioning as dihydroxyindole carboxylic acid oxidase in mice, also passes through endosomes to melanosomes [3, 4]. It has been reported that gp100, a melanosomal structural protein, is transported from Golgi to early-stage melanosomes [5]. In order for these melanosome-specific proteins to be transported in proper cascade of melanin biosynthesis, a large number of cargo molecules or transporters must be involved.

Previously we have reported that Rab7 is localized on the melanosomal membrane of B16 murine melanoma cells [6]. The cells treated with Rab7 antisense-oligonucleotide revealed Tyrp1 confined to the perinuclear area, indicating that Rab7 is involved in the transport of Tyrp1 from TGN to melanosomes, possibly passing through late endosome-delineated compartments. Moreover, we have reported that, through the use of adenovirus vectors, amelanotic melanoma cells with exogenous expression of Tyrp1 and Rab7N125I revealed Tyrp1 to be localized to early endosomes [7]. Thus, it has been suggested that the transport of Tyrp1 may require functional Rab7 and that Tyrp1 may pass through early to late endosomal compartments.

Rab7 is a member of Rab small GTP-binding protein, and is essential for the regulation of endosomal/lysosomal vesicular transport, phagocytosis and autophagocytosis [8-11]. Furthermore, four Rab7-binding molecules have recently been found and, through binding with these molecules,

Rab7 regulates organelle motility, phospholipid signaling pathways and cytosolic degradative machineries [12-16].

Here, we transfected immortal melanocytes with plasmids carrying cDNA of wild-type and mutant Rab7s, and analyzed their differential effects on the intracellular trafficking of tyrosinase, Tyrp1 and gp100 by single cell observation approach with immunofluorescent staining and confocal laser scanning microscopy. We found that the inhibition of Rab7 function resulted in preferential Tyrp1 elimination from melanocytes due to proteasomal degradation. Our results indicate that the vesicular transport pathway of Tyrp1 from Golgi to melanosomes is different from that of tyrosinase and gp100 and that Rab7 is a crucial regulator for Tyrp1 transport in melanogenesis cascade.

Materials and Methods

Vector Constructions

Rab7WT, a cDNA of wild-type Rab7 [17] was amplified from the Rab7 plasmids by using the N-terminal flag sequence-containing primer with *Hin*dIII site and C-terminal primer with the same restriction site. Oligonucleotides used for the amplification were 5'-CCC AAG CTT ACC ATG *GAC TAC AAG GAT GAC GAT GAC AAG* ACC TCT AGG AAG AAA GTG TTG-3' and 5'-CCC AAG CTT TCA GCA ACT GCA GCT TTC CGC-3' (underlines: *Hin*dIII site, italics: flag sequence). cDNAs of Rab7 mutants were constructed by polymerase chain reaction-mediated mutagenesis [18] by which a partial sequence of flag-tagged Rab7WT oligonucleotide was modified as follows; A374T for Rab7N125I; C65A and A66C for Rab7T22N; and C199T, A200T and G201A for

Rab7Q67L. The fragments of flag-tagged-Rab7s were separately inserted into the *Hin*dIII site of pcDNA3.1/Hygro (+) (Invitrogen, Carlsbad, CA). Parts of the inserted cDNAs in pcDNA3.1/Hygro (+) were verified by nucleotide sequencing by the dideoxy termination method.

Cell culture and transient transfection

COS7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) supplemented with 5% fetal calf serum (Sigma) and Penicillin-streptomycin solution (Sigma), and grown in a 5% CO_2 incubator at 37°C. Melan-a cells, kindly provided by Dr D. C. Bennett, UK, were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, 200 nM phorbol myristate acetate and Penicillin-streptomycin solution in 10% CO_2 atmosphere. Transfection was performed using FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Cells grown on round coverslips coated with 1% porcine skin gelatin (Sigma) were transfected with 0.5 μg of plasmid DNA and cultured for 24 or 48 h according to each examination. When cellular protease activities should be inhibited, cells were incubated with 10 μM MG132 (Sigma) or 100 μM leupeptin (Sigma) for 2.5 or 5 h prior to fixation.

Antibodies

Anti-PEP7, rabbit polyclonal antiserum against tyrosinase, was kindly provided by Dr V. J. Hearing, USA. HMSA5, a mouse monoclonal antibody (mAb) against Tyrp1, was developed previously [19]. HMB45, mouse mAb against gp100 was purchased from DAKO (Carpinteria, CA). Anti-flag mouse mAb (clone M2) and rabbit anti-flag polyclonal antibody were purchased from Sigma. Rabbit antiserum against Rab7 was purchased from Cytosignal Research Products

(Irvine, CA). Anti-EEA1 mouse mAb (clone 14) and anti-mannose phosphate receptor mouse mAb (clone 2G11) were purchased from BD-Biosciences (San Diego, CA) and Research Diagnostics (Flanders, NJ), respectively. Goat anti-mouse and anti-rabbit IgG antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 were products of Molecular Probes (Eugene, OR).

Immunofluorescent staining and confocal laser scanning microscopy

Immunofluorescent staining was performed as described previously with a slight modification [7]. Cells were fixed in a mixture of acetone and methanol (1:1) at −20°C for 5 min. After a brief wash with PBS containing 0.5% (w/v) BSA, a pair of monoclonal and polyclonal primary antibody was used simultaneously for double staining. The following antibody dilutions were used; HMSA5, 1:1000; anti-flag M2, 1:1000; polyclonal anti-flag, 1:2000; Rab7 antiserum, 1:2000; anti-EEA1 1:100; and anti-MPR 1:100. The secondary fluorescent-labeled antibodies were used at a dilution of 1:600. The coverslips were mounted with Permafluor (Immunotech, Marseille, France) on slide glasses. Immunofluorescent images were obtained by confocal laser scanning microscopy by using Axiovert 100M (Carl Zeiss, Thornwood, NY) equipped with LSM510 V2.5 (Carl Zeiss).

Statistics

The distribution of melanosomal proteins or the amount of Tyrp1-positive granules was compared by using Pearson's chi-square test (significant level, 1%). When multiple comparisons were needed, significant level was reduced to value divided by a number of comparisons according

to Bonferoni's method.

Results

Subcellular localization of Rab7N125I was different from that of the wild-type protein in COS7 cells

As described in the materials and methods, plasmids encoding Rab7WT or Rab7N125I were constructed with a flag-tag at the N-terminus and introduced into cultured cells by lipofection. The addition of such tag sequences to Rab proteins does not appear to alter their function or subcellular localization [9]. To examine the expression and subcellular distribution of flag-fused Rab7 proteins, COS7 cells were transfected with plasmids carrying Rab7WT or Rab7N125I and carried out immunofluorescent staining, and observed using a confocal laser scanning microscope. All images were taken in such a way that exogenous Rab7-expressing cells and untransfected control cells were obtained in every image. Rab7WT-positive granules were assembled in the perinuclear area and rarely detected in the cell periphery (Figs. 1A and B). Some of the Rab7WT-positive granules appeared to form vesicular structures (Fig. 2A, inset). In contrast, in cells expressing Rab7N125I, the mutant protein was detected in both the cytoplasmic and the perinuclear areas (Figs. 1C and D). Small immuno-positive granules were diffusely scattered throughout the cytoplasm, including its dendrites. Swollen Rab7N125I-positive granules were conglomerated in the perinuclear area without vesicular appearance.

To confirm the localization of the exogenous Rab7 proteins, cells were doubly stained with anti-flag polyclonal antibody and endosomal marker antibody, anti-EEA1 mAb (Figs. 1A and C) for

early endosomes, and anti-MPR mAb (Figs. 1B and D) for late endosomes. Rab7WT was co-localized with MPR, but not with EEA1. The distribution of each organelle marker in Rab7WT-expressing cells was similar to that in control cells (Figs. 1A and B). In contrast, the two forms of Rab7N125I-positive granules, located in the perinuclear area and the cytoplasm, were partly co-localized with EEA1 (Fig. 1C, inset). There was no co-localization of Rab7N125I with MPR, and the distribution of MPR in the Rab7N125I-expressing cells was similar to that of the control cells (Fig. 1D). These results indicate that Rab7N125I was not localized to late endosomes but was partly co-localized with EEA1-positive early endosomes, whereas Rab7WT was localized to late endosomes.

Rab7N125I inhibited the localization of Tyrp1, which was quite different from that of tyrosinase or gp100

To study how Rab7 is involved in the localization of melanosomal proteins, melan-a, an immortal murine melanocyte cell line, cells were transfected with the plasmids encoding Rab7WT or Rab7N125I, and 48 h after the transfection, double-immunofluorescent staining was performed by using anti-flag polyclonal antibody and HMSA5 (Figs. 2A and D), anti-flag mAb and PEP7 antiserum (Figs. 2B and E), and anti-flag polyclonal antibody and HMB45 (Figs. 2C and F). Tyrp1-positive granules in both Rab7WT-expressing and untransfected-control cells were similarly distributed; densely assembled in the perinuclear area and scattered in the peripheral area (Fig. 2A). Approximately 39% of Tyrp1-positive granules were co-localized with Rab7WT (Fig. 2A, inset, and G). In contrast to the cells expressing Rab7WT, Tyrp1 was not detected in the entire

cytoplasm of the cells expressing Rab7N125I (Fig. 2D).

Tyrosinase-positive granules in Rab7WT-expressing cells and control ones were also distributed in both the perinuclear and peripheral areas (Fig. 2B). The co-localization of tyrosinase with Rab7WT was 14%, less apparent than that of Tyrp1 with Rab7WT (Fig. 2B, inset and G). Unlike Tyrp1 which was not detectable in the Rab7N125I-expressing melan-a cells, tyrosinase-positive granules were observed in the Rab7N125I-expressing cells. However, tyrosinase-positive granules in the Rab7N125I-expressing melan-a cells did not show perinuclear aggregation (Fig. 2E).

In Rab7WT-expressing cells and control ones, gp100-positive granules were also distributed in both the perinuclear and peripheral areas (Fig. 2C). The co-localization of gp100-positive granules and Rab7WT was 15%, less apparent than that of Tyrp1 with Rab7WT (Fig. 2C, inset and G). The cells expressing Rab7N125I contained a comparable amount of gp100-positive granules to those expressing Rab7WT. The majority of gp100-positive granules in the Rab7N125I-expressing cells were distributed in the peripheral area of the cytoplasm (Fig. 2F).

Rab7N125I altered the melanosomal protein expression and distribution

To verify if the distribution of the melanosomal proteins is altered by Rab7N125I expression in melan-a cells, the microscopic features of immunostaining results in melan-a cells were statistically analyzed (Fig. 3). Melan-a cells were transfected and immunostained as described in the foregoing paragraph. Approximately 68 digital images (ranging 55-75 images) of the cells expressing exogenous Rab7 were examined for each analysis. The distribution of Tyrp1-, tyrosinase- and gp100-positive granules was categorized into three basic patterns; *perinuclear*, cells

had perinuclearly assembled granules; *peripheral*, cells had no perinuclearly assembled granules but had peripherally scattered granules; and *agranular*, cells had no granules.

The distribution pattern of Tyrp1-positive granules in Rab7N125I-expressing cells was quite different from that of Rab7WT-expressing cells (Fig. 3A, $p<0.00001$). In 73% of Rab7N125I-expressing cells, Tyrp1-positive granules were not detected. From those of remaining cells (27%), that were categorized as *perinuclear* or *peripheral* pattern cells, 24% cells contained a smaller number of Tyrp1-positive granules than did control cells, and only 3% of the cells contained a comparable number of Tyrp1-positive granules to control cells (data not shown).

In contrast, the percentage of cells without detectable tyrosinase-positive granules was 14% and 15% in the cells expressing Rab7WT and Rab7N125I, respectively (Fig. 3B). The percentage of cells showing tyrosinase with *perinuclear* pattern in Rab7WT- and Rab7N125I-expressing cells was 58% and 37%, respectively. The percentage of cells showing tyrosinase with *peripheral* pattern in Rab7WT- and Rab7N125I-expressing cells was 28% and 48%, respectively. The difference in the distribution patterns of tyrosinase between Rab7WT- and Rab7N125I-expressing cells was not statistically significant ($p=0.025$).

The percentage of cells without detectable gp100-positive granules was 7% in the cells expressing either Rab7WT or Rab7N125I (Fig. 3C). The percentage of cells showing gp100 with *perinuclear* pattern in Rab7WT- and Rab7N125I-expressing cells was 67% and 26%, respectively. And the percentage of cells showing gp100 with *peripheral* pattern in Rab7WT- and Rab7N125I-expressing cells was 26% and 67%, respectively. The difference in the distribution

patterns of gp100-positive granules between Rab7WT- and Rab7N125I-expressing cells was statistically significant ($p<0.00001$). These results indicate that Rab7N125I affects the trafficking of tyrosinase but does that of Tyrp1 and gp100 differently and, among them, Tyrp1 required Rab7 function for its proper transport.

Tyrp1 was gradually eliminated in Rab7N125I-expressing melanocytes

To examine if Tyrp1 could be detected at an earlier time point after the transfection of the plasmid encoding Rab7N125I, melan-a cells were fixed and immunostained at the end of 24 or 48 h incubation after the transfection. The cells expressing Rab7WT for both 24 h and 48 h possessed comparable amount of Tyrp1-positive granules to surrounding control melanocytes (Figs. 4A and B). In Rab7N125I-expressing cells after 48 h incubation, Tyrp1-positive granules were eliminated (Fig. 4D). However in melanocytes expressing Rab7N125I for 24 h, Tyrp1-positive granules were detected in the perinuclear area (Fig. 4C).

To make sure that Tyrp1 is detectable at early time point of Rab7N125I expression, thirty of flag-positive melanocytes were randomly selected from each sample; and their immunofluorescent images were digitally recorded. The cells were categorized into three groups according to the amount of Tyrp1-positive granules they had; *rich*, cells contained comparable member of Tyrp1-positive granules to surrounding control cells; *poor*, cells contained reduced member of Tyrp1-positive granules; and *agranular*, cells had no detectable Tyrp1 (Fig. 4E). Among the cells expressing Rab7WT for 24 and 48 h, the percentage of *rich* melanocytes were 73% and 60%, respectively, and most part of the cells contained detectable Tyrp1-positive granules. In contrast,

only 33% of the cells among the ones expressing Rab7N125I for 48 h contained detectable Tyrp1-positive granules. However, in no less than 87% of the cells expressing Rab7N125I for 24 h, Tyrp1-positive granules were detected. These results suggest that Tyrp1 was localized to the perinuclear structures early after the transfection of Rab7N125I and then eliminated from the cell.

Other Rab7 mutants also eliminated Tyrp1 from melan-a cells

To determine if the elimination of Tyrp1 is due to the inhibition of Rab7 not to the specific effect of Rab7N125I, transfection with plasmids encoding other Rab7 mutants were performed. The mutants were Rab7T22N, another dominant negative mutant because of its preferential interaction with GDP, and Rab7Q67L, a constitutively active mutant because of its disability to hydrolyze GTP [9]. The immunostaining results of Tyrp1 in Rab7T22N-expressing cells were similar to those in Rab7N125I-expressing ones (Figs. 5A and B); small amount of Tyrp1-positive granules were distributed in the perinuclear area 24 h after the transfection, and Tyrp1 was eliminated 48 h after the transfection. On the other hand, cells expressing Rab7Q67L for 24 h showed swollen Tyrp1-positive granules in the perinuclear area that were co-localized with Rab7Q67L (Fig. 5C). Interestingly, Tyrp1 was also eliminated in the cells expressing Rab7Q67L for 48 h (Fig. 5D).

Tyrp1 elimination in mutant Rab7-expressing cells was due to proteasomal degradation

Because Tyrp1 was gradually disappeared from the cells expressing mutant Rab7s, we hypothesized that Tyrp1 was mislocalized to other organelles than its proper destination, melanosomes, and then degraded. Firstly, a possibility that Tyrp1 was transported to plasma

membrane was examined. Rab7N125I-expressing cells were fixed in 3% paraformaldehyde, permeabilized in 0.1% triton X-100 in PBS and followed by immunostaining in order not to destruct the membranous structures and membrane proteins. Despite under this condition, Tyrp1 was not detected on the plasma membrane (data not shown). Secondly, a possibility that Tyrp1 elimination requires either a proteasomal or a lysosomal activity was examined by using MG132 and leupeptin, a proteasomal and a lysosomal inhibitor, respectively. Melan-a cells were transfected with the plasmid encoding Rab7N125I or Rab7T22N and incubated for 48 h before fixation and placed in medium containing MG132 for 5 h before the fixation. Although mutant Rab7-expressing cells did not have Tyrp1-positive granules (Figs. 6A and C) in the absence of MG132, the granules were detected in the perinuclear area when cells were incubated with MG132 for 5 h (Figs. 6B and D). On the other hand, mutant Rab7-expressing cells treated with leupeptin did not show the Tyrp1-positive granules (data not shown).

To confirm the significance of the statistic results, 30 cells were randomly selected from each sample in which cells were treated with the inhibitors for 0, 2.5 or 5 h, digitally recorded and categorized into *rich*, *poor*, and *agranular* as described in the foregoing paragraph. In Rab7N125I-expressing cells, the percentages of *rich* and *poor* pattern cells in MG132-treated cells were larger than those in MG132-untreated cells (Fig. 6E). The differences between the cells treated with MG132, for 2.5 or 5 h, and those untreated were statistically significant ($p<0.0025$). In contrast, the differences between the cells treated with leupeptin, for 2.5 or 5 h, and those untreated were not statistically significant. Similarly, in Rab7T22N-expressing cells, the

percentages of *rich* and *poor* pattern cells in MG132-treated cells were larger than those in MG132-untreated cells, and the differences in the ratios of the pattern of Tyrp1-positive granules were statistically significant only between 5 h MG132-treated and untreated cells (Fig. 6F). As a result, Tyrp1 elimination in the cells expressing mutant Rab7s was rescued by the proteasomal inhibitor, but not by the lysosomal one.

Discussion

Tyrosinase, Tyrp1 and gp100 are melanosomal proteins that possess distinct biological and biochemical functions in the melanogenesis cascade. They are all glycoproteins and are sorted from Golgi to melanosomal compartment. A previous study indicated that tyrosinase, TYRP1 and TYRP2 form a melanogenic complex in low pH circumstance [20]. A more recent study indicated that tyrosinase and TYRPs form a complex in late endosomes and are transported to stage II melanosomes [3]. However, it is still unknown whether tyrosinase and Tyrps travel together in the same or independent pathway from TGN to early-stage melanosomes [21]. In this study, we sought to disclose the differential transport pathways of melanosomal proteins by comparing the expression and the intracellular distribution of melanosomal proteins on a single cell level by using confocal laser scanning microscopy.

Experiments of Rab7N125I showed that the vesicular transport of Tyrp1 from Golgi to melanosomal compartments is different from that of tyrosinase and gp100. Rab7WT, which was detected as perinuclear aggregates, was localized at late endosomes and did not alter the distribution

of pre-existing subcellular structures, while Rab7N125I altered the distribution of Rab7-positive granules and perturbed endosomal vesicular transport by inhibiting a proper distribution of Tyrp1. Most of the melan-a melanocytes expressing Rab7N125I contained no or reduced number of Tyrp1-positive granules. Therefore the melan-a cells expressing Rab7N125I appeared to lose Tyrp1. These findings indicate that Tyrp1 may pass through early and late endosomal compartments before being targeted to melanosomes, and that the vesicular transport pathway of Tyrp1 is different from that of tyrosinase and gp100. A previous study indicated that for proper targeting of tyrosinase, di-leucine-based signal motif at the cytoplasmic domain of tyrosinase, to which AP-3 binds, and two acidic amino acid residues four to five proximal to the di-leucine motif are essential [22]. However, Tyrp1 possesses only one of the two acidic amino residues, revealing less affinity to AP-3 than tyrosinase. It was also reported that Tyrp1 resides in the transport vesicles with AP-1 [23]. The AP-1 is involved in the TGN/endosome trafficking while AP-3 regulates the vesicular transport to lysosome-related organelles including melanosomes [24]. Therefore it can be postulated that tyrosinase is transported from TGN to early-stage melanosomes directly or indirectly via late endosomes by AP-3, and that Tyrp1 is transported to early endosomes, then subsequently to late endosomes under the regulation of Rab7, and finally to early-stage melanosomes. Higher rate of co-localization of Tyrp1-positive granules with Rab7WT than that of tyrosinase- or gp100-positive granules also suggests that Tyrp1 passes through early to late endosomal pathway in correlation with Rab7. Tyrosinase and TYRPs may form a complex after reaching late endosomal/melanosomal compartments which have an acidic environment.

In our study, Tyrp1 was gradually eliminated in Rab7N125I-expressing melan-a cells and could be detected at 24 h after the transfection of the plasmid. The Tyrp1-positive granules detected at 24 h after the transfection were mainly distributed in the perinuclear area. It is thus indicated that Tyrp1 is transported to the certain organelle located in the perinuclear area, followed by mislocalization to other organelle than proper targeting sites i.e. melanosomes. Gomez *et al* previously reported that Tyrp1 was confined to the perinuclear region of murine melanoma cells treated with Rab7 antisense-oligonucleotide [6]. Furthermore, Hirosaki *et al* showed that exogenously expressed Tyrp1 was retained at EEA1-positive early endosomes in amelanotic melanoma cells expressing Rab7N125I [7]. Taking these reports into consideration, Tyrp1-positive perinuclear structures we observed at an early time point of Rab7 expression may be early endosomes. There are several possibilities existing as to why the Tyrp1 elimination was not mentioned in the previous reports. We have used immortal melanocytes which were introduced cDNA of mutant Rab7s by lipofection and observed the behavior of endogenous Tyrp1. In these previous study by Hirosaki et al, however, they had used amelanotic melanoma cells exogenously introduced Tyrp1 and Rab7N125I by adenovirus vector. Therefore our current results may reflect more physiological state, compared to adenovirus transfection.

Tyrp1 elimination was caused by not only Rab7N125I but Rab7T22N. This result may strongly indicate that Tyrp1 elimination is caused by the inhibition of Rab7 and is not a specific effect of Rab7N125I. Interestingly, Tyrp1-positive granules were also eliminated in the cells expressing Rab7Q67L. The appearance of Tyrp1-positive granules observed early in the

expression of Rab7Q67L was different from that in the expression of dominant negative mutants. Tyrp1 was localized to perinuclear plump granules and was co-localized with Rab7Q67L. Because the mutant exists preferentially as GTP-bound form due to the disruption of GTPase activity domain, it inhibits the cyclical regulatory process of wild-type Rab7. Therefore Tyrp1-positive endosomes may be fixed in the perinuclear area and fuse each other. Finally Tyrp1 may not be transported to a proper destination, alternatively to a degradation pathway.

Our finding that the degradation of Tyrp1 can be rescued by MG132 provides evidence that the proteasomal pathway plays a role in the Tyrp1 elimination in the cells which express dominant negative mutant Rab7s. Furthermore the finding that leupeptin could not rescue the Tyrp1 elimination does not suggest that lysosomes participate in the Tyrp1 elimination, although we can not deny a possibility that lysosomes are involved in the Tyrp1 degradation in concert with proteasomes. The ubiquitin-proteasome pathway controls a wide variety of regulatory processes via ubiquitin-mediated degradation of cytosolic and nuclear proteins [25]. Growing evidences have been accumulated to indicate that ubiquitin-proteasome pathway is also involved in internalization, intracellular routing and processing of membrane proteins [26]. It has been reported that plasma membrane receptors such as platelet-derived growth factor receptor, Met tyrosine kinase receptor and growth hormone receptor are degraded by proteasomal processing after ubiquitination [27] [28] [29]. From the findings of these ubiquitin-proteasome pathways, a mechanism of Tyrp1 elimination due to dominant negative Rab7s can be hypothesized as follows. Dominant negative Rab7s may block the proper routing of Tyrp1 from early to late endosomes,

which results in the retention at the perinuclear endosomal structures and subsequent proteasomal degradation. Tyrp1 retention in early endosomes may be visualized only when both of the Rab7 function and proteasome activities are inhibited. Although there is no evidence that Tyrp1 can be ubiquitinated, it has been reported that tyrosinase can be ubiquitinated and degraded by proteasomes in/after the ER/Golgi processing [30]. Therefore Tyrp1 may also be degraded by ubiquitin-proteasome pathway.

In conclusion, inhibition of Rab7 results in Tyrp1-specific elimination due to proteasomal activity, not to lysosomal one. This report demonstrates that the transport pathway of Tyrp1 from Golgi to melanosomes is different from that of tyrosinase and gp100, and that Rab7 is a crucial regulator of Tyrp1 transport. These results may indicate that Rab7 is a potent regulator of endosomal vesicular trafficking in melanogenesis and that it is a candidate molecule for future identification of abnormalities in pigmentary diseases.

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Figure legends

Fig. 1. Rab7WT was localized at late endosomes whereas Rab7N125I was distributed in the perinuclear area as well as throughout the cytoplasm. COS7 cells transfected with the plasmid encoding Rab7WT (A and B) or Rab7N125I (C and D) were immunostained with anti-flag antibody (*green*) and endosomal marker antibody (*red*); anti-EEA1 (A and C) and anti-MPR (B and D). Arrowheads point to Rab7WT-positive vesicular structures. Arrows point to Rab7N125I-positive granules which were co-localized with EEA1. Results show one representative experiment of three. Scale bars= 10 μm.

Fig. 2. Rab7N125I inhibited the localization of Tyrp1, which was quite different from that of tyrosinase or gp100. Melan-a cells transfected with the plasmid encoding Rab7WT (A-C) or Rab7N125I (D-F) were immunostained with anti-flag antibody (*green*) and antibody against melanosomal proteins (*red*); anti- Tyrp1 (A and D), anti-tyrosinase (B and E) or anti-gp100 (C and F) antibody. Results show one representative experiment of three. Scale bars= 10 μm. (G) The rate of co-localization of melanosomal protein-positive granules with Rab7WT was calculated. More than 200 melanosomal protein-positive granules (three independent dishes for each plasmid) were counted. Data are expressed as means ± S.D. of three independent experiments.

Fig. 3. Rab7N125I altered the distribution of melanosomal proteins. For each statistical analysis, the immunofluorescent images of Rab7WT- or Rab7N125I-expressing cells were collected. The distribution of melanosomal protein-positive granules in the cells was categorized into three basic

patterns; *perinuclear*; *peripheral*, and *agranular*. Bars are percentages of the cells included in each category. The total number of cell examined is shown in parentheses. *A*, $p<0.00001$; *B*, $p=0.025$; *C*, $p<0.00001$; wt, Rab7wt; N125I, Rab7N125I.

Fig. 4. Tyrp1-positive granules were detected early during the expression of Rab7N125I. Melan-a melanocytes were transfected with the plasmids encoding Rab7WT (A and B) or Rab7N125I (C and D), and incubated for 24 h (A and C) or 48 h (B and D) before fixation. Cells were immunostained with anti-flag antibody (*green*) and anti- Tyrp1 antibody (*red*). Scale bars= 10 μm. (E) Thirty cells from each coverslip were took a photograph digitally and categorized into three basic patterns according to the amount of Tyrp1-positive granules they contained; *rich, poor* and *agranular*. Bars are the percentages of each pattern cell. Results show one representative experiment of three.

Fig. 5. Rab7T22N and Rab7Q67L also eliminated Tyrp1 in melan-a melanocytes. Melan-a melanocytes were transfected with the plasmids encoding Rab7T22N (A and B) or Rab7Q67L (C and D), and incubated for 24 h (A and C) or 48 h (B and D) before fixation. Cells were immunostained with anti-flag antibody (*green*) and anti- Tyrp1 antibody (*red*). Scale bars= 10 μm. Results show one representative experiment of three.

Fig. 6. Proteasomal inhibitor MG132 rescued the Tyrp1-positive granules in mutant Rab7s-expressing cells. Melan-a melanocytes were transfected with the plasmids encoding Rab7N125I (A and B) or Rab7T22N (C and D), and incubated for 48 h. Before fixation, cells were incubated in the absence (A and C) or presence (B and D) of 10 μM MG132 for 5 h. Scale

bars= 10 μm. The cells transfected with plasmids encoding Rab7N125I (E) or Rab7NT22N (F) were incubated with either MG132 for 2.5 h or 5h, or 100 mM leupeption 2.5 h or 5 h. Thirty cells from each coverslip were took a photograph digitally and categorized into three basic patterns described above. *p=0.00015, **p<0.00001, ***p=0.165, ****p=0.076, †p=0.047, ††p=0.00019, †††p=0.343, ††††p=0.028, significant level: 1%. Bars are the percentages of each pattern cell. Results show one representative experiment of three.