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A novel Varp-binding protein, RACK1, regulates dendrite outgrowth through stabilization of Varp protein in mouse melanocytes

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Short title: RACK1 stabilizes Varp protein in melanocytes

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Abbreviations: ANKR, ankyrin repeat; EGFP, enhanced green fluorescent protein; Fsk, forskolin; HRP, horseradish peroxidase; mStr, monomeric Strawberry; RACK1, receptor of activated protein kinase C 1; RT, reverse transcription; siRNA, small interfering RNA; SC, synthetic complete; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; Varp, VPS9-ankyrin repeat protein.

ABSTRACT

Varp (VPS9-ankyrin repeat protein) in melanocytes is thought to function as a key player in pigmentation of mammals. Varp regulates two different melanocyte functions, i.e., transport of melanogenic enzymes to melanosomes by functioning as a Rab32/38 effector, and promotion of dendrite outgrowth by functioning as a Rab21-guanine nucleotide exchange factor. The Varp protein level has recently been shown to be negatively regulated by proteasomal degradation through interaction of the ankyrin repeat 2 (ANKR2) domain of Varp with Rab40C. However, the molecular mechanisms by which Varp escapes from Rab40C and retains its own expression level remain completely unknown. Here, we identified RACK1 (receptor of activated protein kinase C 1) as a novel Varp-ANKR2-binding partner and investigated its involvement in Varp stabilization in mouse melanocytes. The results showed that knockdown of endogenous RACK1 in melanocytes caused dramatic reduction of the Varp protein level and inhibition of dendrite outgrowth, and intriguingly, overexpression of RACK1 inhibited the interaction between Varp and Rab40C and counteracted the negative effect of Rab40C on dendrite outgrowth. These findings indicated that RACK1 competes with Rab40C for binding to the ANKR2 domain of Varp and regulates dendrite outgrowth through stabilization of Varp in mouse melanocytes.

INTRODUCTION

Melanocytes are unique cells that play an important role in producing pigmented organelles, called melanosomes, that protect cells against ultraviolet injury (Raposo and Marks, 2007). Melanocytes originate from the embryonic neural crest cells of mammals, and the embryonic neural crest cells migrate and differentiate into melanocytes in the basal layer of the epidermis and in the hair bulbs (Cichorek *et al*., 2013). Skin and hair become pigmented as a result of a process that consists of several steps. In the first step, mature melanosomes are produced around the nucleus (melanosome biogenesis step). In the next step, the mature melanosomes are transported (melanosome transport step) along the two cytoskeletal components, microtubules and actin filaments, and then transferred to surrounding keratinocytes through the melanocyte dendrites (melanosome transfer step). The melanocyte dendrites are specialized cell structures that form and contact surrounding keratinocytes and hair matrix cells (dendrite outgrowth step) in response to growth factors and ultraviolet irradiation (Ohbayashi and Fukuda, 2012; Wu and Hammer, 2014). Each of these steps is thought to be crucial to pigmentation, because genetic defects in one of these steps have been reported to cause albinism, a group of hereditary diseases that are characterized by hypopigmentation of the hair and skin, e.g., Griscelli syndrome, Hermansky-Pudlak syndrome, and Chédiak-Higashi syndrome (Tomita and Suzuki, 2004; Di Pietro and Dell'Angelica, 2005; Van Gele *et al*., 2009). Genetic analysis of these diseases and their corresponding coat color mutant mice in the past few decades

have revealed their causative genes, and further characterization of the gene products has enabled identification of a variety of their binding proteins and regulators that participate in skin and hair pigmentation, e.g., BLOC (biogenesis of lysosome-related organelles complex) (Wei and Li, 2013) and small GTPase Rab38 (Loftus *et al.*, 2002).

Varp (VPS9-ankyrin repeat protein) is one such protein that functions as an effector molecule for the small GTPase Rab38 (Tamura *et al*., 2009), a deficiency of which causes the diluted coat color of *chocolate* mice (Loftus *et al.*, 2002), one of the mouse models of Hermansky-Pudlak syndrome (Wei and Li, 2013). Varp is a pleiotropic regulator of melanogenesis, because it promotes dendrite formation through activation of Rab21 via its N-terminal VPS9 domain (Zhang *et al*., 2006; Ohbayashi *et al*., 2012b), and melanogenic enzyme transport to melanosomes via its ankyrin repeat 1 (ANKR1) domain, which binds Rab32/38 (Wang *et al.*, 2008; Tamura *et al*., 2009; Tamura *et al*., 2011). In addition, binding of VAMP7 to a VAMP7-interaction domain (VID) of Varp (Burgo *et al*., 2009; Schäfer *et al*., 2012) is required for both dendrite outgrowth and melanogenic enzyme transport (Tamura *et al*., 2011). The results of recent proteomic analyses have revealed the presence of additional Varp binding partners, including retromer, GolginA4, and Kif5A, in non-melanocytic cells (Burgo *et al.*, 2012; Hesketh *et al.*, 2014; McGough *et al.*, 2014), but their involvement in melanogenesis is poorly understood. More recently, we have shown that Rab40C binds to a C-terminal ANKR2 domain of Varp and that the binding promotes proteasomal degradation of Varp through recruiting a Cullin-type ubiquitin ligase complex (Yatsu *et al*., 2015). However, the molecular mechanism by which Varp

escapes from Rab40C-mediated degradation remains completely unknown, and the presence of an additional as yet unknown Varp binding partner(s) that regulates the interaction between Rab40C and the ANKR2 domain of Varp in melanocytes is assumed.

In this study, we screened for a novel Varp ANKR2-domain-binding protein by performing yeast-two hybrid assays and succeeded in identifying RACK1 (receptor of activated protein kinase C 1: also known as Gnb2l1) as a candidate. RACK1 was originally described as an anchoring protein for activated PKCβII (Ron *et al*., 1994), and it has subsequently been shown to function as a scaffold protein that is involved in a variety of cellular events, including translation, apoptosis, and migration (Serrels *et al*., 2011; Gandin *et al*., 2013; Li and Xie, 2014). The results of our biochemical assays showed that the interaction between RACK1 and Varp was increased by forskolin, which increased the intracellular cAMP concentration and induced dendrite outgrowth of melanocytes (Ohbayashi *et al.*, 2012b), and that RACK1 had the ability to reduce Rab40C binding to the ANKR2 domain of Varp by competitively binding to it. Moreover, knockdown of endogenous RACK1 in melanocytes in our study caused a dramatic reduction in the amount of Varp protein and in forskolin-induced dendrite outgrowth. Based on our findings, we discuss a novel role of RACK1 in protecting Varp protein from Rab40C during dendrite outgrowth of melanocytes and its relation to albinism.

RESULTS

Identification of RACK1 as a novel Varp-binding protein

To identify novel Varp ANKR2-domain-binding partners, we performed yeast two-hybrid screening with the Varp ANKR2 domain used as bait (Figure 1a). We screened approximately 1.8×10^6 colonies of a mouse cDNA library and obtained 40 positive clones. We especially focused on the candidate protein, RACK1, because three independent prey clones were obtained for RACK1 (Figure 1b; clone #2 was not depicted because of its incomplete sequence), whereas only one or two positive clones were obtained for other candidate proteins. Because the two completely sequenced (#1 and #3) of the three RACK1 clones contained part of the C-terminal sequence of RACK1, we prepared a RACK1ΔN mutant (amino acids 204–317) and performed yeast two-hybrid assays to investigate its binding activity in relation to three Varp truncated mutants (Figure 1a). As expected, the RACK1ΔN construct specifically interacted with the ANKR2 domain and did not interact at all with the N+VPS9 domain or the ANKR1 domain (Figure 1c). In addition, we confirmed the interaction between full-length Varp and RACK1 in mammalian cultured cells by performing co-immunoprecipitation assays (Figure 1d, lane 4 in the middle panel). We also confirmed the endogenous interaction between Varp and RACK1 by using specific antibodies (Figure 1f). Because RACK1 has previously been shown to be involved in several signaling pathways, including a cAMP pathway and a PKC pathway (Adams *et al*., 2011), we treated cells with a PKC activator, 12-*O*-tetradecanoylphorbol 13-acetate

(TPA), and a PKA activator, forskolin. Intriguingly, the interaction between Varp and RACK1 was significantly increased by forskolin, whereas TPA had no effect at all (Figure 1d, lanes 5 and 6 in the middle panel, and e).

Knockdown of RACK1 in melanocytes induces Varp degradation and inhibition of dendrite outgrowth

The finding that the Varp–RACK1 interaction was increased by forskolin led us to investigate the involvement of RACK1 in the forskolin-induced dendrite outgrowth of melanocytes (Ohbayashi *et al*., 2012b), because melanocyte dendrite outgrowth is the only forskolin-dependent phenomenon in which Varp has been reported to be involved in the literature. First, we investigated whether RACK1 is endogenously expressed in melanocytes. Since the results of immunoblotting and RT (reverse transcription)-PCR analysis indicated that RACK1 is actually expressed in mouse melan-a cells (Figure 2a, lane 1 in the top panel, and c, left graph), we proceeded to investigate whether RACK1 is involved in dendrite outgrowth of melanocytes. To do so, we prepared two independent sites of *RACK1* siRNAs, both of which efficiently knocked down endogenous RACK1 protein (Figure 2a, top panel) as well as *RACK1* mRNA in the melanocytes (Figure 2c, left graph), and the results showed that the knockdown of RACK1 strongly inhibited forskolin-induced dendrite outgrowth (Figure 3a and b), the same as Varp knockdown did (Ohbayashi *et al.*, 2012b). Furthermore, knockdown of RACK1 also resulted in a significant decrease in the number of dendrites/cell (Control siRNA, 2.22 ± 0.11; *RACK1* siRNA st1, 1.62 ± 0.09; and *RACK1* siRNA st2, 1.27 ±

0.10 (mean ± SEM); $P<0.01$). To our surprise, there was a concomitant decrease in the amount of Varp protein in the RACK1-knockdown melanocytes (Figure 2a, middle panel, and b), and yet RACK1 knockdown had no significant effect on the level of *Varp* mRNA expression (Figure 2c, right graph). The lower level of Varp protein in the RACK1-knockdown cells is likely to have been attributable to proteasomal degradation, because treatment of the cells with proteasome inhibitor MG132 resulted in partial recovery of its protein level (Figure 2d, middle panel). These results indicated that RACK1 is most likely involved in dendrite outgrowth of forskolin-stimulated melanocytes through regulation of the Varp protein expression level.

RACK1 inhibits the interaction between Varp and Rab40C

Because the Varp protein expression level is negatively controlled by Rab40C through proteasomal degradation and because Rab40C interacts with the ANKR2 domain of Varp (Yatsu *et al.*, 2015), the same as RACK1 does (Figure 1c), we hypothesized that RACK1 protects Varp protein from proteasomal degradation by inhibiting the interaction between Rab40C and Varp. To test our hypothesis, we expressed T7-tagged Varp and FLAG-tagged Rab40C (or EGFP as a control) with or without HA-tagged RACK1 in COS-7 cells and performed co-immunoprecipitation assays (Figure 4a). The results showed that expression of HA-RACK1 dramatically decreased the amount of Varp that co-immunoprecipitated with Rab40C (Figure 4a, lanes 2 and 3 in the second panel, and b), indicating that RACK1 effectively inhibits the interaction between Varp and Rab40C in vitro. Consistent with this result, the effect

of the RACK1 knockdown on the Varp protein level (i.e., decreased Varp protein level) in melanocytes was clearly attenuated by simultaneous knockdown of Rab40C (Figure 4c, lanes 2 and 4 in the middle panel, and d).

Finally, we investigated whether RACK1 antagonizes the effect of Rab40C on the Varp protein expression level during dendrite outgrowth. To do so, we expressed RACK1 with or without Rab40C in forskolin-stimulated melanocytes and evaluated its effect on dendrite outgrowth. Expression of mStr-RACK1 alone (+ EGFP as mock control) in forskolin-stimulated melanocytes had no effect on dendrite outgrowth (Figure 5a, second row, and b), whereas expression of EGFP-Rab40C alone (+ mStr as mock control) significantly inhibited forskolin-induced dendrite outgrowth (Figure 5a, third row, and b). By contrast, simultaneous expression of EGFP-Rab40C and mStr-RACK1 in forskolin-stimulated melanocytes completely restored dendrite outgrowth to the mock control (EGFP/mStr) level (Figure 5a, bottom row, and b), and the rescue effect of RACK1 on dendrite outgrowth was canceled when endogenous Varp was knocked down in EGFP-Rab40C+mStr-RACK1-expressing cells (Supplementary Figure S1). A similar tendency was observed in regard to the numbers of dendrites of the forskolin-stimulated cells: expression of EGFP-Rab40C alone slightly decreased the number of dendrites/cell, but the decrease was not statistically significant (mStr+EGFP, 2.15 ± 0.10; mStr-RACK1+EGFP, 2.15 ± 0.09; mStr+EGFP-Rab40C, 2.01 ± 0.07; and mStr-RACK1+EGFP-Rab40C, 2.25 ± 0.07 (mean ± SEM)). Taken together, these results indicated that RACK1 has the ability to protect Varp protein from Rab40C and promotes forskolin-induced dendrite outgrowth

of melanocytes.

DISCUSSION

We previously identified Rab40C as a negative regulator of the Varp protein expression level in melanocytes that induces proteasomal degradation of Varp through interaction with the ANKR2 domain (Yatsu *et al.*, 2015). However, the molecular mechanism by which Varp protein escapes from Rab40C remained unknown, and no attempt had ever been made to identify a positive regulator of the Varp protein expression level that counteracts the Varp degradation caused by Rab40C. In the present study, we identified RACK1 as a novel Varp ANKR2-domain-binding protein by yeast two-hybrid screening and demonstrated that forskolin, which increases the intracellular cAMP level by activating adenylate cyclase, increases the interaction between RACK1 and Varp (Figure 1). Because Varp has been shown to regulate forskolin-induced dendrite outgrowth of melanocytes (Ohbayashi *et al.*, 2012b), we proceeded to investigate the functional involvement of RACK1 in this process by knockdown and overexpression experiments. The results showed that RACK1 is actually required for stabilization of Varp protein (i.e., for prevention of Varp proteasomal degradation) but does not affect the *Varp* mRNA level (Figure 2) and that knockdown of RACK1 inhibited forskolin-induced dendrite outgrowth (Figure 3), the same as Varp knockdown did (Ohbayashi *et al.*, 2012b). More importantly, RACK1 has the ability to compete with Rab40C for binding to the Varp ANKR2 domain in vitro (Figure 4), and the inhibitory effect of Rab40C on dendrite outgrowth in forskolin-stimulated melanocytes was fully restored by simultaneous expression of RACK1 (Figure 5). These results taken

together indicated that RACK1 is a positive regulator of the Varp protein expression level in melanocytes that directly competes with a negative regulator Rab40C for binding to the ANKR2 domain (see model in Supplementary Figure S2). The molecular mechanism by which the RACK1–Varp interaction is regulated by forskolin is completely unknown, but because RACK1 interacts with many kinases, including PKC, Src, and MAPK (Adams *et al.*, 2011), phosphorylation is likely to regulate the interaction. Further research will be necessary to determine whether phosphorylation (or post-translational modification(s)) of RACK1 (or Varp) actually regulates the interaction between Varp and RACK1 or Rab40C during skin pigmentation.

Although in the present study we focused on the role of RACK1 in forskolin-induced dendrite outgrowth, RACK1 may also contribute to forskolin-independent functions of Varp, e.g., melanogenic enzyme transport, in melanocytes (Tamura *et al.*, 2009; Tamura *et al.*, 2011; Marubashi *et al.*, 2016), because the RACK1–Varp interaction occurs even in the absence of forskolin (Figure 1d). Actually, RACK1 has previously been reported to be present on melanosomes in human primary melanocytes with an unknown mechanism and to regulate tyrosinase activity by recruiting PKCβ (Park *et al.*, 2004). Moreover, knockdown of RACK1 in melan-a cells caused disappearance of tyrosinase signals from melanosomes without altering the *tyrosinase* mRNA level and decreased melanin content as well, the same as Varp knockdown did (Supplementary Figure S3), suggesting that protection of Varp protein by RACK1 from Rab40C-mediated degradation is also crucial for melanogenic enzyme transport to melanosomes in melanocytes. Intriguingly, it has recently been reported

that RACK1 heterozygous mice exhibit skin pigmentation defects characterized by a white belly spot and hypopigmented tail and paws, while RACK1 homozygous mice die at an early embryonic stage (Volta *et al*., 2013). The hypopigmented phenotype may be mainly attributable to defects in dendrite outgrowth (Figure 3) and in melanin synthesis (Park *et al.*, 2004; Supplementary Figure S3), both of which are crucial for pigmentation and are regulated by Varp protein (Tamura *et al.*, 2009; Ohbayashi *et al.*, 2012b).

Because both RACK1 and Varp are expressed in non-melanocytic cells, RACK1-dependent stabilization of Varp protein may be retained in cells other than melanocytes and their interaction may be involved in retromer-dependent cargo transport pathways (Hesketh *et al.*, 2014; McGough *et al.*, 2014) and neurite outgrowth, both of which are positively regulated by Varp (Burgo *et al*., 2009; Burgo *et al*., 2012). It is noteworthy that RACK1 is highly expressed in mouse brain (Ashique *et al*., 2006) and has been shown to regulate neurite outgrowth through interaction with molecules other than Varp (Ensslen and Brady-Kalnay, 2004; Ceci *et al.,* 2012; Dwane *et al.,* 2014). Further investigation of the RACK1–Varp complex in neurite outgrowth will clarify the universal role of RACK1 in Varp protein stabilization.

In conclusion, the results of this study have revealed a novel function of the ANKR2 domain of Varp, i.e., RACK1-mediated stabilization of Varp protein in melanocytes. Based on our findings, we propose that the ANKR2 domain of Varp fine-tunes the level of Varp protein expression through interaction with either a positive regulator, RACK1 (this study), or a negative regulator, Rab40C (Yatsu *et al.*, 2015)

rather than directly regulating melanogenesis. Dysregulation of this process may lead to skin pigmentation defects, including impaired melanin synthesis and impaired dendrite outgrowth.

MATERIALS AND METHODS

Materials

Anti-Varp guinea pig polyclonal antibody and anti-tyrosinase rabbit polyclonal antibody were prepared as described previously (Beaumont *et al.*, 2011; Yatsu *et al.*, 2015). The following antibodies used in this study were obtained commercially: anti-RACK1 mouse monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX), anti-β-actin mouse monoclonal antibody (Applied Biological Materials, Richmond, BC, Canada), HRP (horseradish peroxidase)-conjugated anti-FLAG tag (M2) mouse monoclonal antibody, anti-FLAG tag antibody-conjugated agarose (Sigma-Aldrich Corp., St. Louis, MO), HRP-conjugated anti-T7 tag mouse monoclonal antibody (Merck Millipore, Billerica, MA), and HRP-conjugated anti-HA tag (3F10) rat monoclonal antibody (Roche Applied Science, Penzberg, Germany). Forskolin and TPA were from Sigma-Aldrich. The proteasome inhibitor MG132 and *N*-ethylmaleimide were obtained from Peptide Institute (Osaka, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively.

Plasmid construction

Plasmids encoding mouse Varp (N+VPS9, ANKR1, and ANKR2), Rab40C, and EGFP were prepared as described previously (Tamura *et al.*, 2009; Yatsu *et al.*, 2015). The cDNA of Varp-ANKR2 (amino acid residues 730–1048) (Yatsu *et al.*, 2015) was subcloned into the pGBKT7 vector (Clontech-Takara Bio Inc., Shiga, Japan) for yeast

two-hybrid screening. The cDNA of mouse RACK1 was amplified from Marathon-Ready mouse brain and testis cDNAs (Clontech-Takara Bio Inc.) by performing PCR with the following pair of oligonucleotides containing a *Bam*HI linker (underlined) or a stop codon (boldface): forward primer, 5'-GGATCCATGACCGAGCAGATGACCCT-3' and reverse primer, 5'-**TTA**GCGGGTACCAATAGTTA-3'. The RACK1 cDNAs obtained were subcloned into the pEF-T7 tag vector (Fukuda *et al.*, 1999), pEF-HA tag vector (Fukuda, 2002), pmStr-C1 vector (Ohbayashi *et al.*, 2012a), and pGBD-C1 vector (James *et al.*, 1996). A RACK1ΔN mutant (deletion of amino acid residues 1–203 of mouse RACK1) was prepared by removing the *Bam*HI insert of the pGBD-C1-RACK1 vector (an intrinsic *Bam*HI site in the RACK1 cDNA and *Bam*HI site in the multi-cloning site of the vector) and by self-ligating the vector. Small interfering RNAs (siRNAs) against mouse *RACK1* (target site 1: 5'-GGATGAGAGTCATTCAGAA-3' and target site 2; 5'-GACCAACTATGGCATACCA-3') and mouse Rab40C (target site: 5'-CTGCATGACCTTCTTTGAA-3') were chemically synthesized by Nippon Gene Co., Ltd. (Toyama, Japan). Knockdown efficiency of the *Rab40c* siRNA has already been shown by RT-PCR in our previous study (Yatsu *et al.*, 2015).

Forskolin-induced dendrite formation of melanocytes

The black-mouse-derived immortal melanocyte cell line melan-a (generous gift of Dorothy C. Bennett) was cultured on 12-mm diameter coverslips as described previously (Bennett *et al.*, 1987; Kuroda *et al.*, 2003). Plasmids and siRNAs were

transfected into melan-a cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. One day after transfection the cells were treated with 20 μM forskolin or DMSO alone (control), and 20 hours later they were fixed with 4% paraformaldehyde and examined for fluorescence with a confocal laser-scanning fluorescence microscope (Fluoview FV1000-D; Olympus, Tokyo, Japan). The images of the EGFP-expressing cells were captured at random with the confocal microscope, and the total length of their dendrites was measured with ImageJ software (version1.48u4; NIH, Bethesda, MD) as described previously (Ohbayashi *et al.*, 2012b). In brief, the length of each dendrite of a cell was measured from the edge of the cell body (cell body–dendrite boundary was determined to be a cross point by extrapolating the cell membrane) to the tip of dendrite, and total dendrite length means the sum of the lengths of all of the dendrites of the cell. Each experiment was performed independently at least three times, and the numbers of cells analyzed in each experiment are stated in the figure legends. The statistical analyses were performed by using Dunnett's test, Tukey's test, or Student's unpaired t-test, and P values <0.05 were considered statistically significant (*, P<0.05; **, P<0.01).

Immunoblotting

siRNAs were transfected into melan-a cells by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. At 48 hours after transfection the cells were washed with ice-cold PBS and lysed with the lysis buffer (50 mM HEPES-KOH pH7.2, 150 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100

supplemented with complete EDTA-free protease inhibitor cocktail (Roche Applied Science)). In Figure 2d, at 6 hours after transfection the cells were treated with 1 nM proteasome inhibitor MG132 or DMSO and then were cultured for 48 hours. The cells were washed with ice-cold PBS and lysed with 50 mM HEPES-KOH pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% (w/v) sodium deoxycholate, 1% (w/v) SDS, and 10 mM *N*-ethylmaleimide supplemented with complete EDTA-free protease inhibitor cocktail. The lysates were subjected to 7.5% (or 8.8%) SDS-PAGE and transferred to a PVDF membrane (Merck Millipore) by electroblotting. The blots were blocked with 1% skim milk or 1% BSA in PBS containing 0.1% Tween-20 and incubated at room temperature with primary antibodies for 1 hour and then with appropriate HRP-conjugated secondary antibodies for 1 hour. Immunoreactive bands were detected by enhanced chemiluminescence (ECL, GE Healthcare Ltd., Little Chalfont, UK). The intensity of the immunoreactive bands was measured with ImageJ software. The positions of the molecular mass markers (in kDa) are shown on the left of each figure.

Co-immunoprecipitation assays

COS-7 cells (4×10^5 cells/60-mm dish) were co-transfected with plasmids (indicated in Figures 1 and 4) by using Lipofectamine LTX Plus (Invitrogen) according to the manufacturer's instructions. At 36 hours after transfection the cells were lysed with the lysis buffer. Co-immunoprecipitation assays with anti-FLAG tag antibody-conjugated agarose beads (Sigma-Aldrich) were performed as described

previously (Fukuda *et al.*, 1999; Fukuda and Kanno, 2005), and proteins bound to the beads were analyzed by immunoblotting as described above. Endogenous interaction between Varp and RACK1 in melan-a cells that had been treated with 20 μM forskolin for 3 hours was also investigated by co-immunoprecipitation assays with anti-RACK1 antibody as described previously (Fukuda and Kanno, 2005).

Other methods, including yeast two-hybrid assays, RT-PCR analysis, melanin assay, and tyrosinase staining, are available at online supplementary material.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. RACK1 is a novel Varp-interacting protein. (**a**) Schematic representation of Varp and its truncated mutants used in this study. (**b**) Schematic representation of RACK1 protein and its corresponding mRNA. (**c**) Interaction of RACK1ΔN with Varp truncated mutants as revealed by yeast two-hybrid assays. (**d**) Interaction between T7-RACK1 and full-length FLAG-Varp. Before harvesting, the transfected COS-7 cells were stimulated for 3 hours with 20 μM forskolin (Fsk), 20 nM TPA, or DMSO alone. FLAG-EGFP was used as a negative control. (**e**) Quantification of T7-RACK1 bands shown in the middle panel in **d**. The bars represent the means and SEM of three independent experiments. *, $P<0.05$ in comparison with DMSO (Dunnett's test). (**f**) Endogenous interaction between RACK1 and Varp (arrowhead) in FSK-treated mouse melanocytes (melan-a cells).

Figure 2. Knockdown of RACK1 in melanocytes decreases their level of Varp protein expression. (**a**) Reduced expression of Varp in RACK1-knockdown melan-a cells as revealed by immunoblotting with the antibodies indicated. (**b**) Quantification of Varp bands shown in the middle panel in **a**. The bars represent the means and SEM of six independent experiments. *, $P<0.05$; **, $P<0.01$ in comparison with the control (Dunnett's test). (**c**) Knockdown efficiency of *RACK1* siRNAs as revealed by real-time quantitative RT-PCR analysis. **, $P<0.01$ (Dunnett's test). (**d**) Exposure of RACK1-knockdown melan-a cells to MG132 or DMSO for 48 hours. Representative

data from one of three independent experiments with similar results are shown.

Figure 3. Knockdown of RACK1 inhibits dendrite outgrowth of melanocytes. (**a**) Melan-a cells were cotransfected with *RACK1* siRNA (st1 or st2) or control siRNA and pEGFP-C1 (transfection marker) and then stimulated for 20 hours with 20 μM forskolin (Fsk) or DMSO alone. Images of EGFP-expressing cells were captured at random, and typical images of RACK1-knockdown cells (EGFP-expressing cells) are shown. Scale bars, 50 μm. (**b**) Quantification of total dendrite length/cell shown in **a**. The bars represent the means and SEM of data from three independent experiments (totally 145 cells were analyzed). *, $P<0.05$; **, $P<0.01$ in comparison with the control (Dunnett's test).

Figure 4. RACK1 inhibits the interaction between Varp and Rab40C. (**a**) COS-7 cells transiently expressing T7-Varp and FLAG-Rab40C (or FLAG-EGFP as a control) with or without HA-RACK1 were lysed, and the interaction between T7-Varp and FLAG-Rab40C was evaluated by immunoprecipitation with anti-FLAG tag antibody-conjugated agarose beads followed by immunoblotting with the antibodies indicated. (**b**) Quantification of Varp bands shown in the second panel in **a** (normalized to the input). The bars represent the means and SEM of data from four independent experiments. (**c**) The Varp protein level in RACK1-, Rab40C-, and RACK1/Rab40C-knockdown melan-a cells. (**d**) Quantification of Varp bands shown in **c** (normalized to actin bands). The bars represent the means and SEM of data from

five independent experiments (normalized to control). *, $P<0.05$ (Student's unpaired t-test).

Figure 5. RACK1 rescues Rab40C inhibition of dendrite outgrowth of melanocytes. (**a**) Melan-a cells co-expressing EGFP-Rab40C (or EGFP alone) and mStr-RACK1 (or mStr alone) were stimulated for 20 hours with 20 μM forskolin (Fsk) or DMSO alone. Images of the cells expressing both EGFP-tagged and mStr-tagged proteins were captured at random, and typical images are shown. Scale bars, 50 μm. (**b**) Quantification of total dendrite length/cell shown in **a**. The bars represent the means and SEM of data from one representative experiment (n=110 cells). **, $P<0.01$ (Tukey's test).

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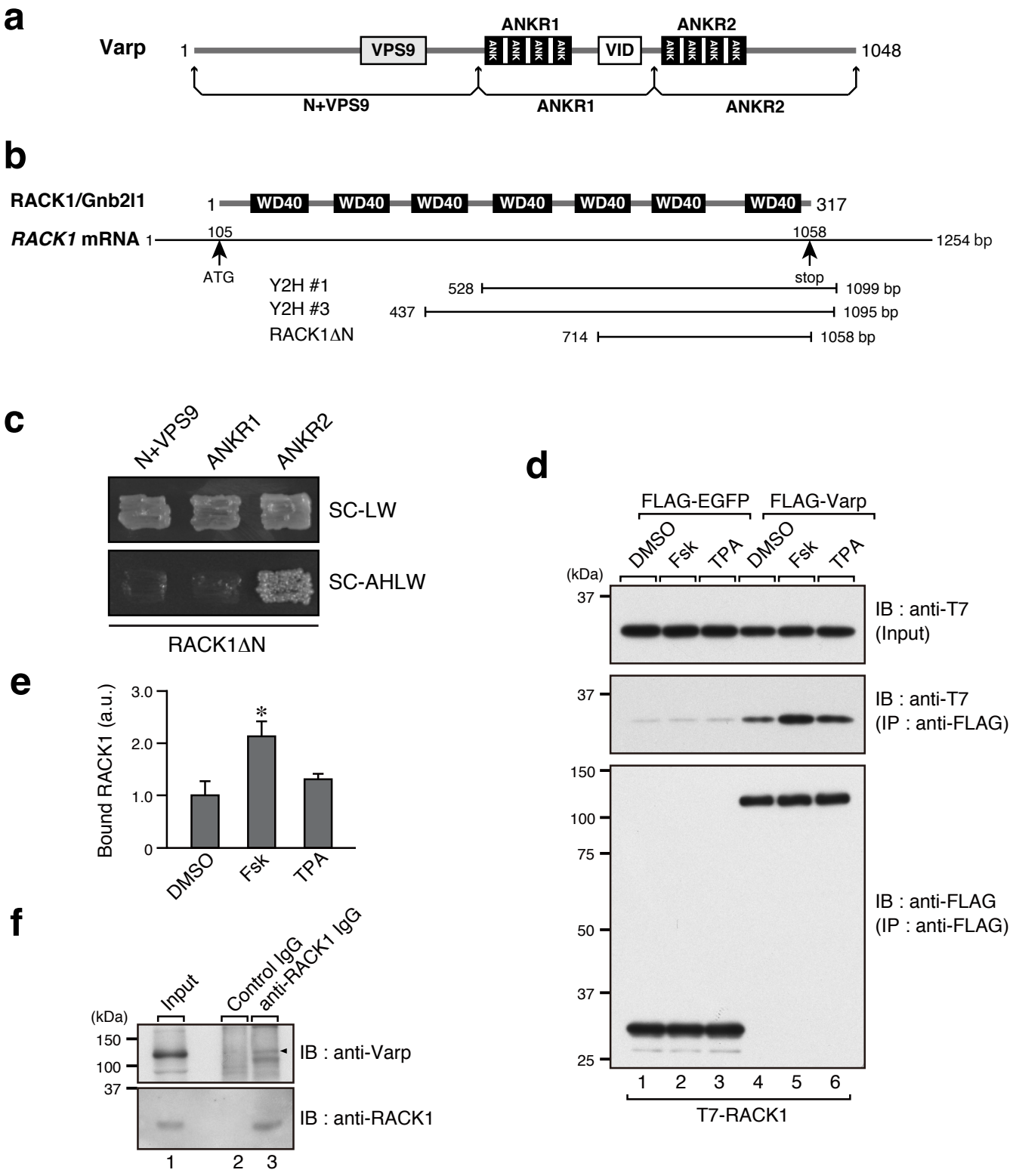

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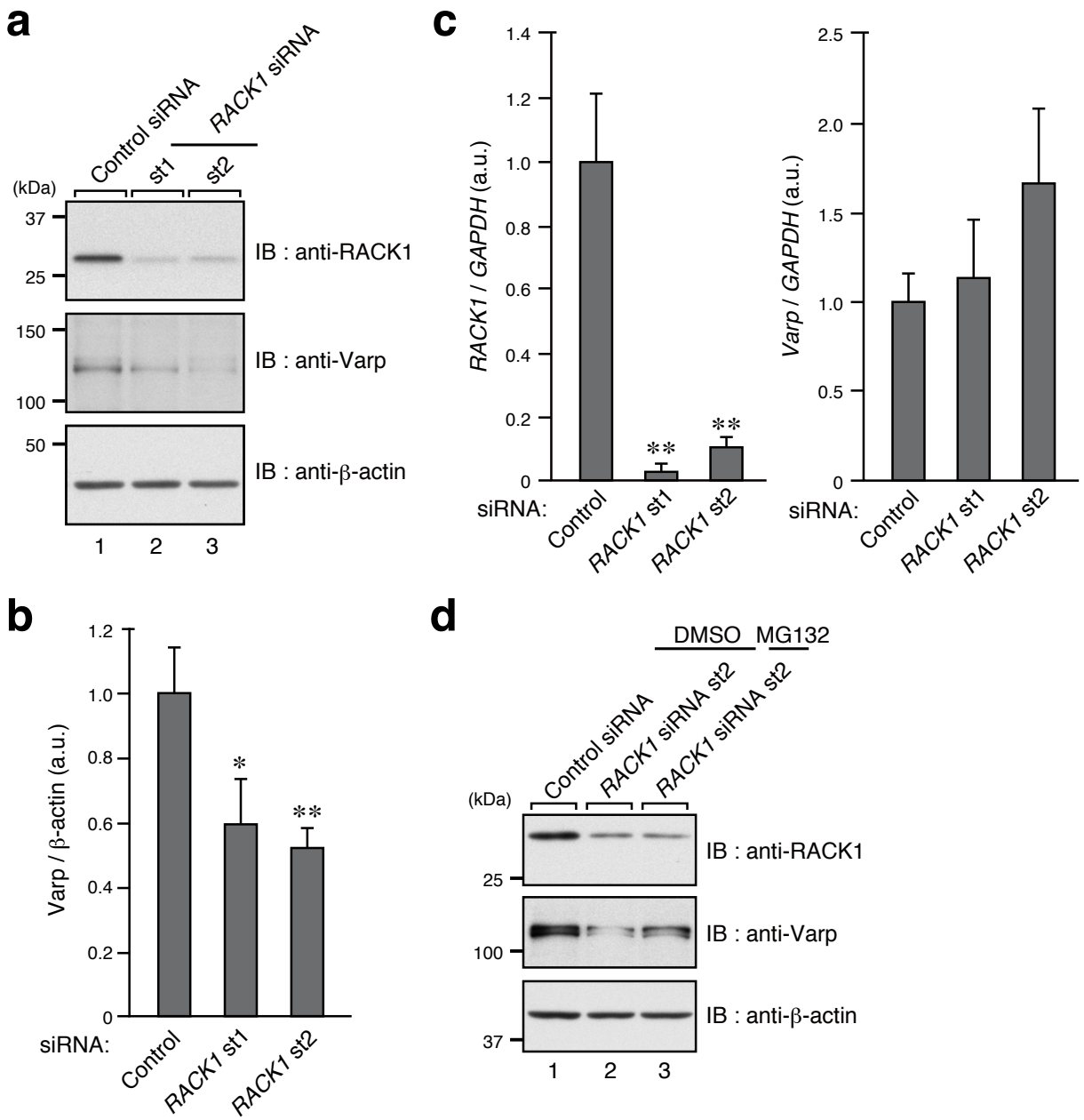

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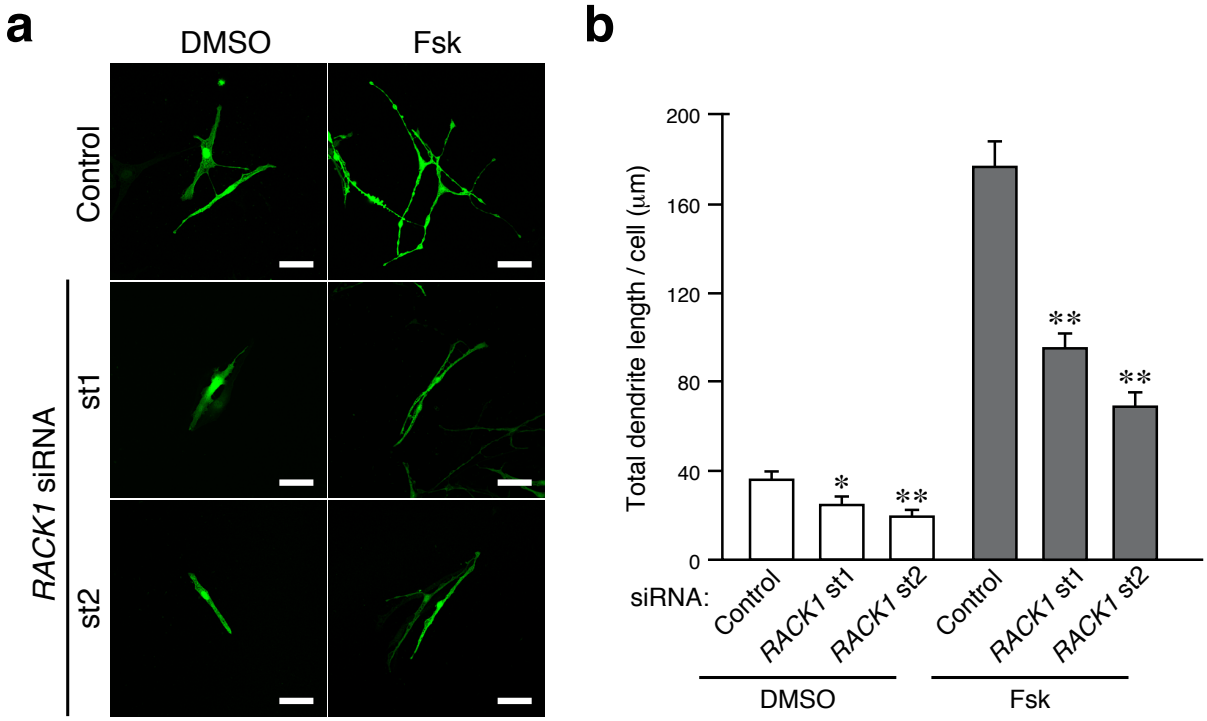

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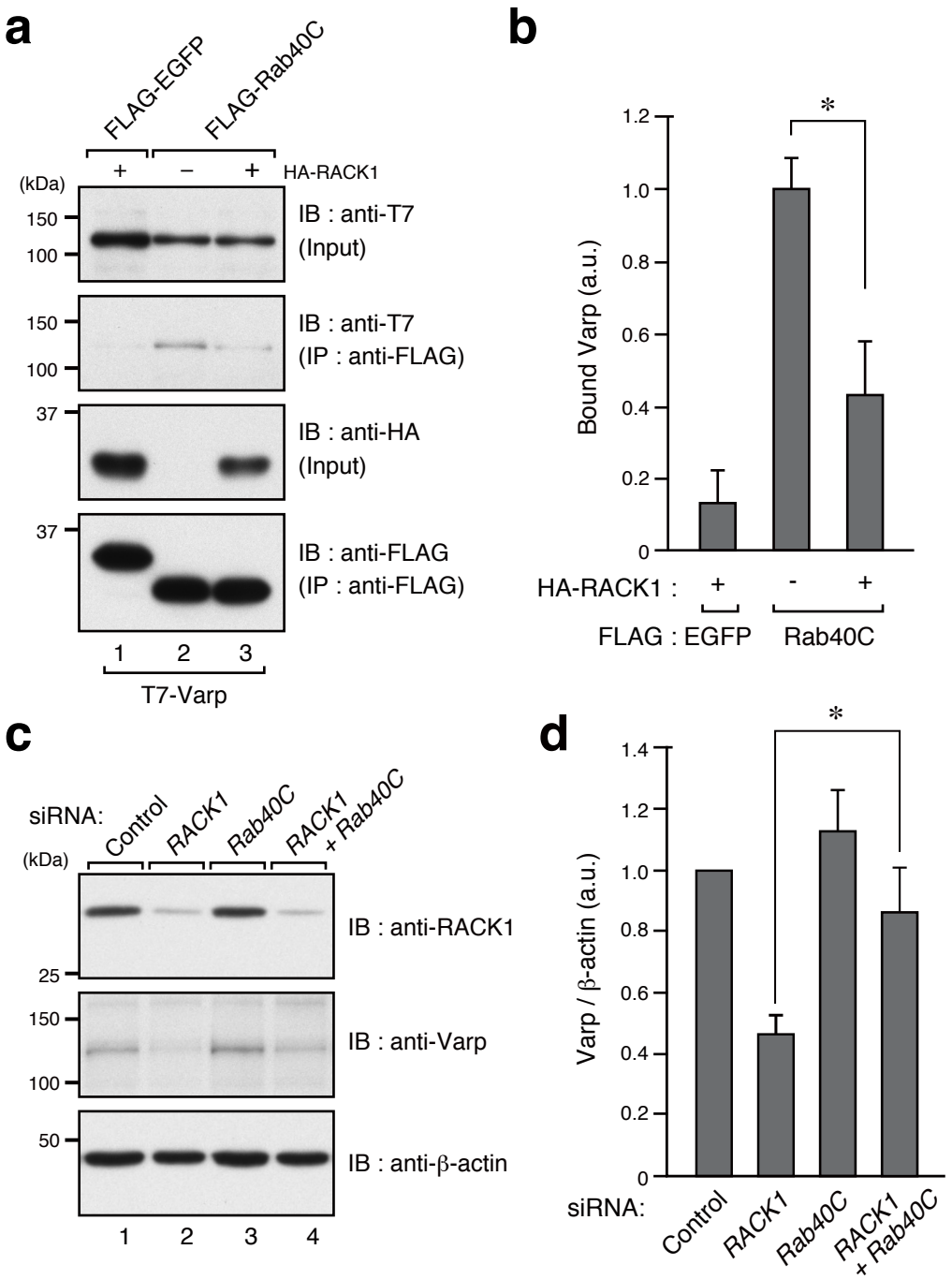

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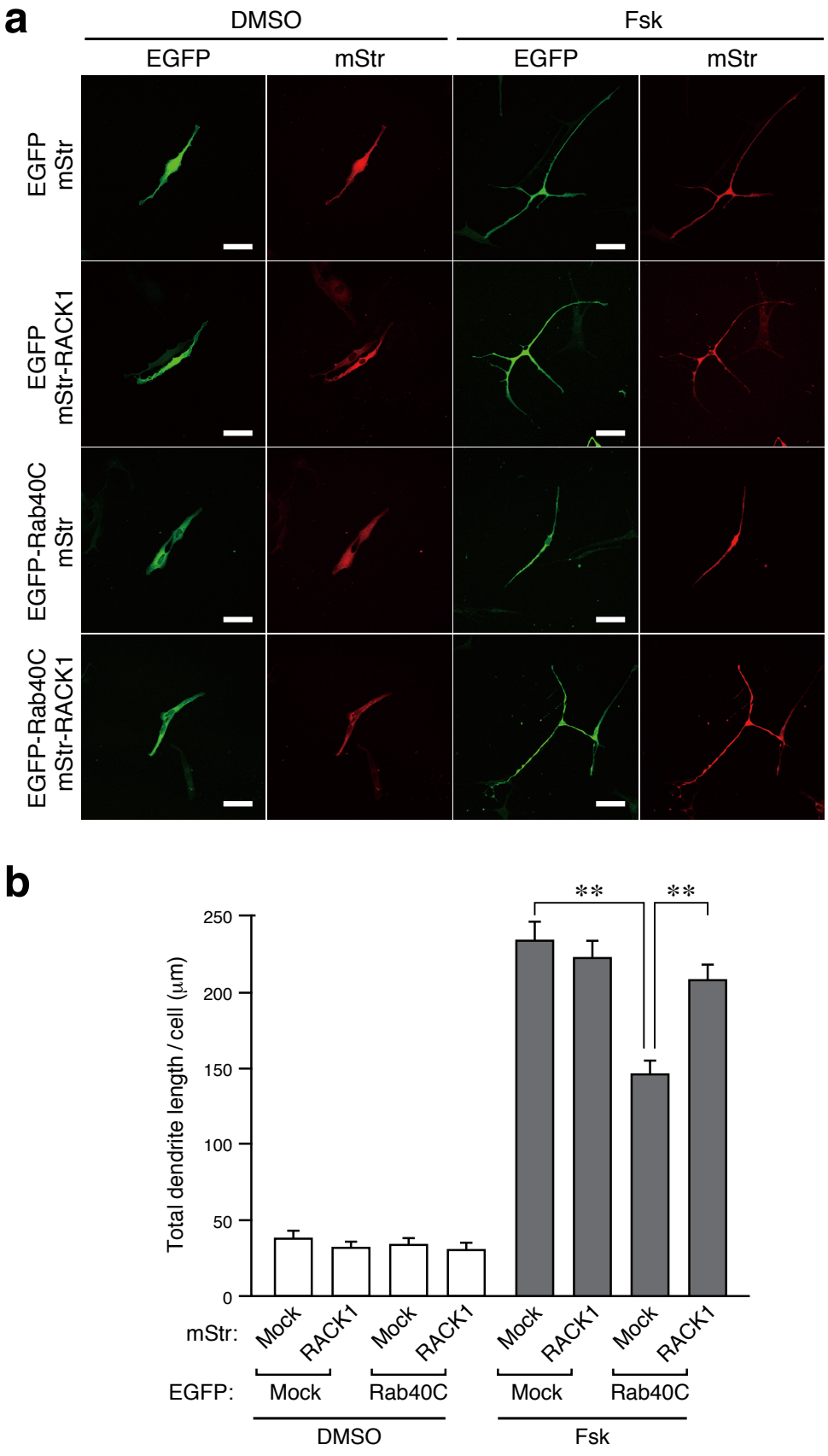

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SUPPLEMENTARY MATERIAL

Supplementary Methods

Yeast two-hybrid assays

Yeast two-hybrid screening was performed by using pGBKT7-Varp-ANKR2 as bait and mouse testis and mouse 11-day embryo mixed cDNA libraries (Clontech-Takara Bio Inc.) as prey according to the manufacturer's instructions. Yeast two-hybrid assays were performed by using pGBD-C1-RACK1ΔN and pAct2-Varp-N+VPS9, pAct2-Varp-ANKR1, or pAct2-Varp-ANKR2 as described previously (Tamura *et al.*, 2009). The yeast strain (pJ69-4A), SC-LW medium (synthetic complete medium lacking leucine and tryptophan), selection medium SC-AHLW (synthetic complete medium lacking adenine, histidine, leucine, and tryptophan), culture conditions, and transformation protocol used are described elsewhere (James *et al.*, 1996; Kobayashi *et al.*, 2015). Yeast cells containing both pGBD-C1-RACK1ΔN and each of the pAct2 plasmids expressing a Varp mutant were each streaked on both SC-LW medium and SC-AHLW medium and incubated at 30˚C for 1 day and 1 week, respectively.

RT (reverse transcription)-PCR analysis

The total RNA of melan-a cells transfected with *RACK1* siRNA (st1 or st2) or control siRNA was prepared with TRI-reagent (Sigma-Aldrich), and reverse transcription was performed by using ReverTra Ace® (Toyobo, Osaka, Japan) according to the

manufacturer's instructions. Real-time quantitative PCR was performed with SYBR Green and Light Cycler 2.0 (Roche Applied Science) according to the manufacturer's instructions. The following pairs of oligonucleotides were used for amplification: for RACK1, forward primer, 5'-GTACACGGTCCAGGATGAG-3' and reverse primer, 5'-GGCCAATGTGGTTGGTCTTTA-3'; for Varp, forward primer, 5'-ACATCCTGAACAAGAGGCAGTA-3' and reverse primer, 5'-CCTGATCTCAACAGTCACGTA-3'; for tyrosinase, forward primer, 5'-GGGATGAGAACTTCACTGTTCCATA-3' and reverse primer, 5'-TGATCTGCTACAAATGATCTGCC-3'; and for GAPDH, forward primer, 5'-CATGGCCTTCCGTGTTCCTA-3' and reverse primer, 5'-GAGTTGCTGTTGAAGTCGC-3'. qPCR data were normalized to *GAPDH* and expressed as means and SEM of four independent experiments.

Melanin assay and tyrosinase staining

The melanin content was assayed as described previously (Tamura *et al.*, 2009). In brief, melan-a cells that had been transfected with *RACK1* siRNAs were cultured for 4 days and their melanin content normalized to protein content was measured as optical density at 490 nm. Tyrosinase staining was also performed as described previously (Yatsu *et al.*, 2015).

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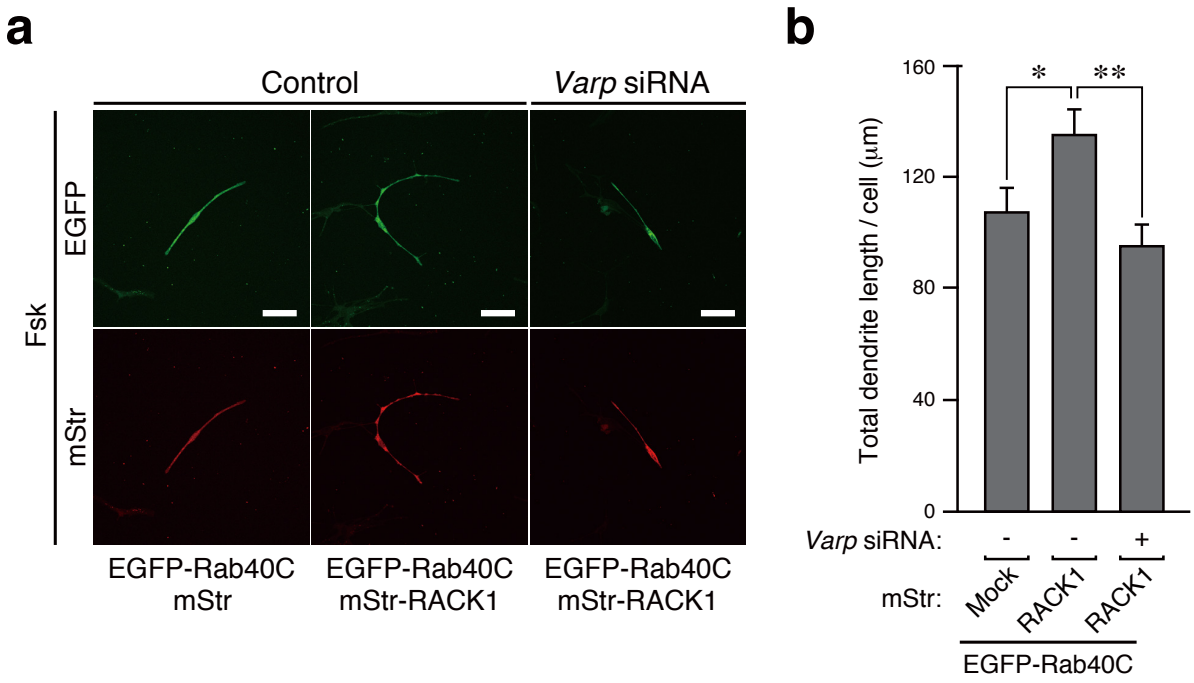
Supplementary Figure legends

Supplementary Figure S1. RACK1 is unable to rescue Rab40C inhibition of dendrite outgrowth of Varp-knockdown melanocytes. (a) Melan-a cells co-expressing EGFP-Rab40C and mStr-RACK1 (or mStr alone) or Varp-knockdown cells co-expressing EGFP-Rab40C and mStr-RACK1 were stimulated for 20 hours with 20 μM forskolin (Fsk). Images of the cells expressing both EGFP-tagged and mStr-tagged proteins were captured at random, and typical images are shown. Scale bars, 50 μm. (b) Quantification of total dendrite length/cell shown in **a**. The bars represent the means and SEM of data from three independent experiments (totally 90

cells were analyzed). *, $P<0.05$; **, $P<0.01$ (Tukey's test).

Supplementary Figure S2. A proposed model of the regulation of the Varp protein expression level by RACK1 and Rab40C in melanocytes. In this model, Rab40C (negative regulator; Yatsu *et al.*, 2015) and RACK1 (positive regulator; this study) competitively bind to the ANKR2 domain of Varp, and this competitive mechanism fine-tunes the level of Varp protein expression in melanocytes. Forskolin (Fsk) increases the interaction between RACK1 and Varp (Figure 1), and the stabilization of Varp protein contributes to promoting dendrite outgrowth of melanocytes.

Supplementary Figure S3. Knockdown of RACK1 decreases the protein expression level of tyrosinase in melanocytes. (**a**) Reduced expression of tyrosinase in RACK1-knockdown cells as revealed by immunoblotting with the antibodies indicated. (**b**) Unaltered expression level of *tyrosinase* mRNA in RACK1-knockdown cells as revealed by real-time quantitative RT-PCR analysis. (**c**) Reduced signals of tyrosinase in RACK1-knockdown melanocytes. Scale bars, 20 μm. (**d**) Quantification of tyrosinase intensity shown in **c**. The bars represent the means and SEM of data from one representative experiment (n=120 cells). **, $P<0.01$ in comparison with the control (Dunnett's test). (**e**) Reduced melanin content in RACK1-knockdown melanocytes. The bars represent the means and SEM of data from five independent experiments. **, $P<0.01$ (Student's unpaired t-test).



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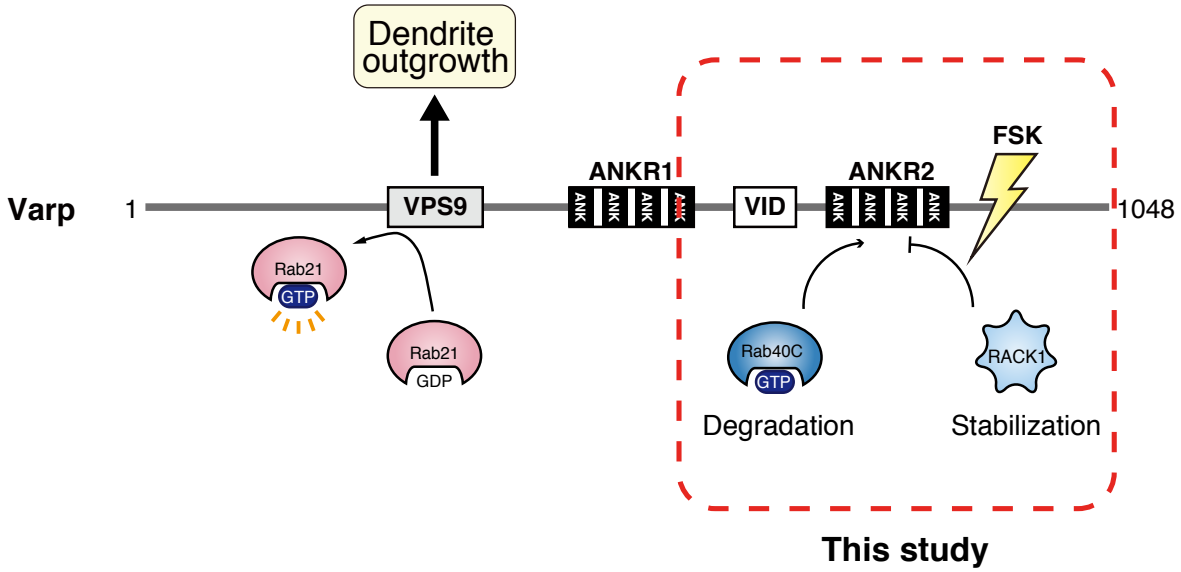

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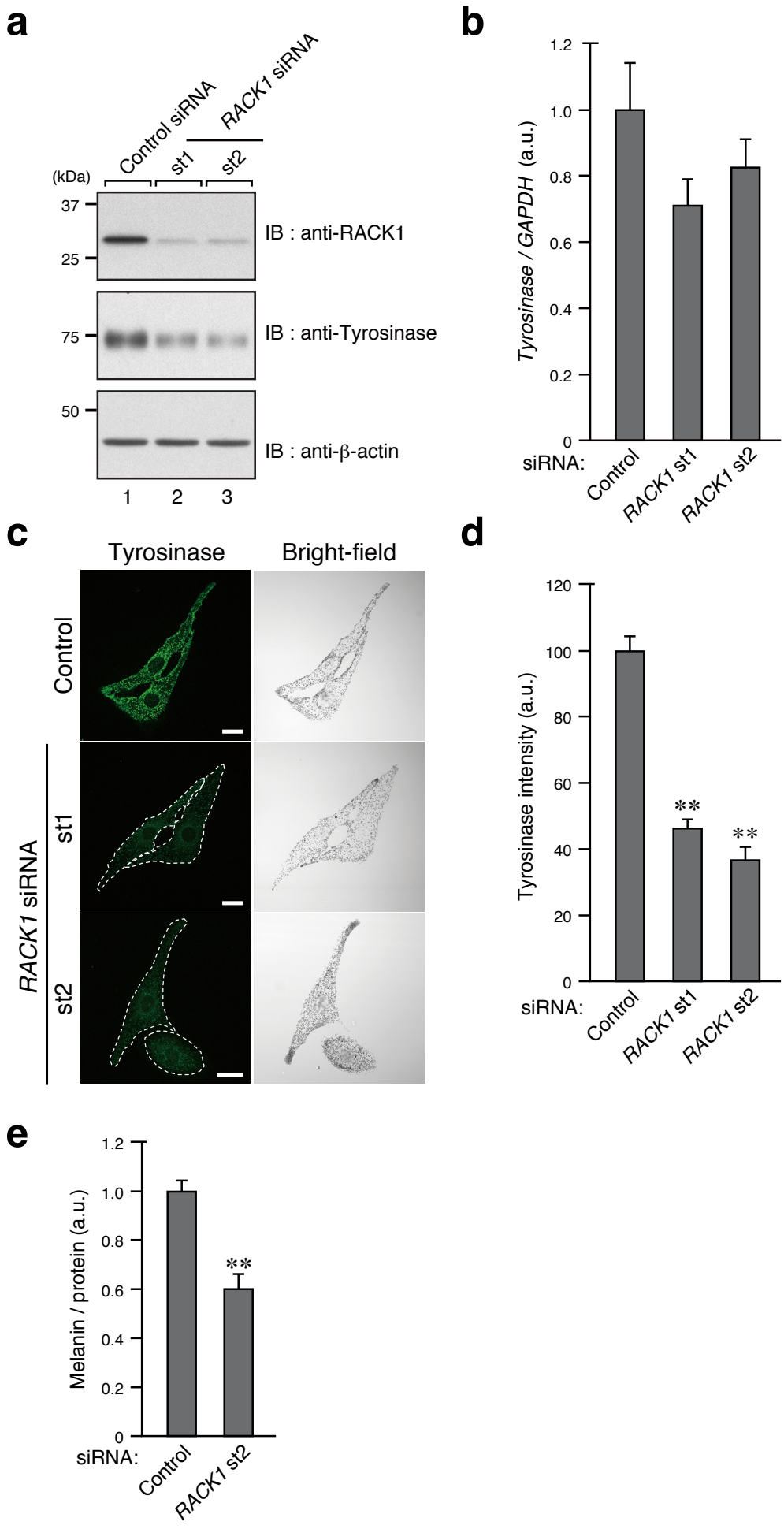

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