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RUTBC1 Functions as a GTPase-activating Protein for Rab32/38 and Regulates Melanogenic Enzyme Trafficking in Melanocytes*

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Two cell type-specific Rab proteins, Rab32 and Rab38 (Rab32/38), have been proposed as regulating the trafficking of melanogenic enzymes, including tyrosinase and tyrosinase-related protein 1 (Tyrp1), to melanosomes in melanocytes. Like other GTPases, Rab32/38 function as switch molecules that cycle between a GDP-bound inactive form and a GTP-bound active form; the cycle is thought to be regulated by an activating enzyme, guanine nucleotide exchange factor (GEF), and an inactivating enzyme, GTPase-activating protein (GAP), which stimulates the GTPase activity of Rab32/38. Although BLOC-3 has already been identified as a Rab32/38-specific GEF that regulates the trafficking of tyrosinase and Tyrp1, no physiological GAP for Rab32/38 in melanocytes has ever been identified, and it has remained unclear whether Rab32/38 is involved in the trafficking of dopachrome tautomerase, another melanogenic enzyme, in mouse melanocytes. In this study we investigated RUTBC1, which was originally characterized as a Rab9-binding protein and GAP for Rab32 and Rab33B *in vitro*, and the results demonstrated that RUTBC1 functions as a physiological GAP for Rab32/38 in the trafficking of all three melanogenic enzymes in mouse melanocytes. The results of this study also demonstrated the involvement of Rab9A in the regulation of the RUTBC1 localization and in the trafficking of all three melanogenic enzymes. We discovered that either excess activation or inactivation of Rab32/38 achieved by manipulating RUTBC1 inhibits the trafficking of all three melanogenic enzymes. These results collectively indicate that proper spatiotemporal regulation of Rab32/38 is essential for the trafficking of all three melanogenic enzymes in mouse melanocytes.

Mammalian epidermal melanocytes are specialized cells that produce the pigment melanin, which is synthesized and stored in specialized organelles called melanosomes. Melanosomes have several features in common with lysosomes, but because melanosomes possess other specific characteristics of their own, such as containing the melanogenic enzymes that are responsible for melanin synthesis, *i.e.* tyrosinase, tyrosinase-related protein 1 (Tyrp1),[3] and dopachrome tautomerase (Dct), they are generally considered to be lysosome-related organelles (1, 2). Tyrosinase and Tyrp1 clearly have been demonstrated to be transported from the *trans*-Golgi network by way of endosomes into immature premelanosomes, away from degradative lysosomes (1). By contrast, relatively little is known about the transport of Dct, although, to our knowledge, one study has shown that Dct is transported directly from the *trans*-Golgi network to immature malanosomes (3). Defects in the trafficking of these melanogenic enzymes have been shown to cause a number of pigment disorders, including Hermansky-Pudlak syndrome (HPS), which is characterized by prolonged bleeding, immunodeficiency, and pulmonary fibrosis as well as hypopigmentation of the hair, skin, and eyes (4, 5). The genes responsible for HPS encode the subunits of five large cytoplasmic complexes, *i.e.* adaptor protein complex-3 (AP-3), vacuolar protein sorting (VPS)-C, and biogenesis of lysosome-related organelles complex (BLOC)-1, BLOC-2, and BLOC-3 (4, 5).

Rab proteins are small GTPases that are well recognized as membrane-trafficking regulators in all eukaryotes (6–8). They function as switch molecules that cycle between a GDP-bound inactive form and GTP-bound active form, which interacts with effector molecules to promote membrane trafficking events (6–8). Two regulatory enzymes, a guanine nucleotide exchange factor (GEF) and a GTPase-activating protein (GAP), control the spatiotemporal Rab cycle by activating and inactivating, respectively, the Rab proteins (9, 10). The discovery of dramatically lower levels of tyrosinase and Tyrp1 in Rab32 knockdown melan-cht cells (mutation in the *Rab38* locus) has revealed that Rab32 and Rab38 redundantly regulate the trafficking of melanogenic enzymes, at least of tyrosinase and Tyrp1 (11). Interestingly, however, Rab32, and not Rab38, has

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[3] The abbreviations used are: Tyrp1, tyrosinase-related protein 1; BLOC, biogenesis of lysosome-related organelles complex; Dct, dopachrome tautomerase; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; HPS, Hermansky-Pudlak syndrome; TBC, Tre-2/Bub2/Cdc16; Varp, VPS9-ankyrin repeat protein.

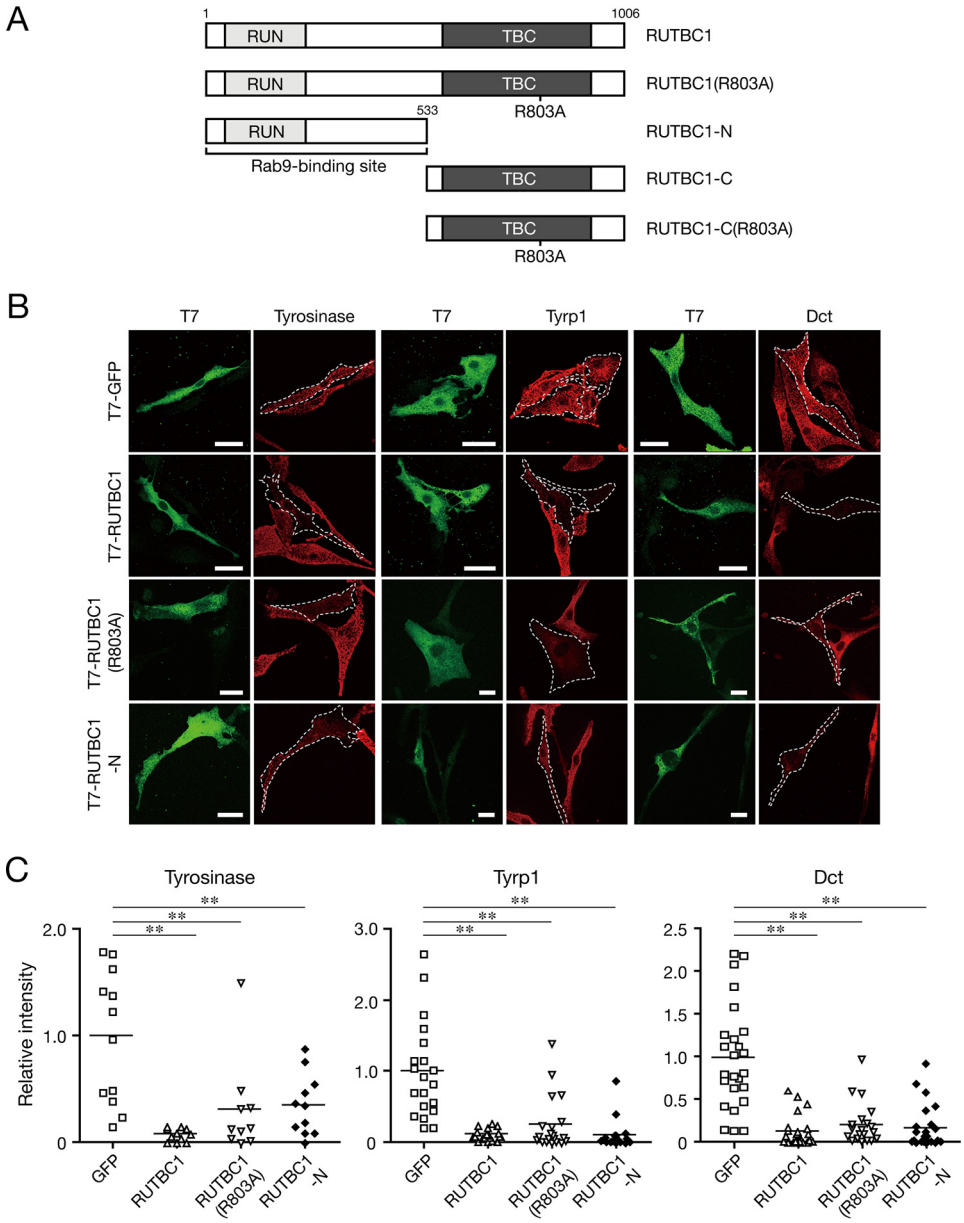

FIGURE 1. **Effect of overexpression of RUTBC1 on the melanosomal localization of melanogenic enzymes in melanocytes.** *A*, schematic representation of the human RUTBC1 and its truncated mutants (RUTBC1-N and RUTBC1-C) used in this study. RUTBC1 contains an N-terminal RUN domain (*light gray boxes*), which possesses Rab9A/B binding activity, and a C-terminal TBC domain (*dark gray boxes*), which exhibits GAP activity toward Rab32 and Rab33B *in vitro* (18, 19). R803A mutants lack a catalytic Arg residue that is crucial for GAP activity (31). *B*, typical images of melanocytes expressing T7-RUTBC1, T7-RUTBC1(R803A), T7-RUTBC1-N, or T7-GFP (as a negative control). Melan-a cells were transfected with each T7-tagged protein-expressing plasmid and stained with anti-tyrosinase antibody, anti-Tyrp1 antibody, or anti-Dct antibody. Cells expressing T7-tagged protein are outlined with a *broken line*. *Scale bars*, 20 μm. *C*, quantification of the tyrosinase/Tyrp1/Dct signals shown in *B*. The *bars* represent the means of the data ($n = 20$). The symbols represent the relative intensity of the tyrosinase/Tyrp1/Dct signals in cells expressing T7-GFP (*squares*), T7-RUTBC1 (*triangles*), T7-RUTBC1(R803A) (*inverted triangles*), and T7-RUTBC1-N (*diamonds*). **, $p < 0.01$, Dunnett's test.

recently been reported responsible for the trafficking of Dct in human MNT-1 melanoma cells, suggesting the existence of trafficking pathways for tyrosinase/Tyrp1 and Dct in human melanoma cells (3). A physiological GEF and an effector molecule of Rab32/38 have already been identified in melanocytes. BLOC-3, a heterodimer of HPS1 and HPS4, functions as a Rab32/38 GEF (12), and mutations of either of these subunits are known to cause HPS (13). The VPS9-ankyrin repeat protein (Varp; official name is Ankrd27) is a Rab32/38 effector that regulates the trafficking of tyrosinase and Tyrp1 in melanocytes (14–16). Moreover, BLOC-2 has been reported to be an effector molecule complex of Rab32/Rab38 (3) with the function of targeting recycling endosomal intermediates containing the melanogenic enzymes to melanosomes (17). Nevertheless, no physiological GAP for Rab32/38 has ever been identified in melanocytes, although a Rab9-binding protein, RUTBC1, has recently been reported to possess *in vitro* GAP activity toward Rab32 and Rab33B (18, 19).

In this study we investigated the physiological function of RUTBC1 in melanogenic enzyme trafficking in mouse melanocytes. The results showed that RUTBC1 is a physiological GAP for Rab32/38 in melanocytes and that either excess activation of Rab32/38 or inactivation of Rab32/38, achieved by manipulating RUTBC1, inhibited the trafficking of all three melanogenic enzymes.

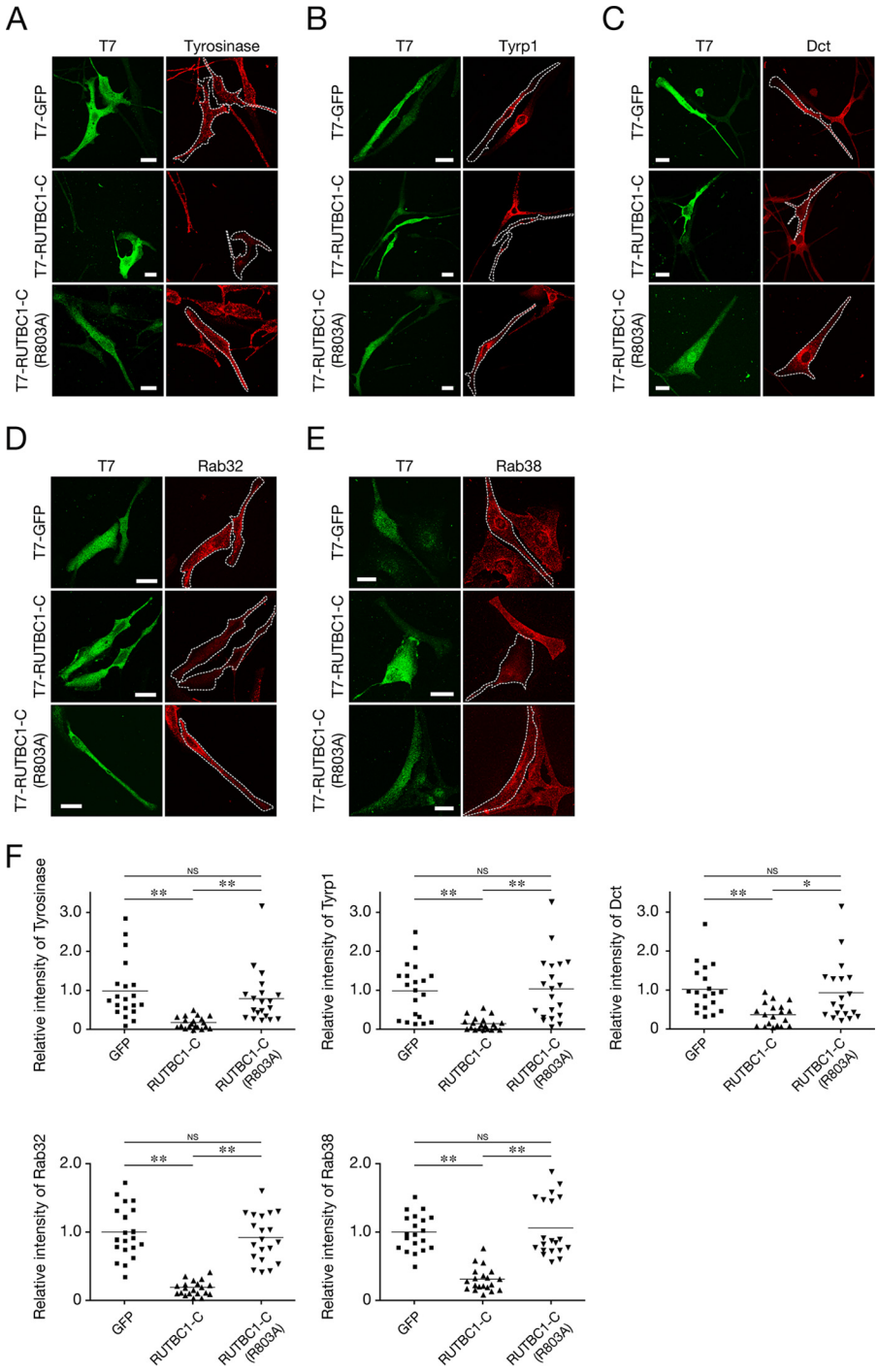

FIGURE 2. **The GAP activity of the C-terminal TBC domain of RUTBC1 is involved in tyrosinase/Tyrp1/Dct trafficking in melanocytes.** *A–E*, typical images of melanocytes expressing T7-GFP, T7-RUTBC1-C, or T7-RUTBC1-C(R803A). Melan-a cells were transfected with each T7-tagged protein-expressing plasmid and stained with antibodies against tyrosinase (*A*), Tyrp1 (*B*), Dct (*C*), Rab32 (*D*), or Rab38 (*E*). Cells expressing T7-tagged proteins are outlined with a *broken line*. *Scale bars*, 20 μm. *F*, quantification of the tyrosinase/Tyrp1/Dct/Rab32/Rab38 signals shown in *A–E*. The *bars* represent the means of the data ($n = 20$). The symbols represent the relative intensity of the tyrosinase/Tyrp1/Dct/Rab32/Rab38 signals in cells expressing T7-GFP (*squares*), T7-RUTBC1-C (*triangles*), and T7-RUTBC1(R803A)-C (*inverted triangles*). *, $p < 0.05$; **, $p < 0.01$, Dunnett's test. *NS,* not significant.

Based on our findings we discuss the possible molecular mechanism responsible for the spatiotemporal regulation of Rab32/38 by RUTBC1 and its binding partner, Rab9A, in melanocytes.

Experimental Procedures

Materials—The following antibodies used in this study were obtained commercially: anti-GFP rabbit polyclonal antibody (MBL, Nagoya, Japan); anti-FLAG tag rabbit polyclonal antibody, anti-FLAG tag mouse monoclonal (M2) antibody, and anti-FLAG tag antibody-conjugated agarose beads (Sigma-Aldrich); horseradish peroxidase (HRP)-conjugated anti-T7 tag mouse monoclonal antibody and anti-T7 tag antibody-conjugated agarose beads (Novagen™, Merck, Darmstadt, Germany); anti-HA tag rat monoclonal (3F10) antibody (Roche Diagnos-

tics); anti-β-actin mouse monoclonal antibody (Applied Biological Materials, Richmond, British Columbia, Canada); HRP-conjugated anti-GST rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA); and Alexa Fluor 488/594-conjugated anti-mouse/rabbit IgG goat antibody (Invitrogen). Rabbit polyclonal antibodies against Rab32, Rab38, tyrosinase, and Tyrp1 were prepared as described previously (14, 20, 21). Anti-Dct rabbit polyclonal antibody was raised against a peptide corresponding to the C-terminal sequence (amino acid residues 504–517) of mouse Dct and affinity-purified essentially as described previously (21). Anti-Rab9A rabbit polyclonal antibody was produced by using purified GST-tagged mouse Rab9A (22). Glutathione-Sepharose beads were purchased from GE Healthcare.

Plasmid Construction—cDNA encoding the open reading frame of human RUTBC1 was amplified from an RUTBC1/KIAA0397 clone (Kazusa DNA Research Institute, Chiba, Japan) by PCR, performed with specific primers containing a BglII linker (underlined) or a stop codon (bold) plus a SalI linker (underlined) as follows: Met primer, 5′-GGGGAGATCTATGGGCAGCGCAGAGGACGC-3′; and stop primer, 5′-GGGGGTCGAC**TCA**CTTGTTCTCTATGAGCA-3′. cDNA encoding the open reading frame of mouse HPS4 was amplified from Marathon-Ready adult mouse brain/testis cDNA (Clontech-Takara Bio, Shiga, Japan) by PCR using specific primers containing a BamHI linker (underlined) or a stop codon (bold) as follows: Met primer, 5′-GGATCCATGGCCACCACCACTCCCCC-3′; and stop primer, 5′-**TCA**CAGCAGGTTTACCCCGT-3′. The purified PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI). The cDNA inserts were excised with the appropriate restriction enzymes and subcloned into the pEF-T7 tag, pEF-HA tag, or pEF-T7-GST tag mammalian expression vector or the pEGFP-C1 vector (Clontech-Takara Bio) as described previously (23, 24). An RUTBC1 mutant carrying an Arg-to-Ala mutation at amino acid position 803 (R803A) was prepared by conventional PCR techniques performed with mutagenic oligonucleotides as described previously (25). Truncated mutants of RUTBC1 (Fig. 1*A*) were also prepared by conventional PCR techniques performed with specific pairs of oligonucleotides. pEF-T7-GST-Varp was constructed by transferring the cDNA insert of pEF-T7-Varp-ANKR1 (amino acid residues 451–729) (14) into the pEF-T7-GST vector (referred to as pEF-T7-GST-Varp in this article). pEF-FLAG-Rab32, pEF-FLAG-Rab32(Q83L), pEF-FLAG-Rab38, and pEF-FLAG-Rab38 (Q69L) were prepared as described previously (26, 27).

RNAi—The siRNAs against mouse *RUTBC1* (target site 1, 5′-CCAGGAAATTCGTGCATGA-3′; and target site 2, 5′-GCAGCCACATCATTGCTCT-3′), mouse *Rab9A* (target site, 5′-CAAGACTGACATAAAAGAA-3′), and mouse *Rab38* (target site, 5′-CAATGGACTCAAGATGGAC-3′) were chemically synthesized by Nippon Gene (Toyama, Japan). The siRNA against mouse *Rab32* has been described previously (15). The Stealth RNAi™ siRNAs against mouse *Varp* were obtained from Invitrogen (catalogue No. MSS217316).

RT-PCR—The total RNA of melan-a cells transfected with *RUTBC1* siRNA and control siRNA were prepared with TRI Reagent (Sigma-Aldrich), and reverse transcription was performed by using ReverTra Ace® (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The following pairs of oligonucleotides were used for amplification: for mouse *RUTBC1*, forward primer, 5′-GCATCCAGTCCAGCCTAGAT-3′, and reverse primer, 5′-GCAGGAACCAGCGATAACAG-3′; and for *GAPDH*, forward primer, 5′-ATGGTGAAGGTCGGAGTCAA-3′, and reverse primer, 5′-GCCATGTAGACCATGAGGTC-3′. The cDNAs of *RUTBC1* and *GAPDH* were amplified by PCR performed with Ex-Taq DNA polymerase (Clontech-Takara Bio).

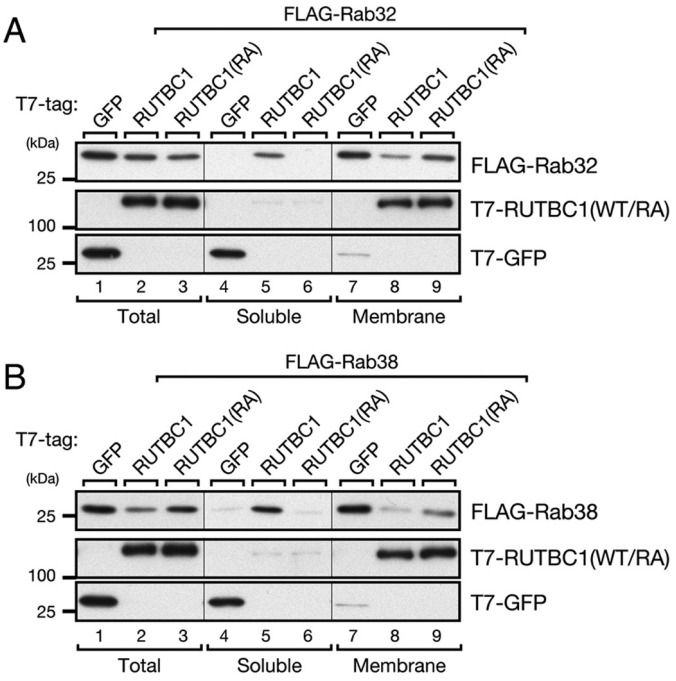

FIGURE 3. **Both Rab32 and Rab38 are present in the cytosolic fraction of RUTBC1-expressing melanocytes.** *A* and *B*, B16-F1 cells expressing FLAG-Rab32 (*A*) or FLAG-Rab38 (*B*) together with T7-GFP, T7-RUTBC1, or T7-RUTBC1(R803A) (*RA*) were homogenized, and the soluble and membrane fractions were separated as described under "Experimental Procedures." Note that the amount of FLAG-Rab32/38 in the soluble fraction was greatly increased in the RUTBC1-expressing cells when compared with the GFP-expressing cells or RUTBC1(R803A)-expressing cells. The positions of the molecular mass markers (in kDa) are shown on the *left*.

Immunofluorescence Analysis—The black mouse-derived immortal melanocyte cell line melan-a (generous gift of Dorothy C. Bennett) was cultured as described previously (28, 29). Plasmids were transfected into melan-a cells for 48 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were fixed with 10% (w/v) trichloroacetic acid, permeabilized with 0.1% Triton X-100, stained with specific primary antibodies, and then visualized with Alexa Fluor 488/594-conjugated secondary antibodies. The stained cells were examined for fluorescence with a confocal fluorescence microscope (FluoView 1000-D, Olympus, Tokyo, Japan) through an objective lens (×60 magnification, N.A. 1.40, Olympus) and with FluoView software (version 4.1a, Olympus). The images were processed with Adobe Photoshop software (CS5). The fluorescent signals of the melanogenic enzymes and Rab32/38 were captured at random (20 cells each) with the confocal microscope and quantified with MetaMorph software (Molecular Devices, Sunnyvale, CA). The sta-

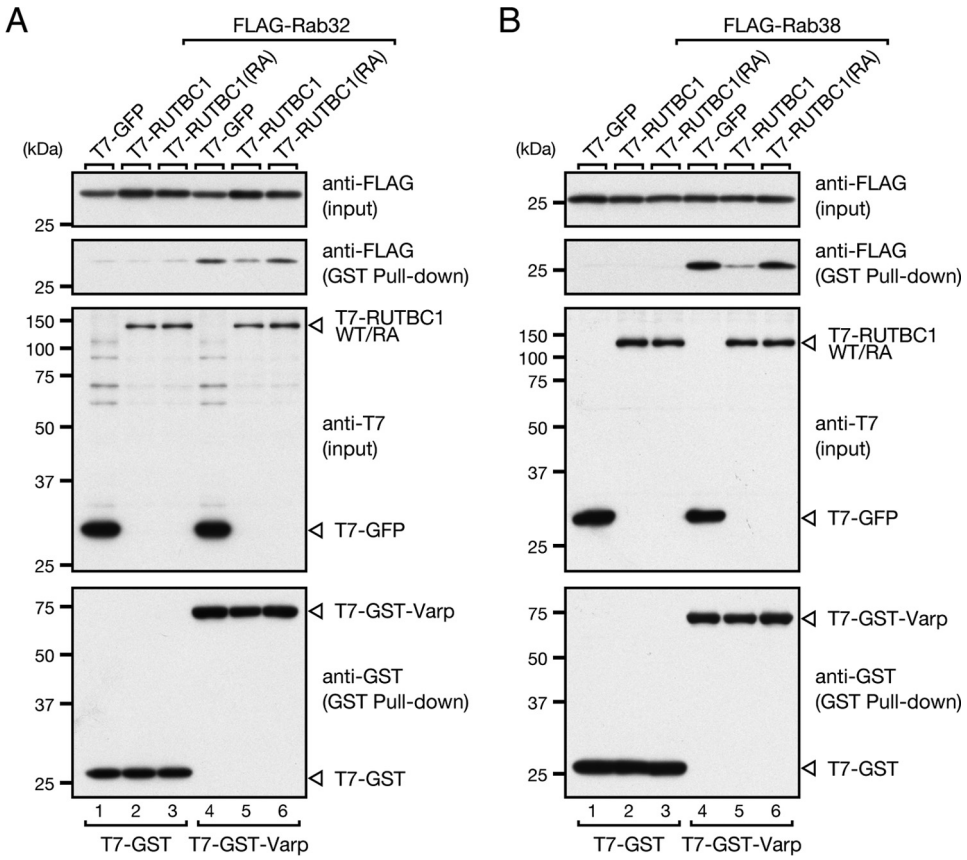

FIGURE 4. **RUTBC1 inactivates both Rab32 and Rab38 in living cells.** *A*, COS-7 cells expressing FLAG-Rab32 together with T7-GFP, T7-RUTBC1, or T7-RUTBC1(R803A) (*RA*) were lysed, and the GTP-bound active Rab32 was pulled down with glutathione-Sepharose beads that had been coupled with T7-GST (as a negative control) or T7-GST-Varp, which contains the Rab32/38-binding region of Varp. *B*, COS-7 cells expressing FLAG-Rab38 together with T7-GFP, T7-RUTBC1, or T7-RUTBC1(R803A) were lysed, and the GTP-bound active Rab38 was pulled down in the same way as described in *A*. Note that RUTBC1, but not its R803A mutant, dramatically reduced the amount of GTP-Rab32 and GTP-Rab38 (compare *lanes 5* and *6* in the *second panel* from the *top* in *A* and *B*, respectively). *Input*, 0.8% of the volume of the reaction mixture (*top* and *third panels*). *Arrowheads* indicate the positions of T7-tagged and T7-GST-tagged proteins. The positions of the molecular mass markers (in kDa) are shown on the *left*.

tistical analyses were performed using Dunnett's test or Student's unpaired *t* test, and *p* values <0.05 were considered statistically significant.

Co-immunoprecipitation and GST Pulldown Assays—COS-7 cells were transfected for 36 h with pEF-T7-GST-Varp or pEF-T7-GST by using Lipofectamine-LTX Plus (Invitrogen). The transfected cells were lysed with lysis buffer (50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100 supplemented with complete EDTA-free protease inhibitor mixture (Roche)), and the cell lysates were incubated for 2 h at 4 °C with glutathione-Sepharose beads. The beads were washed three times with washing buffer (50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, and 0.2% Triton X-100), and COS-7 cell extracts that had been co-transfected with pEF-T7-RUTBC1 wild-type (WT)/R803A and pEF-FLAG-Rab32/38 were incubated with the glutathione-Sepharose beads coupled with T7-GST-Varp or T7-GST.

COS-7 cell extracts that had been transfected with pEF-T7-Varp were lysed with the lysis buffer and allowed to react with anti-T7 tag antibody-conjugated agarose beads for 2 h at 4 °C. The beads were washed three times with the washing buffer and then incubated for 2 h at 4 °C with the lysates from RUTBC1 knockdown melan-a cells or control melan-a cells. The proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with the indicated antibodies.

Subcellular Fractionation—B16-F1 melanoma cells that had been transfected with pEF-FLAG-Rab32/38 together with pEF-T7-RUTBC1(WT/R803A) or pEF-T7-GFP were harvested and homogenized in 0.32 M sucrose, 5 mM HEPES-KOH, pH 7.2, 1 mM EGTA, 1 mM β-mercaptoethanol, and complete EDTA-free protease inhibitor mixture (Roche) in a glass-Teflon Potter homogenizer as described previously (21). The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C; then the supernatant was centrifuged at 100,000 × *g* for 1 h at 4 °C, and the supernatant and precipitate obtained were used as the cytosolic fraction and the membrane fraction, respectively. Equal proportions of the cytosolic fraction and membrane fraction were subjected to 10 or 7.5% SDS-PAGE and analyzed by immunoblotting.

Results

Functional Involvement of RUTBC1 in Melanogenic Enzyme Trafficking in Melanocytes—It had been demonstrated previously that Rab32 and Rab38 redundantly regulate the trafficking of tyrosinase and Tyrp1 to melanosomes and that knockdown of either Rab isoform alone in melanocytes has no effect

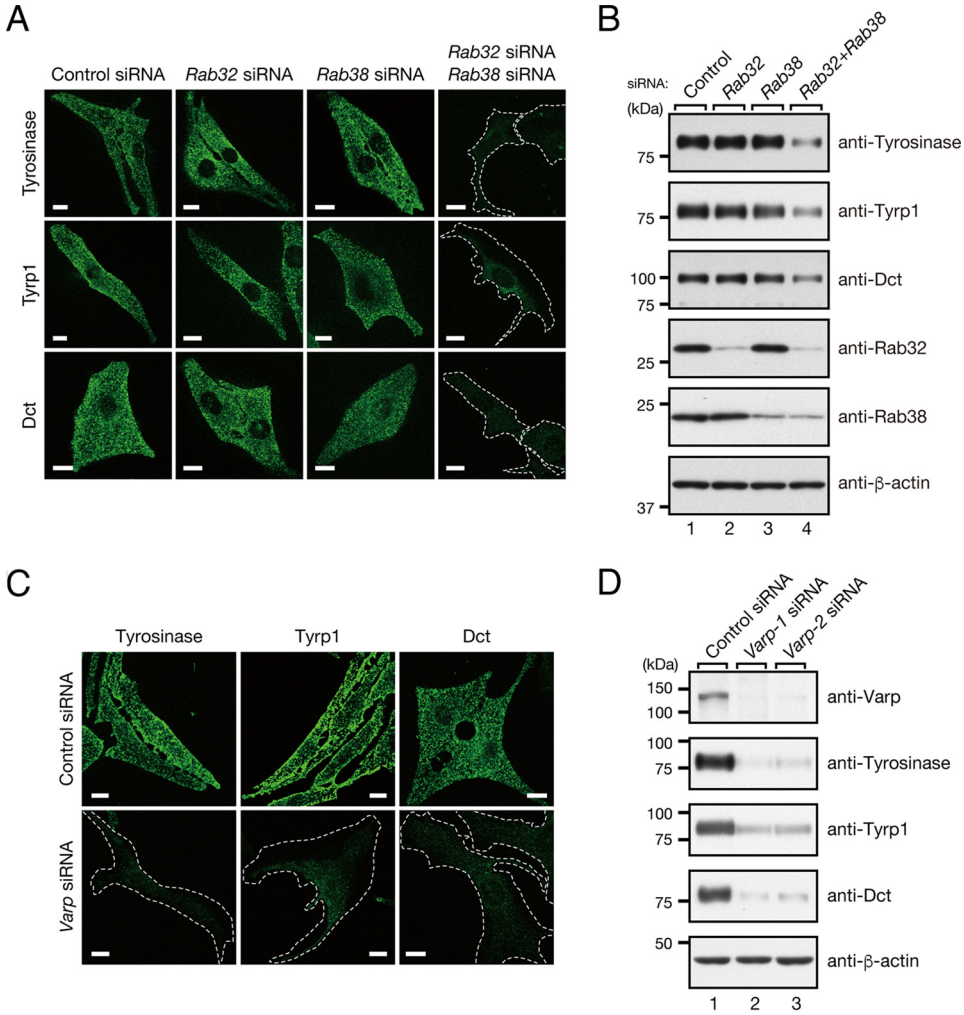

FIGURE 5. **Rab32/38 and Varp regulate the trafficking of all three melanogenic enzymes.** *A*, typical images of Rab32 knockdown and/or Rab38 knockdown melanocytes (tyrosinase, Tyrp1, and Dct images). Melan-a cells were transfected with siRNAs against Rab32 and/or Rab38 and then stained with anti-tyrosinase antibody, anti-Tyrp1 antibody, or anti-Dct antibody. *Scale bars*, 20 μm. *B*, reduced expression of all three melanogenic enzymes in Rab32/38 double knockdown melanocytes as revealed by immunoblotting with the antibodies indicated. *C*, typical images of Varp knockdown melanocytes (tyrosinase, Tyrp1, and Dct images). Melan-a cells were transfected with Stealth RNAi^{TM} siRNAs against Varp and then stained with the indicated antibodies. *Scale bars*, 20 μm. Cells exhibiting reduced melanogenic enzyme signals are outlined with a *broken line* in *A* and *C*. *D*, reduced expression of all three melanogenic enzymes in Varp knockdown melanocytes as revealed by immunoblotting with the antibodies indicated. The positions of the molecular mass markers (in kDa) are shown on the *left* in *B* and *D*.

on the melanosomal localization of either of these melanogenic enzymes (3, 11, 15). Because a Tre-2/Bub2/Cdc16 (TBC) domain-containing protein, RUTBC1, has been shown to possess GAP activity toward Rab32 but with roughly 10-fold lower activity toward Rab38 *in vitro* (18), overexpression of RUTBC1 in melanocytes was expected to have no or little effect on the expression of tyrosinase and Tyrp1, the same as in Rab32 single knockdown cells (3, 11). Surprisingly, however, when T7-tagged RUTBC1 was expressed in melan-a cells, the results of an immunofluorescence analysis showed that it had strongly reduced expression of all three melanogenic enzymes, tyrosinase, Tyrp1, and Dct (Fig. 1*B*, *top two rows*, and *C*). As demonstrated previously by us and other groups (3, 16, 30), defects in the trafficking of melanogenic enzymes usually cause loss of their expression, mainly as a result of lysosomal degradation, because melanogenic enzymes that are not properly transported to melanosomes are redirected to lysosomes for degradation. Actually, when RUTBC1-overexpressing melanocytes were treated with bafilomycin A1, a V-ATPase inhibitor that blocks lysosomal degradation, tyrosinase signals were clearly recovered, consistent with findings in the previous studies (3, 16, 30) (data not shown). To determine whether the reduction in tyrosinase/Tyrp1/Dct signals was attributable to the GAP activity of the RUTBC1 TBC domain, we prepared two truncated mutants lacking an N-terminal RUN domain, named T7-RUTBC1-C and T7-RUTBC1-C(R803A), the latter of which lacks a catalytic Arg residue for the GAP activity of TBC proteins (Fig. 1*A*) (31). As shown in Fig. 2, *A*–*C* and *F*, RUTBC1-C reduced the expression of all three melanogenic enzymes, whereas RUTBC1-C(R803A) did not. These results indicated that the reduction in tyrosinase/Tyrp1/Dct signals in RUTBC1-C-expressing melanocytes are likely attributable to the forced inactivation of substrate Rab proteins of RUTBC1. Unexpectedly, however, overexpression of the full-length RUTBC1(R803A) mutant still reduced the tyrosinase/Tyrp1/Dct signals (Fig. 1, *B* and *C*), suggesting the existence of an

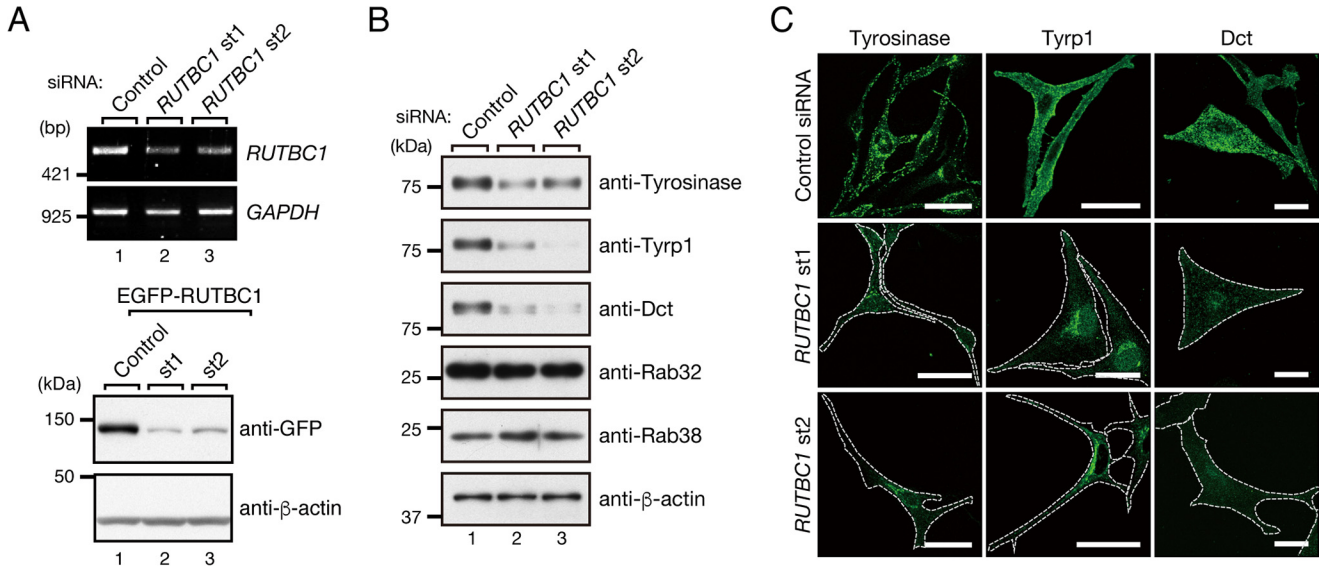

FIGURE 6. **RUTBC1 knockdown in melanocytes reduces the expression of all three melanogenic enzymes.** *A*, knockdown efficiency of *RUTBC1* siRNAs in melanocytes as revealed by RT-PCR analysis (*top two panels*) and in COS-7 cells as revealed by immunoblotting (*bottom two panels*). Both *RUTBC1* siRNAs (*st1* and *st2*) efficiently suppressed expression of *RUTBC1* mRNA (*upper top panel*) and EGFP-RUTBC1 protein (*upper bottom panel*). *GAPDH* mRNA expression is shown as a control to ensure that equivalent amounts of first-strand cDNA were used for the RT-PCR analysis. The size of the molecular weight markers (in bp, *top two panels*) is shown on the *left side* of these *panels*. *B*, RUTBC1 knockdown dramatically suppressed the expression of all three melanogenic enzymes in melanocytes without altering the expression of Rab32/38. The positions of the molecular mass markers (in kDa) are shown on the *left* in *A* (*bottom two panels*) and *B*. *C*, typical images of RUTBC1 knockdown melanocytes stained with the indicated antibodies. *Scale bars*, 20 μm.

additional GAP activity-independent mechanism that presumably is mediated by the N-terminal half of RUTBC1 (for details, see the last two paragraphs under "Results").

RUTBC1 Possesses GAP Activity toward Both Rab32 and Rab38—The inhibitory effect of the RUTBC1 overexpression on the trafficking of all three melanogenic enzymes led us to hypothesize that in living cells RUTBC1 possesses GAP activity toward Rab32 and Rab38, both of which redundantly regulate the trafficking of tyrosinase and Tyrp1 (3, 11, 14, 15). To test our hypothesis, we investigated the endogenous Rab32/38 expression in T7-RUTBC1-C-expressing cells by performing immunostaining. Intriguingly, T7-RUTBC1-C dramatically reduced both the Rab32 signals and the Rab38 signals in melan-a cells, whereas T7-RUTBC1-C(R803A) had no effect (Fig. 2, *D–F*). Because GDP-Rab proteins are generally thought to be present in the cytosol and to be lost during permeabilization with Triton X-100, we hypothesized that the reduced Rab32/38 signals in RUTBC1-expressing cells were attributable to the increased level of the GDP-bound form of Rab32/38. To test this hypothesis, we performed a subcellular fractionation experiment with B16-F1 melanoma cells that had been transfected with a FLAG-Rab32/38 expression vector together with a T7-RUTBC1(WT/R803A) expression vector. As anticipated, the results showed that both Rab32 and Rab38 were present in the cytosolic fraction of RUTBC1(WT)-overexpressing cells rather than in their membrane fraction, whereas they were present mainly in the membrane fraction in the control GFP-expressing cells and RUTBC1(R803A)-expressing cells (Fig. 3). Thus, the reduced Rab32 and Rab38 signals in RUTBC1(WT)-overexpressing cells shown in Fig. 2 are likely attributable mainly to the loss of GDP-Rab32/38 from the cells during the permeabilization process.

To confirm the GAP activity of RUTBC1 toward Rab32/38 biochemically, we proceeded to perform GTP-Rab32/38 pull-down assays by using a specific GTP-Rab32/38-binding domain of Varp (14). In brief, we coexpressed T7-RUTBC1(WT/R803A) and FLAG-Rab32/38 in cultured mammalian cells and then incubated their lysates with GST-tagged Varp, a specific GTP-Rab32/38 trapper. As anticipated, the amount of GTP-Rab32/38 was dramatically decreased when FLAG-Rab32/38 were coexpressed with T7-RUTBC1 (Fig. 4, *A* and *B*, *lane 5*, *second panel* from *top*), whereas T7-RUTBC1(R803A) failed to reduce the amount of GTP-Rab32/38 at all (Fig. 4, *A* and *B*, *lane 6*, *second panel* from *top*). Taken together, these results allowed us to conclude that RUTBC1 possesses GAP activity toward both Rab32 and Rab38 in living cells, even though RUTBC1 had been found to exhibit much lower Rab38-GAP activity *in vitro* (18).

Rab32 and Rab38 Regulate the Trafficking of All Three Melanogenic Enzymes in Melanocytes—Because it has been reported previously that Rab32 is involved specifically, and Rab38 is not involved, in the trafficking of Dct to melanosomes in human MNT-1 cells (3), and because RUTBC1 inhibits Dct trafficking to melanosomes (Fig. 1), we reinvestigated the involvement of Rab32/38 in the trafficking of tyrosinase/Tyrp1/Dct in mouse melan-a cells. We did so by using specific siRNAs to knock down Rab32 alone, Rab38 alone, or both Rab32 and Rab38 in melan-a cells. Knockdown of either Rab32 or Rab38 alone in the melan-a cells did not alter the signals of the three melanogenic enzymes at all, whereas double knockdown of Rab32/38 dramatically reduced the signals of tyrosinase/Tyrp1/Dct according to the results obtained from both immunostaining (Fig. 5*A*) and immunoblotting (Fig. 5*B*). Consistent with these results, the knockdown of Varp, a Rab32/38 effector (14, 15), in melan-a cells also resulted in a reduction of Dct signals as well as tyrosinase/Tyrp1 signals according to both the immunostaining (Fig. 5*C*) and the immunoblotting (Fig. 5*D*) findings. These results indicate that Rab32/38 and Varp are

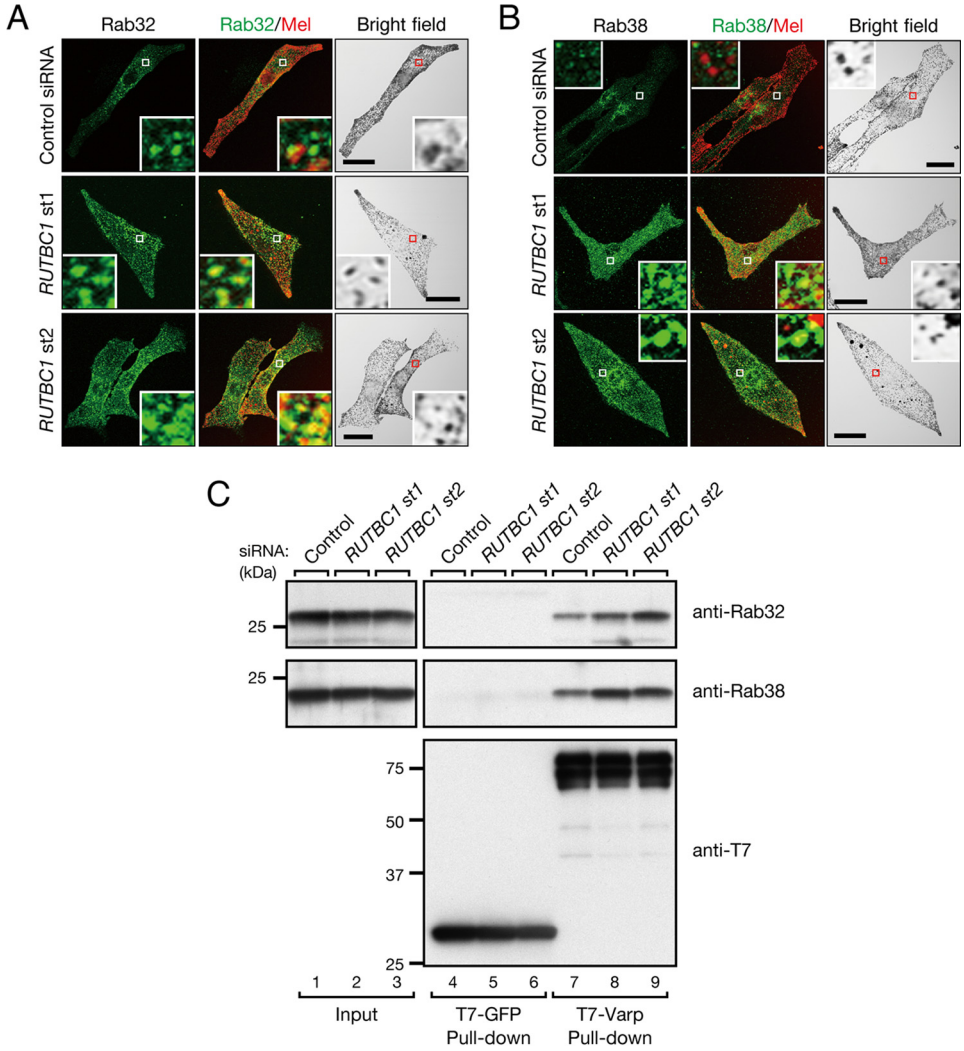

FIGURE 7. **RUTBC1 knockdown increases the melanosomal expression of Rab32/38.** *A* and *B*, typical images of RUTBC1 knockdown melanocytes stained with anti-Rab32 antibody (*A*) or anti-Rab38 antibody (*B*) and their corresponding bright field images. The *insets* show magnified views of the *boxed areas*. Note that only a few Rab32/38-positive signals colocalized with melanosomes (*Mel*, pseudo-colored in *red*) in the control siRNA-treated melanocytes, whereas the melanosomal Rab32/38 signals were clearly increased in the RUTBC1 knockdown melanocytes. *Scale bars*, 20 μm. *C*, excess activation of Rab32/38 in RUTBC1 knockdown melanocytes as revealed by immunoblotting with the indicated antibodies. Anti-T7 tag antibody-conjugated agarose beads that had been coupled with T7-GFP or T7-Varp (ANKR1, an active Rab32/38 trapper) were incubated with the lysates of RUTBC1 knockdown melanocytes. Immunoprecipitated T7-GFP or T7-Varp and co-immunoprecipitated endogenous Rab32/38 were detected with the antibodies indicated. *Input*, 1% of the volume of the reaction mixture (*two panels* on the *left*). The positions of the molecular mass markers (in kDa) are shown on the *left*.

required for proper trafficking of all three melanogenic enzymes, at least in mouse melanocytes.

Excess Activation of Rab32 and Rab38 Caused Inhibition of Melanogenic Enzyme Trafficking—Next, we investigated the function of endogenous RUTBC1 in melanocytes by knocking down RUTBC1 with specific siRNAs. First, we demonstrated endogenous expression of *RUTBC1* by performing an RT-PCR analysis and validated the knockdown efficacy of two independent *RUTBC1* siRNAs in melan-a cells (Fig. 6*A*). If RUTBC1 is a crucial factor for inactivating Rab32/38 during melanogenic enzyme trafficking, RUTBC1 knockdown should result in reduced Rab32/38 inactivation, *i.e.* an increase in GTP-Rab32/38 level, and it affects melanogenic enzyme trafficking in the RUTBC1 knockdown cells. As anticipated, the level of endogenous GTP-Rab32/38 that co-immunoprecipitated with T7-Varp was higher in the RUTBC1 knockdown melan-a cells, but there was no change in the total level of Rab32/38 expression (Fig. 7*C*). Moreover, immunostaining clearly revealed greater melanosomal Rab32/38 signals in the RUTBC1 knockdown melan-a cells (Fig. 7, *A* and *B*), suggesting that Rab32/38 were excessively activated and continuously localized on melanosomal membranes in the RUTBC1 knockdown melan-a cells.

Because activation of Rab32/38 is essential for the trafficking of tyrosinase/Tyrp1/Dct (3, 11, 12, 15) (Fig. 5), we initially thought that RUTBC1 knockdown, *i.e.* an increase in the GTP-Rab32/38 level, would increase the level of expression of all three melanogenic enzymes. Unexpectedly, however, the signals of all three melanogenic enzymes were dramatically reduced in the RUTBC1 knockdown melan-a cells (Fig. 6, *B* and *C*), despite the excess activation of Rab32/38 (Fig. 7*C*). To investigate the effect of excess activation of Rab32/38 on melanogenic enzyme trafficking by a different approach, we expressed a constitutively active mutant of Rab32(Q83L) or Rab38(Q69L) in melan-a cells and quantified the intensity of

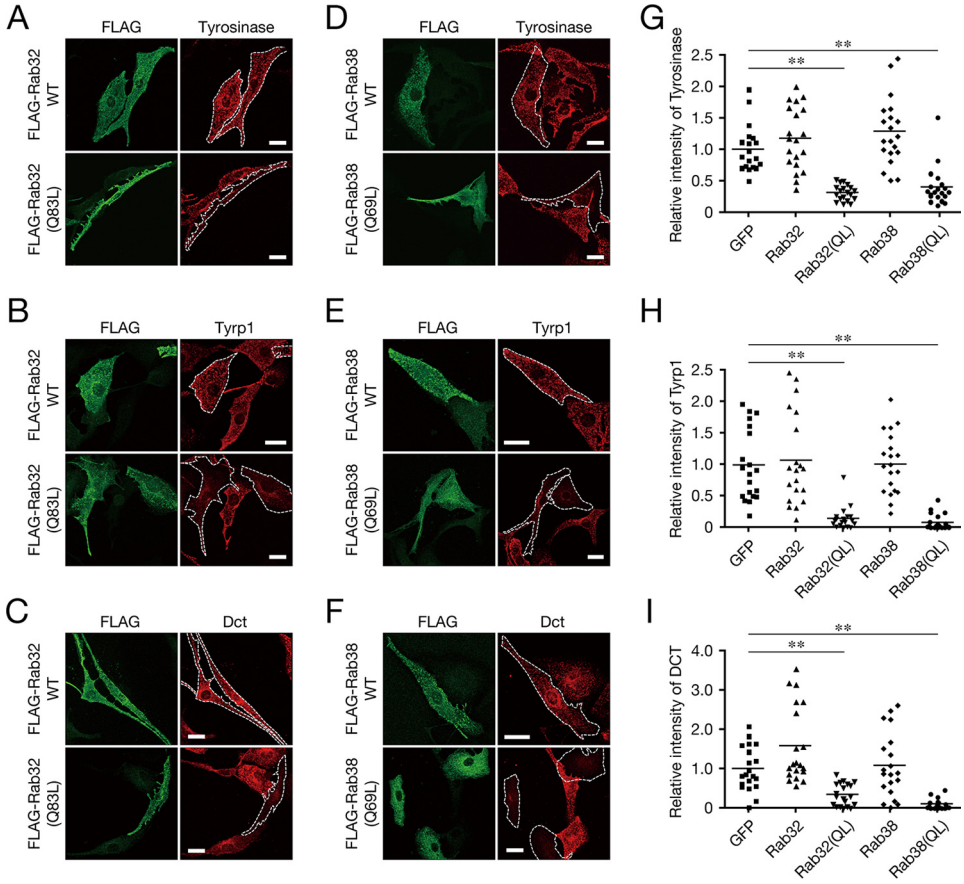

FIGURE 8. **Overexpression of a constitutively active form of Rab32/38 in melanocytes reduces the expression of all three melanogenic enzymes.** *A–C*, typical images of melanocytes expressing FLAG-Rab32(WT/Q83L) (tyrosinase, Tyrp1, and Dct images). Melan-a cells were transfected with pEF-FLAG-Rab32(WT/Q83L) and then stained with the indicated antibodies. Cells expressing FLAG-tagged protein are outlined with *broken lines*. *Scale bars*, 20 μm. *D–F*, typical images of melanocytes expressing FLAG-Rab38(WT/Q69L) (tyrosinase, Tyrp1, and Dct images). *G–I*, quantification of the tyrosinase/Tyrp1/Dct signals shown in *A–F*. The *bars* represent the means of the data ($n = 20$). The symbols represent the relative intensity of the tyrosinase/Tyrp1/Dct signals in cells expressing FLAG-GFP (*squares*), FLAG-Rab32 (*triangles*), FLAG-Rab32(Q83L) (*inverted triangles*), FLAG-Rab38 (*diamonds*), and FLAG-Rab38(Q69L) (*circles*). **, $p < 0.01$, Dunnett's test.

the tyrosinase/Tyrp1/Dct signals. Although expression of wild-type Rab32/38 in melan-a cells had no significant effect on the tyrosinase/Tyrp1/Dct signals, expression of Rab32(Q83L) or Rab38(Q69L) dramatically reduced their signals (Fig. 8), the same as knockdown of Rab32/38 or Varp did (Fig. 5).

Functional Involvement of Rab9A in Melanogenic Enzyme Trafficking—Finally, we attempted to determine the molecular basis for the reduction in expression of all three melanogenic enzymes as a result of overexpression of the GAP activity-deficient T7-RUTBC1(R803A) mutant, the same as occurred in response to overexpression of the wild-type T7-RUTBC1 (Fig. 1, *B* and *C*). As T7-RUTBC1-C(R803A) did not exert an inhibitory effect (Fig. 2), the N-terminal half of RUTBC1, which contains a RUN domain, is very likely to have an additional role in melanogenic enzyme trafficking. Interestingly, RUTBC1 has also been identified as a Rab9 effector, and the N-terminal half of RUTBC1 has been shown to be responsible for Rab9 binding (18, 19). We therefore turned our attention to Rab9 and investigated its involvement in the trafficking of tyrosinase/Tyrp1/Dct in melan-a cells. Because melan-a cells express only the Rab9A isoform and do not express the Rab9B isoform (data not shown), we expressed Rab9A together with RUTBC1(WT/R803A) in melan-a cells. As shown in Fig. 9*A* (*left panels*), Rab9A and RUTBC1(WT/R803A) were often co-localized at melanosomes. This melanosomal localization of RUTBC1 presumably is mediated by Rab9A, because most RUTBC1 itself appears to be present in the cytoplasm (Fig. 9*A*, *right panels*). Intriguingly, melan-a cells expressing both RUTBC1 and Rab9A contained dramatically reduced tyrosinase signals, whereas expression of both RUTBC1(R803A) and Rab9A or Rab9A alone had no effect on tyrosinase signals (Fig. 9*A, left panels,* and *B*). We also knocked down endogenous Rab9A by using a specific siRNA, and the results show dramatic reductions in the expression of all three melanogenic enzymes (Fig. 10).

Finally, to investigate the functional relationship between Rab9 and RUTBC1, we focused on HPS4, a subunit of a Rab32/38 GEF, BLOC-3 (12), because HPS4 is also known to be a Rab9 effector molecule (32). Consistent with previous reports (18, 19, 32), we confirmed the existence of HPS4-Rab9A and RUTBC1-Rab9A interactions (Fig. 11, *lane 4*, *second panel* from *top*, and *lane 5*, *top panel*). We then performed a competition experiment and found that RUTBC1 expression is able to disrupt the interaction between Rab9A and HPS4 (Fig. 11, *lane 6*, *second panel* from *top*). These results indicated that overexpression of RUTBC1 has at least two impacts: direct inactiva-

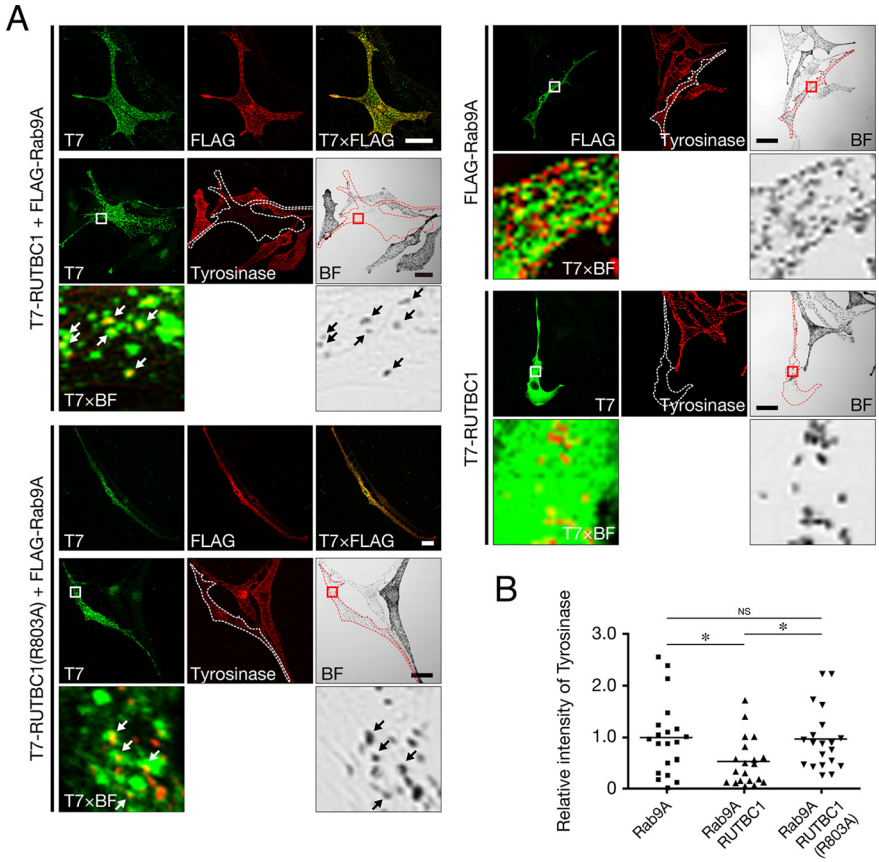

FIGURE 9. **Rab9A is involved in the proper trafficking of tyrosinase to melanosomes.** *A*, typical images of melanocytes expressing FLAG-Rab9A together with T7-RUTBC1 or T7-RUTBC1(R803A), FLAG-Rab9A alone, or T7-RUTBC1 alone. The cells were stained with anti-T7/anti-FLAG antibodies, anti-T7/anti-tyrosinase antibodies, or anti-FLAG/anti-tyrosinase antibodies, and their corresponding bright field (*BF*) images are shown. Cells expressing T7-RUTBC1(WT/R803A) or FLAG-Rab9A are outlined with *broken lines*. The *insets* show magnified views of the *boxed areas*. *Scale bars*, 20 μm. Note that the tyrosinase signals were decreased in the melanocytes expressing T7-RUTBC1/FLAG-Rab9A but not in the melanocytes expressing T7-RUTBC1(R803A)/FLAG-Rab9A. *B*, quantification of the tyrosinase signals shown in *A*. The *bars* represent the means of the data ($n = 20$). The symbols represent the relative intensity of the tyrosinase/Tyrp1/Dct signals in cells expressing FLAG-Rab9A (*squares*), FLAG-Rab9A/T7-RUTBC1 (*triangles*), and FLAG-Rab9A/T7-RUTBC1(R803A) (*inverted triangles*). *, $p < 0.05$, Dunnett's test. *NS*, not significant.

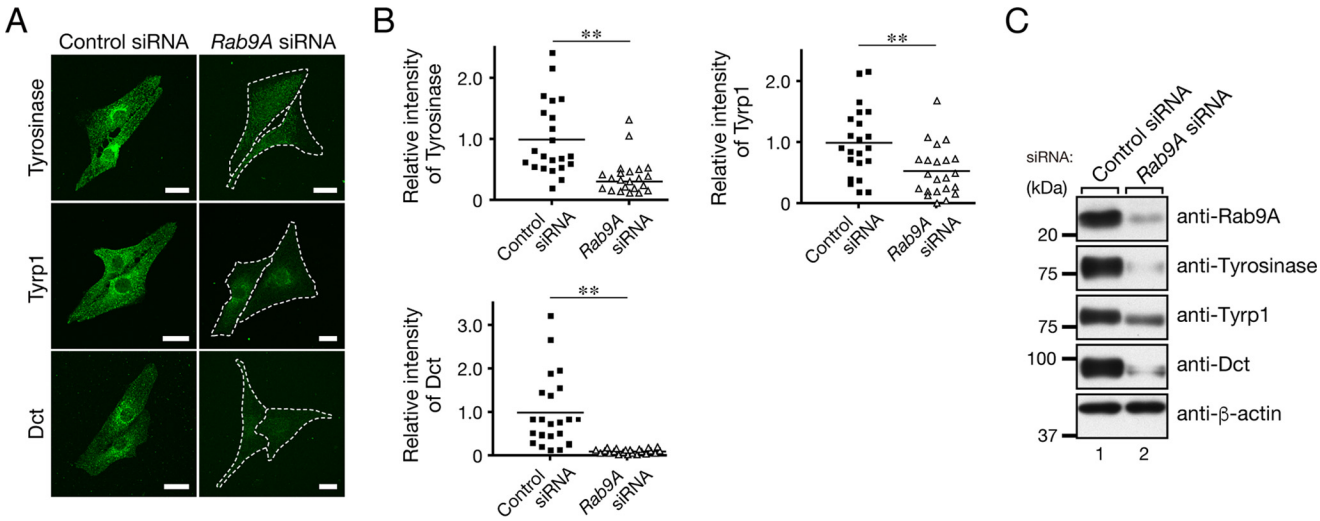

FIGURE 10. **Rab9A is involved in the proper trafficking of all three melanogenic enzymes to melanosomes.** *A*, typical images of Rab9A knockdown melanocytes stained with anti-tyrosinase antibody, anti-Tyrp1 antibody, and anti-Dct antibody. *Scale bars*, 20 μm. *B*, quantification of the tyrosinase/Tyrp1/Dct signals shown in *A*. The *bars* represent the means of the data ($n = 20$). The symbols represent the relative intensity of the tyrosinase/Tyrp1/Dct signals in control cells (*squares*) and Rab9A knockdown cells (*triangles*). **, $p < 0.01$, Student's unpaired *t* test. *C*, reduced expression of all three melanogenic enzymes in Rab9A knockdown melanocytes as revealed by immunoblotting with the indicated antibodies. The positions of the molecular mass markers (in kDa) are shown on the *left*.

tion of Rab32/38 by its TBC/Rab-GAP domain and indirect inactivation of Rab32/38 by disrupting a complex composed of Rab9 and BLOC-3 (see Discussion below). All these results taken together indicated that spatiotemporal regulation of Rab32/38 by RUTBC1 and a RUTBC1 binding partner, Rab9A, is involved in the trafficking of melanogenic enzymes in melanocytes.

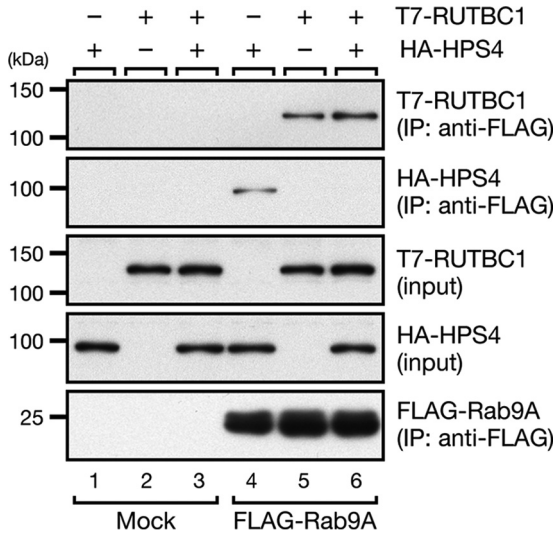

FIGURE 11. **RUTBC1 competes with HPS4 for binding to Rab9A.** COS-7 cell lysates expressing FLAG-Rab9A or nothing (*Mock*) together with HA-HPS4 and/or T7-RUTBC1 were immunoprecipitated with anti-FLAG tag antibody-conjugated agarose beads, and the proteins bound to the beads were analyzed by immunoblotting with the indicated antibodies. *Input*, 0.8% volume of the reaction mixture (*third* and *fourth panels*). The positions of the molecular mass markers (in kDa) are shown on the *left*.

Discussion

Although RUTBC1 had been reported to possess GAP activity toward Rab32 and Rab33B, but roughly 10-fold lower activity toward Rab38, *in vitro* (18), its physiological function had remained unknown. In this paper we have presented several lines of evidence that RUTBC1 is a physiological GAP for Rab32/38 that is crucial for melanogenic enzyme transport to melanosomes in melanocytes. The first line of evidence is that overexpression of the C-terminal TBC/Rab-GAP domain of RUTBC1, but not overexpression of its catalytic Arg mutant (R803A), in melanocytes induced a dramatic reduction in the amount of melanogenic enzymes (tyrosinase/Tyrp1) (Fig. 2, *A* and *B*). Similarly, double knockdown of Rab32/38 in melanocytes induced the same phenotype, whereas single knockdown of Rab32 or Rab38 alone had no effect on tyrosinase/Tyrp1 signals (Fig. 5, *A* and *B*). The second line of evidence is that decreased Rab32/38 signals were observed in RUTBC1-C-overexpressing melan-a cells (Fig. 2, *D* and *E*) and increased Rab32/38 signals were observed in RUTBC1 knockdown melan-a cells (Fig. 7, *A* and *B*). The final line of evidence is that RUTBC1, but not RUTBC1(R803A), dramatically decreased the amount of GTP-Rab38 as well as GTP-Rab32 in COS-7 cells (Fig. 4) and that the amount of GTP-Rab32/38 was increased in RUTBC1 knockdown melan-a cells (Fig. 7*C*). These findings allowed us to conclude that RUTBC1 is a functional Rab32/38 GAP in melanocytes. Nottingham *et al.* (18), however, report finding that RUTBC1 exhibits roughly 10-fold lower Rab38-GAP activity than Rab32-GAP activity *in vitro*. Although the reason for the discrepancy between their findings and our own is unknown, this lower *in vitro* Rab38-GAP activity of RUTBC1 may be sufficient to inactivate endogenous Rab38 in living cells. Alternatively, the recombinant Rab38 protein purified from bacteria (18) that they used may not fold correctly and consequently may fail to function as an efficient substrate of RUTBC1.

Because both overexpression and knockdown of RUTBC1 induced a dramatic reduction in the amount of all three melanogenic enzymes (tyrosinase/Tyrp1/Dct) (Figs. 1 and 6) and overexpression of constitutively active Rab32/38 mutants induced the same phenotype (Fig. 8), the proper activation and inactivation of Rab32/38 (*i.e.* GTP-GDP cycling) must be necessary for melanogenic enzyme transport to occur in melanocytes (Fig. 12*A*, control cells, and Fig. 12*B*, RUTBC1 knockdown cells). The importance of GTP-GDP cycling of Rab27A in actin-based melanosome transport in melanocytes has also recently been reported (33). Thus, in the future it would be interesting to determine the mechanism by which spatiotemporal regulation of Rab32/38 is achieved by RUTBC1 (or Rab27A by EPI64 (34)) in melanocytes.

Because Rab32, and not Rab38, is reportedly responsible for the trafficking of Dct in "human" MNT-1 melanoma cells (3), the discovery in the present study that RUTBC1 and its substrate Rab32/38 are also involved in the trafficking of Dct in mouse melan-a cells was unexpected (Figs. 1*C*, 5, *A* and *B*, 6, *B* and *C*, and 8*I*). Although we do not know the reason for this species difference, the Dct trafficking pathways in human melanoma cells and mouse melanocytes may be different. In the future it would be interesting to investigate the differences between melanogenesis in human and mouse melanocytes. At any rate, because the Rab32/38 effector Varp is also involved in the trafficking of all three melanogenic enzymes (tyrosinase/Tyrp1/Dct) the same as Rab32/38 (Fig. 5), the Rab32/38-mediated membrane trafficking pathway is required for proper tyrosinase/Tyrp1/Dct transport to melanosomes in mouse melanocytes.

Another unexpected finding in our study was that overexpression of the full-length GAP activity-deficient RUTBC1 (R803A) mutant in melanocytes also caused a reduction in the signals of all three melanogenic enzymes, the same as overexpression of wild-type RUTBC1 did (Fig. 1), whereas the C-terminal RUTBC1-C(R803A) mutant had no effect at all (Fig. 2). Because the N-terminal domain of RUTBC1 interacts with Rab9 (Fig. 1*A*) (18, 19), the inhibitory effect of the RUTBC1 (R803A) mutant is likely to be caused by trapping endogenous Rab9A, resulting in inhibition of the endogenous Rab9A-RUTBC1 interaction in melanocytes. Consistent with this possibility, no significant difference in tyrosinase signals was observed when RUTBC1(R803A) was coexpressed together with Rab9A in melanocytes (Fig. 9*A*). Rab9 is known to be expressed ubiquitously and to regulate the transport of the mannose 6-phosphate receptor from late endosomes to the *trans*-Golgi network (35); however, the physiological function of Rab9 in melanocytes had never been satisfactorily elucidated. The results of the present study demonstrate that Rab9A has the ability to recruit RUTBC1 to melanosomes and that Rab9A is required for the trafficking of all three melanogenic enzymes (Fig. 10). Consistent with our results, very recently, during the course of revising this paper, Mahanty *et al.* (36) have reported the involvement of Rab9A in melanogenic enzyme trafficking. Moreover, we demonstrated that RUTBC1 is able to disrupt the interaction between Rab9A and HPS4, a component of BLOC-3, a Rab32/38 GEF (12) (Fig. 11). Thus, RUTBC1 overexpression inhibits Rab32/38 activation both directly by pro-

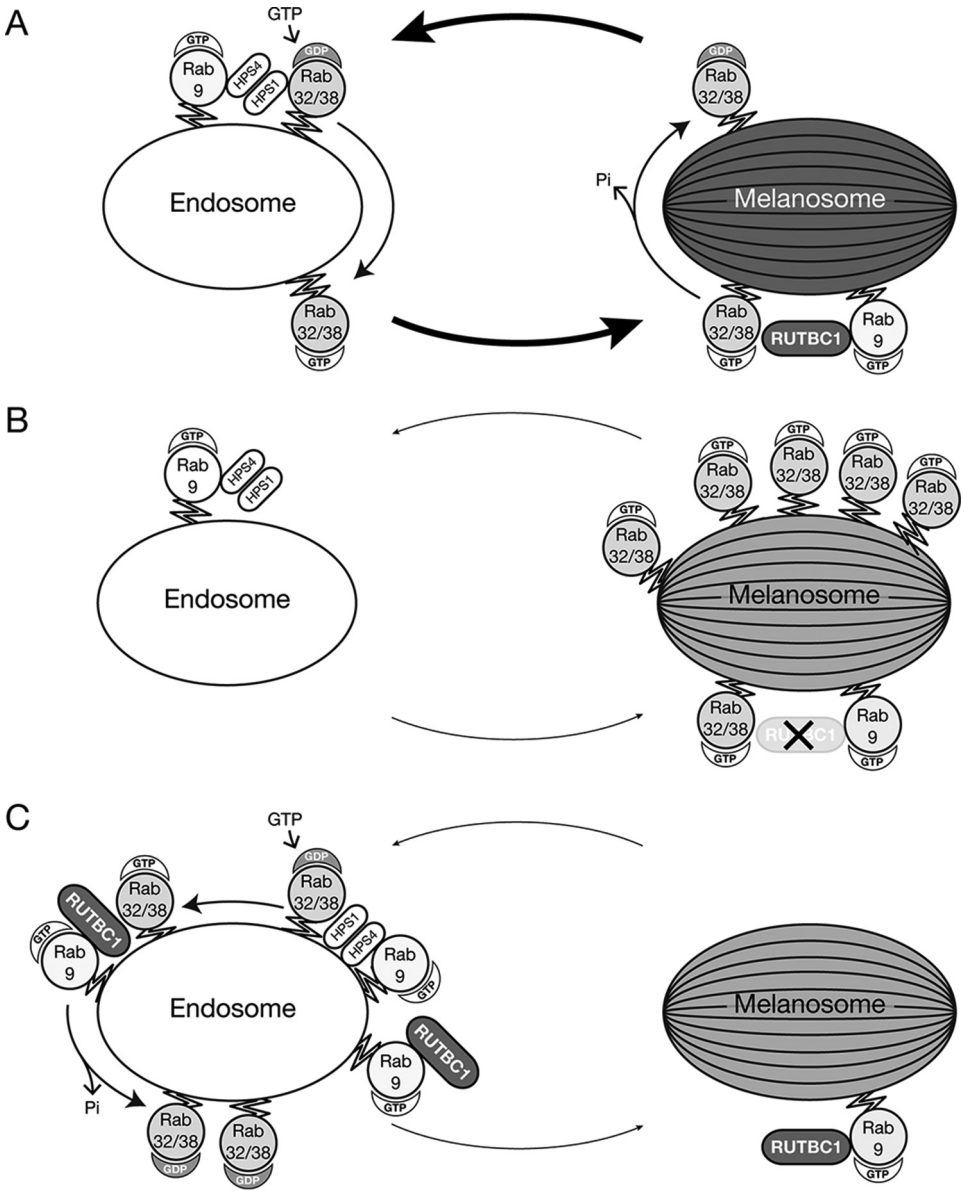

FIGURE 12. **Proposed models of Rab32/38 activation and inactivation in melanocytes.** *A*, in wild-type cells Rab32/38 is activated by the Rab9 effector BLOC-3, and GTP-Rab32/38 is then inactivated by another Rab9 effector, RUTBC1, through promoting the GTPase activity of both Rab32 and Rab38. A proper activation-inactivation cycle between GTP-Rab32/38 and GDP-Rab32/38 is essential for melanogenic enzyme trafficking to melanosomes, as both knockdown of RUTBC1 (see *B*) and expression of constitutively active Rab32/38 mutants caused a dramatic reduction in melanogenic enzyme signals (Figs. 6 and 8). To simplify the model, Varp (Rab32/38 effector) and its cargo (melanogenic enzymes) have not been depicted. *B*, because Rab32 and Rab38 are constantly activated at the melanosomes in RUTBC1 knockdown cells, melanogenic enzymes are not properly transported to the melanosomes and, for the most part, are degraded in lysosomes. *C*, in RUTBC1-overexpressing cells, RUTBC1 inactivates Rab32 and Rab38 immediately after activation by BLOC-3, which prevent the trafficking of melanogenic enzymes. If the level of Rab9A expression is insufficient, RUTBC1 can also disrupt the formation of the Rab9A·BLOC-3 complex, which is required for Rab32/38 activation. If the Rab9A expression level is sufficient because of Rab9A coexpression, RUTBC1(R803A) is unable to disrupt the formation of Rab9A·BLOC-3 complex and does not inactivate Rab32/38 (Fig. 9). Thus, melanogenic enzymes in RUTBC1(R803A)+Rab9A-expressing cells are correctly transported to melanosomes.

moting Rab32/38 GAP activity and indirectly by disrupting the Rab9-HPS4 interaction. RUTBC1 is unlikely to disrupt the Rab9-HPS4 interaction when RUTBC1 and Rab9A are coexpressed in melanocytes, because a sufficient amount of Rab9A is present in the cells and no competition between RUTBC1 and HPS4 would occur. However, the overexpressed RUTBC1 still has the ability to inactivate Rab32/38, because the Rab32/38 activated by BLOC-3 is rapidly inactivated by the overexpressed RUTBC1 (Fig. 12*C*). By contrast, when RUTBC1(R803A) is expressed together with Rab9A, the Rab9A-HPS4 interaction would occur normally, and RUTBC1(R803A) cannot inactivate Rab32/38. Actually, there was no change at all in tyrosinase signals in Rab9A+RUTBC1(R803A)-expressing cells, when compared with Rab9A+RUTBC1(WT)-expressing cells (Fig. 9). Our findings suggest that the proper activation of Rab32/38 by Rab9-BLOC-3 and their subsequent inactivation by Rab9-RUTBC1 (*i.e.* cycling between GDP-Rab32/38 and GTP-Rab32/38) are required for melanogenic enzyme trafficking to melanosomes. Further extensive research will be necessary to determine the exact mechanism of the coordination between the activation and inactivation of Rab32/38 by Rab9A.

Because RUTBC1 has also been found to possess GAP activity toward Rab33B *in vitro* (18), the possibility remains that RUTBC1 participates in the trafficking of melanogenic enzymes through the inactivation of Rab33B rather than Rab32/38. That possibility is very unlikely, however, because of the following observations. First, in contrast to Rab32/38 being colocalized well with Tyrp1 (14), most EGFP-tagged Rab33B is present at the Golgi (37, 38) and not at melanosomes or the tyrosinase-containing vesicles at the cell periphery (data not shown). Second, we found that in sharp contrast to the results of expression of the constitutively active mutants of Rab32/38, which decreased the signals of all three melanogenic enzymes (Fig. 8), expression of a constitutively active mutant of Rab33B(Q92L) affected the signals of the three melanogenic enzymes differently: it increased the Tyrp1 signals slightly and had almost no effect on the Dct signals, whereas it decreased the tyrosinase signals (data not shown). Thus, the observed effects of RUTBC1 knockdown on tyrosinase/Tyrp1/Dct signals are most likely attributable to excess activation of Rab32/38, rather than of Rab33B. However, because Rab33B has been shown to be involved in the autophagosome maturation step (22, 38) and the retrograde Golgi transport pathway (39), and because decreased tyrosinase signals were observed in Rab33B(Q92L)-expressing cells, Rab33B may participate in melanogenic enzyme trafficking in melanocytes in a different way from Rab32/38, *i.e.* by regulating autophagy or retrograde Golgi transport. It seems noteworthy that an autophagy regulator, WIPI1, has been shown to regulate the biogenesis of melanosomes (40). Further investigation of Rab33B in the future should clarify its function in melanogenesis.

In conclusion, this study is the first to demonstrate that the Rab9-binding protein RUTBC1 regulates the trafficking of all three melanogenic enzymes in melanocytes by inactivating Rab32/38. The results of this study also demonstrated that excess activation of Rab32/38, either by RUTBC1 knockdown or by overexpression of Rab32(Q83L)/38(Q69L), inhibits the trafficking of all three melanogenic enzymes. Our findings suggest that a proper GTP-GDP cycle for Rab32/38 is necessary for the normal trafficking of all three melanogenic enzymes to melanosomes to occur.

Author Contributions—S. M. and N. O. designed, performed, and analyzed the experiments and wrote the paper. H. S. performed and analyzed the experiments. M. F. designed and analyzed the experiments and wrote the paper.

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