



# Uncoupling of ER/Mitochondrial Oxidative Stress in mTORC1 Hyperactivation-Associated Skin Hypopigmentation

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Accumulating evidence has described the involvement of mTORC1 signaling in pigmentation regulation; however, the precise mechanism is not fully understood. Here, we generated mice with conditional deletion of the mTORC1 suppressor *Tsc2* in melanocytes. It resulted in constitutive hyperactivation of mTORC1 and reduced skin pigmentation. Mechanistically, neither the number of melanocytes nor the expression of melanogenesis-related enzymes was decreased; however, endoplasmic reticulum and mitochondrial oxidative stress and lower melanization in melanosomes were observed. By contrast, abrogation of mTORC1 by rapamycin completely reversed the reduced pigmentation, and alleviation of endoplasmic reticulum stress by SMER28 or 4-phenylbutyrate (PBA) or alleviation of mitochondrial oxidative stress by administration of adenosine triphosphate partially reversed the reduced pigmentation in these mice. In addition, we showed that these mechanisms were involved in reduced pigmentation of *TSC2* small interfering RNA-transfected cultured human primary melanocytes and skin lesions of patients with the *TSC* gene mutation.

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## INTRODUCTION

The mammalian serine/threonine kinase mTOR was implicated in the regulation of pigmentation (Busca et al., 1996; Jeong et al., 2011; Ohguchi et al., 2005; Tsao et al., 2016). mTOR is found in two functionally and structurally distinct complexes named mTORC1 and mTORC2. mTORC1 is sensitive to rapamycin, but mTORC2 is not inhibited by rapamycin. mTORC1 controls protein synthesis, ribosome biogenesis, cell growth, and autophagy. mTORC2 regulates cellular metabolism and the cytoskeleton (Saxton and Sabatini, 2017). mTORC1 activation, via *TSC* gene inactivation, disrupted pigmentation (Cao et al., 2017). Furthermore, rapamycin, an inhibitor of mTORC1, was reported to up-regulate melanogenesis in MNT-1 melanoma cells (Hah et al., 2012; Ho et al., 2011). We reported that rapamycin was effective for treatment of hypopigmented macules of patients with tuberous sclerosis complex (TSC) (Wataya-Kaneda et al., 2012, 2015), which supported a role for mTORC1 in pigmentation.

The mTORC1 pathway has been shown to control protein synthesis, cell growth, proliferation, cell metabolism, insulin

resistance, autophagy, and stress responses (Laplante and Sabatini, 2009, 2012). Dysregulation of the mTOR pathway has been implicated in various diseases (Guertin and Sabatini, 2007; Saxton and Sabatini, 2017; Wullschlegel et al., 2006). The mTOR pathway has attracted broad scientific and clinical interest. Besides, both the PI3K-mTOR and Ras-MAPK-ERK pathways are well known as the cell's chief mechanisms for controlling growth, survival, and metabolism in response to extracellular cues (Mendoza et al., 2011). The Ras-MAPK-ERK signaling pathway is known to inhibit melanogenesis by phosphorylating MITF and posttranscriptionally modulating the activity of MITF in melanocytes (Lin and Fisher, 2007). However, little is known about the mechanism of mTOR in the regulation of skin pigmentation.

In human cells, mTORC1 is activated by amino acids, insulin, growth factors, and stress mediators like starvation and hypoxia (Saxton and Sabatini, 2017); mTORC1 is negatively regulated by a heterodimeric complex consisting of two protein molecules: TSC1 (or hamartin) and TSC2 (or tuberin) (Kwiatkowski, 2003b). Inactivation of either *TSC1* or *TSC2* induces hyperactivation of mTOR. Furthermore, mutations in *TSC1* or *TSC2* cause the TSC disorder (Crino et al., 2006; van Sleightenhorst et al., 1997; Wienecke et al., 1995). TSC is a multisystemic disease characterized by formation of benign tumors in multiple organs and hypopigmented macules on the skin that occur along with seizures or autism (Ess, 2009). Hyperactivation of mTOR is thought to be the most likely trigger for lesion formation in TSC patients (Crino et al., 2006). In TSC, uncontrolled protein synthesis and cell growth from constitutive mTOR activation contribute to formation of benign tumors in multiple systems (Inoki et al., 2006; Kenerson et al., 2002). By contrast, in the melanocyte lineage in TSC, tumor formation has not been observed; however, hypopigmented macules arise at birth or in early

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Abbreviations: ATP, adenosine triphosphate; ER, endoplasmic reticulum; p-, phosphorylated; PBA, 4-phenylbutyrate; TSC, tuberous sclerosis complex

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infancy (Gold and Freeman, 1965; Jimbow, 1997). TSC was initially described approximately 150 years ago, and the molecular mechanisms underlying formation of these hypopigmented macules still are not fully understood. Based on our previous studies that showed topical treatment with rapamycin-reversed hypopigmentation in patients with TSC, we hypothesized that mutated *TSC1*- or *TSC2*-induced hyperactivation of mTORC1 leads to hypopigmented macules in TSC.

Studies of *Tsc*/mTORC1 regulation of pigmentation *in vivo* have been limited by the lack of an animal model, because homogeneous *Tsc* knockout is embryonically lethal and *Tsc* heterozygotes do not develop skin abnormalities (Kobayashi et al., 1999). To investigate whether *Tsc*/mTORC1 signaling in melanocytes plays a role in the regulation of pigmentation, we generated conditional *Tsc2*-knockout mice. As expected, deletion of *Tsc2* caused reduced skin pigmentation due to elevated mTORC1 activity in melanocytes. This mouse model provides a pathogenic mechanism for mTORC1 hyperactivation-mediated hypopigmentation.

## RESULTS

### Generation of mice deficient for *Tsc2* in melanocytes

To activate mTORC1 signaling in melanocytes and address the potential role of mTORC1 signaling in the pigmentation of skin melanocytes, we generated mice with conditional deletion of *Tsc2* in melanocytes. As shown in Figure 1a and b, mice containing a floxed *Tsc2* allele were crossed with transgenic mice expressing Cre under the control of the melanocyte-specific *Mitf* promoter (i.e., MITF-M), which is abundant and specific in neural crest-derived melanocytes (Tachibana, 2000). The resultant Cre-mediated deletion of *Tsc2* exons 3, 4, and 5 inactivated expression of *Tsc2*. *Tsc2*<sup>fllox/fllox</sup>*Mitf*::Cre mice, referred to as mcTsc2<sup>ko/ko</sup>, were used for experiments, and *Tsc2*<sup>+/+</sup>*Mitf*::Cre littermates, referred to as mcTsc2<sup>+/+</sup>, were used as controls. In C57BL6 mice, epidermal melanocytes are mainly distributed in the tail, ear, and sole skin (Reynolds, 1954); therefore, mouse tail skin was analyzed in this study.

Epidermal melanocytes were isolated from mouse tail skin and cultured. In Western blot analyses of lysates from these cells, the product of the *Tsc2* gene, tuberlin, was readily detected in control wild-type melanocytes but was absent in melanocytes from mcTsc2<sup>ko/ko</sup>, which indicated successful deletion of the *Tsc2* gene from melanocytes (Figure 1c). We further investigated the expression of downstream targets of mTORC1 (phosphorylated S6 and 4EBP1), because such phosphorylation suggests mTORC1 activation (Kwiatkowski et al., 2002; Onda et al., 2002). Consistent with loss of *Tsc2*, the expression of p-S6 and p-4EBP1 were markedly increased in mcTsc2<sup>ko/ko</sup> melanocyte lysates (Figure 1c), which suggested that mTORC1 was hyperactivated. Furthermore, mouse tail skin samples were examined via immunohistochemistry staining with anti-Pmel17 (melanocyte marker) and anti-p-S6 antibodies. Increased expression of p-S6 was observed in melanocytes from mcTsc2<sup>ko/ko</sup> tail skin compared with littermate controls (Figure 1e). These results showed that the deletion of *Tsc2* in melanocytes resulted in activation of mTORC1 signaling.

### mcTsc2<sup>ko/ko</sup> mice exhibited reduced skin pigmentation

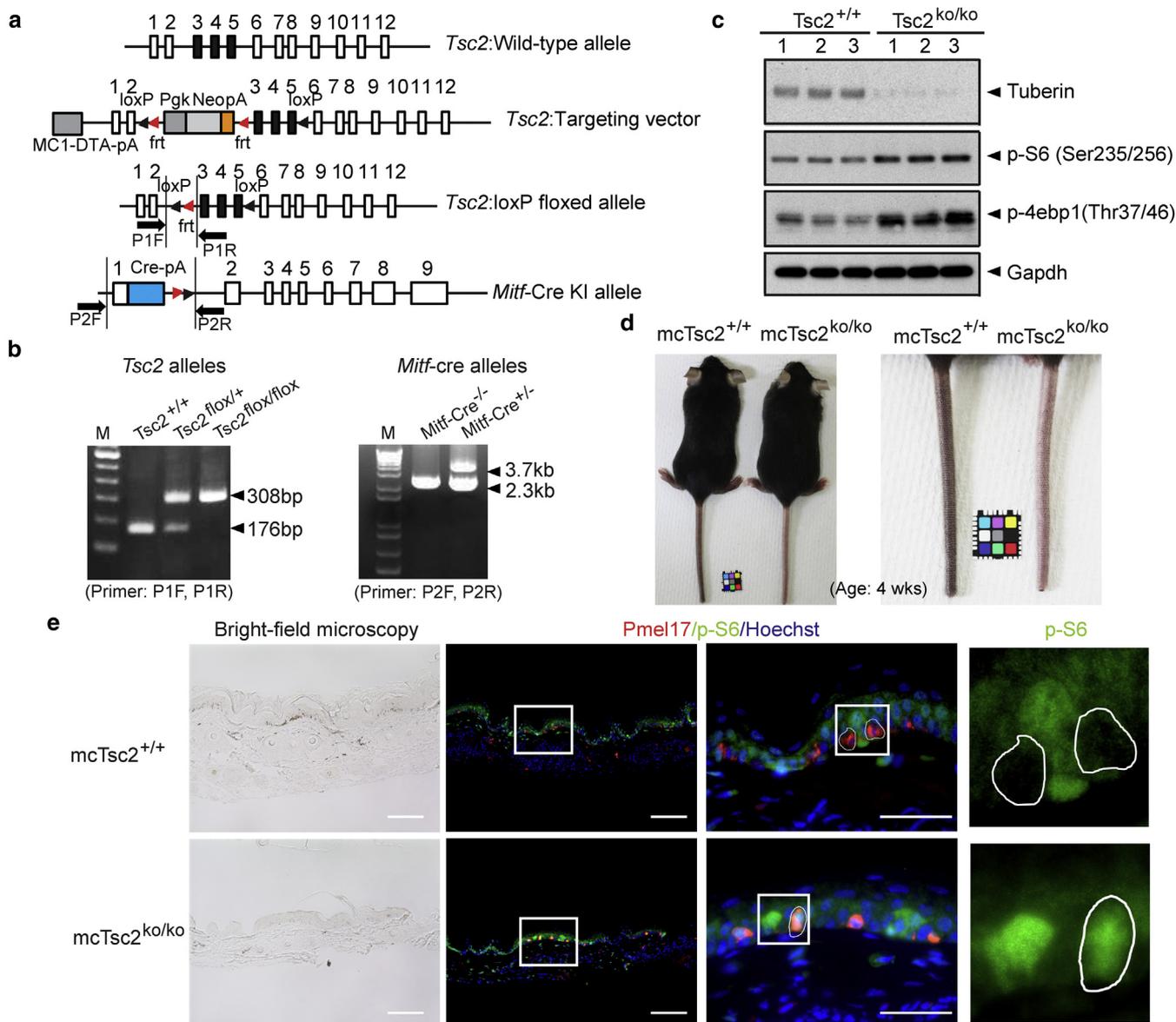
Four weeks after birth, mcTsc2<sup>ko/ko</sup> mice exhibited profoundly lighter tail skin pigmentation than their mcTsc2<sup>+/+</sup> littermates (Figure 1d). Skin sections from mcTsc2<sup>ko/ko</sup> mice had a markedly reduced number of scattered pigment granules in the epidermis compared with control mice (Figure 1e, left panel). Double staining for p-S6 and Pmel17 showed that epidermal melanocytes in tail skin were present but that mTORC1/S6 signaling was constitutively activated in mcTsc2<sup>ko/ko</sup> mice (Figure 1e, right panels). These observations indicated that melanocyte-specific deletion of *Tsc2* induced mTORC1 hyperactivation and resulted in reduced skin pigmentation.

### Deletion of *Tsc2* disrupted melanosome maturation in melanocytes

There was less pigmentation in tail skin from mcTsc2<sup>ko/ko</sup> mice than that from littermate controls, which was confirmed again via hematoxylin staining (Figure 2a) and bright field microscopy (Figure 2b, left panel). Decreased melanin is known to result from decreased numbers or absence of melanocytes in the epidermis or normal melanocyte numbers with little or no melanin production. Therefore, to determine whether less pigmentation resulted from reduced numbers of melanocytes, immunofluorescence staining was used to label skin sections using an antibody against the melanocyte-specific marker Pmel17. The numbers of epidermal melanocytes were similar between the mcTsc2<sup>ko/ko</sup> and control mice (Figure 2b). This result suggested that melanocyte dysfunction was the major reason for the observed tail skin phenotype in mcTsc2<sup>ko/ko</sup> mice.

To clarify the mechanisms involved in regulation of melanocyte function by *Tsc2*/mTORC1, transmission electron microscopy using ultrathin skin sections was performed. Marked reduction of mature melanosomes in melanocytes was detected in *Tsc2*<sup>ko/ko</sup> mice (Figure 2c) compared with samples from littermate controls. Moreover, the number of mature melanosomes was decreased; however, the number of immature melanosomes (indicated by the red arrows in Figure 2d) was greatly increased in melanocytes from mcTsc2<sup>ko/ko</sup> mice.

Next, to confirm whether the enzyme for melanin synthesis was correctly transported into melanosomes, immunoelectron microscopy was performed (Figure 2e). Spherical organelles without melanin deposition and a fibrillar internal matrix but with strong expression of Hmb45 indicate pre-melanosomes; organelles with melanin deposition are mature melanosomes (Raposo et al., 2001). However, we have also found many organelles without melanin deposition but with a fibrillar internal matrix and strong expression of Hmb45 and Tyrp1 (Figure 2e) or Hmb45 and tyrosinase (data not shown); we named this *premature melanosomes*. We found that premature melanosomes were increased but that mature melanosomes were decreased significantly in melanocytes from mcTsc2<sup>ko/ko</sup> mouse tail skin (Figure 2e, right panel;  $P < 0.01$ ) compared with melanocytes from littermate controls. This suggests that the melanogenesis-related enzyme was successfully transported into melanosomes but that melanin synthesis in these melanosomes was disrupted in melanocytes of mcTsc2<sup>ko/ko</sup> mice. These results show that

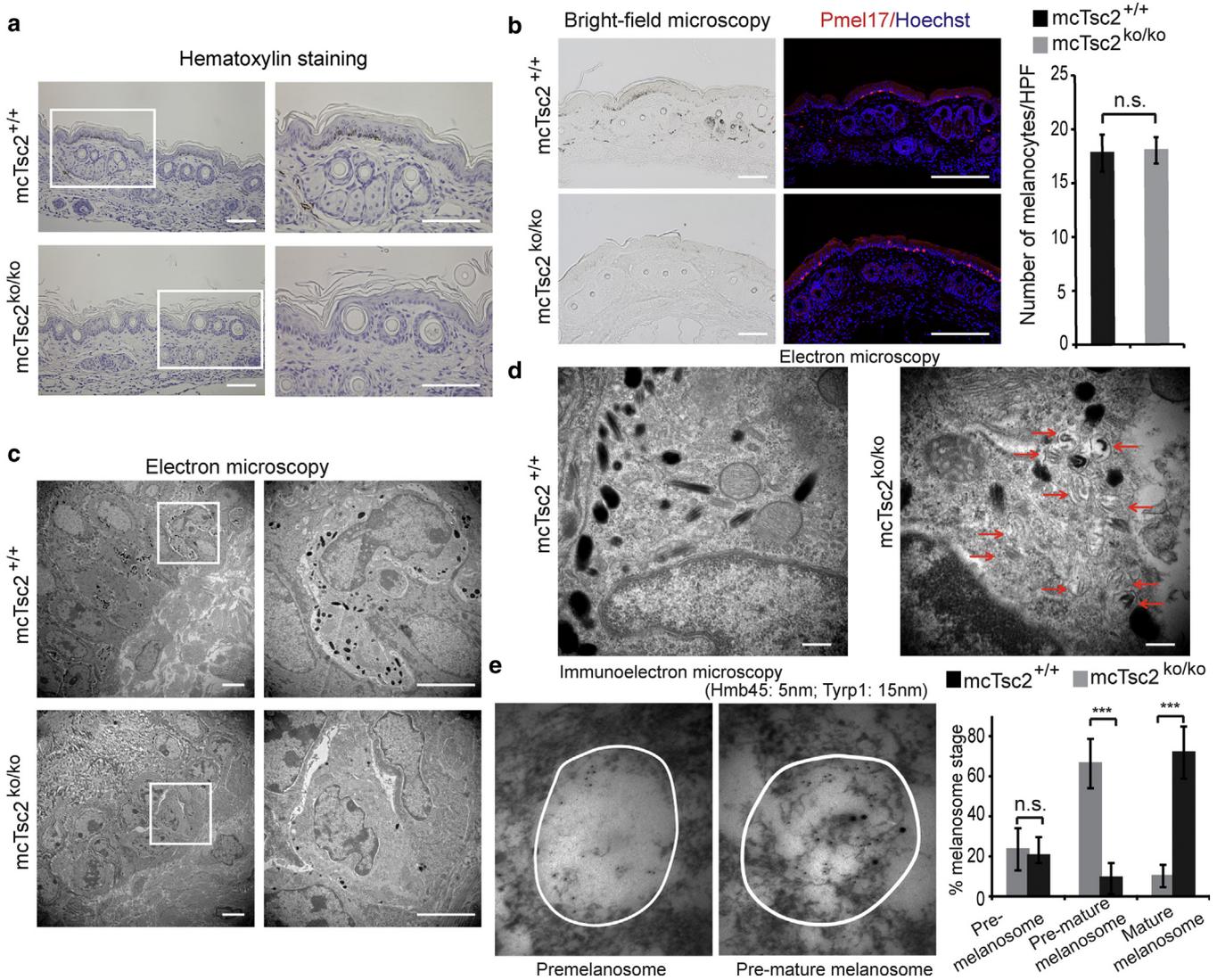


**Figure 1. Melanocyte-specific ablation of *Tsc2* reduced mouse tail skin depigmentation.** (a) Schematic illustration. (b) *Tsc2* allele PCR products from mouse DNA using primers P1F and P1R (left panel); *Mitf* allele PCR products from mouse DNA using primers P2F and P2R (right panel). (c) Western blot analysis of lysates from isolated mouse epidermal melanocytes from tail skin. (d) Mouse tail skin pigmentation at 4 weeks of age. (e) Mouse tail skin sections were stained with anti-p-S6 antibody (green), anti-Pmel17 antibody (red), and Hoechst 33342 (blue). The corresponding bright field microscopy image is shown in the left panel. Scale bar = 100  $\mu$ m. The white rectangle outlines the area that is shown in the corresponding right panel. The white line-circled area indicates melanocytes. bp, base pairs; kb, kilo base pairs; p-, phosphorylated; wks, weeks.

melanocyte-specific deletion of *Tsc2* did not affect melanosome formation or melanogenesis-related enzyme transportation but disrupted melanin synthesis in melanosomes.

To determine whether the abnormality in melanin synthesis resulted from abnormal expression of melanogenesis-related catalytic proteins, the activity of tyrosinase, *Tyrp1*, and *Pmel17* were investigated. No difference was observed in the expression levels of tyrosinase and *Tyrp1* by tissue immunohistochemistry (see [Supplementary Figure S1a](#) online). In addition, Western blot analyses of cell lysates from tail skin epidermal melanocytes also showed no difference in the expression levels of *Pmel17*, tyrosinase, and *Tyrp1* (see [Supplementary Figure S1b](#)).

To examine whether lower tyrosinase activity or insufficient levels of the tyrosinase coactivator copper was involved, skin sections were assessed for L-3,4-dihydroxyphenylalanine (i.e., L-DOPA) in the presence or absence of copper. However, tyrosinase activity was nearly identical in the presence or absence of copper in samples from mc*Tsc2*<sup>ko/ko</sup> mice compared with littermate controls (see [Supplementary Figure S1c](#)). In addition, after L-DOPA staining, the previously observed melanosomes without melanin in *Tsc2*<sup>ko/ko</sup> melanocytes appeared to be filled with melanin (see [Supplementary Figure S1d](#)), which suggested that tyrosinase activity was not affected in the melanosomes of *Tsc2*<sup>ko/ko</sup> melanocytes.

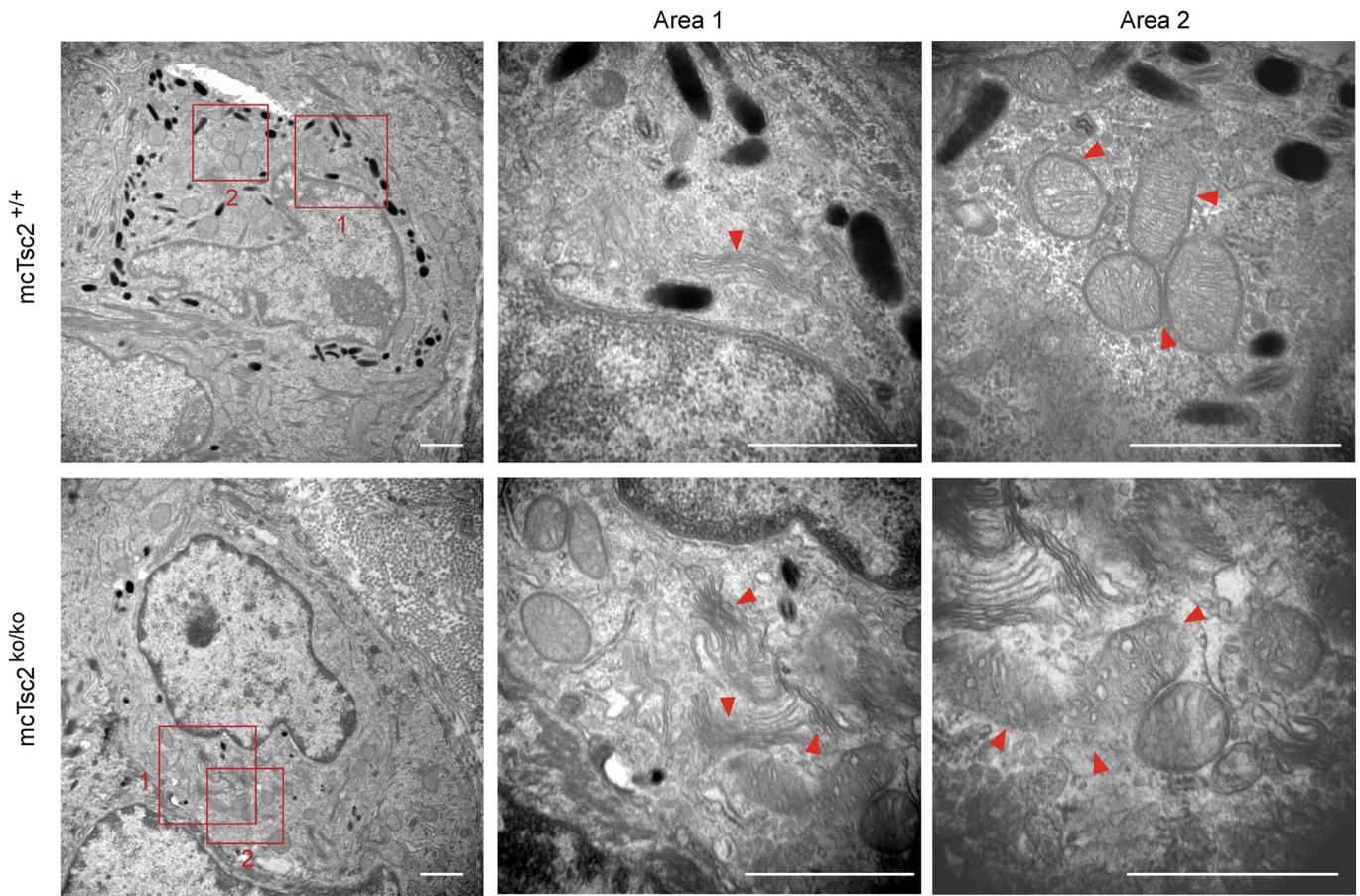


**Figure 2. Melanocyte-specific ablation of Tsc2 disrupted maturation of mouse melanosomes.** (a) Representative sections stained with hematoxylin. The white rectangle outlines the area shown in the corresponding right panel. (b) Representative sections stained by anti-Pmel17 antibody. Scale bars in **a** and **b** = 100  $\mu$ m. The number of melanocytes in each high-power magnification field were counted. (c) Representative ultrathin sections viewed by electron microscopy. The white rectangle outlines the area of melanocytes, and the detail is shown in the corresponding right panel. Scale bar = 3  $\mu$ m. (d) Representative higher-magnification images. Immature melanosomes are shown by red arrows. Scale bar = 200 nm. (e) The expression of melanogenesis-related enzyme in melanosomes (left panel) and the number of melanosomes in melanocytes (right panel). Data in **b** and **e** are shown as mean  $\pm$  standard deviation. \*\*\* $P$  < 0.01. n.s., no significant difference.

### Melanocyte-specific deletion of Tsc2 damaged mitochondria and endoplasmic reticulum

The subcellular alterations in melanocytes in Tsc2<sup>ko/ko</sup> mouse tail skin were further analyzed using transmission electron microscopy. Abnormally enlarged endoplasmic reticulum (ER) was observed in melanocytes from mcTsc2<sup>ko/ko</sup> mice (Figure 3, area 1). Similarly, mitochondria in melanocytes from mcTsc2<sup>ko/ko</sup> mice were generally swollen and pale, with disorganized cristae and loss of membrane density (Figure 3, area 2, lower panel). In contrast, mitochondria in melanocytes from littermate controls showed normal morphology with intact cristae and electron-dense bodies in the mitochondrial matrix (Figure 3, area 2, upper panel). These data show that Tsc2-deficient melanocytes had defective ER and mitochondrial morphology, which may indicate cellular stress in melanocytes.

Cells react to ER stress by initiating a defensive process called the *unfolded protein response* (Senft and Ronai, 2015). In addition to the well-established role of mitochondria in energy metabolism, mitochondria also are known to protect cells from oxidative stress (Lindholm et al., 2017). Therefore, to determine whether ER stress and mitochondrial stress contributed to mouse tail skin depigmentation in mcTsc2<sup>ko/ko</sup> mice, unfolded protein response-related and mitochondrial oxidative stress-related genes and proteins were examined in melanocytes isolated from mouse tail skin. ER stress-related Hsf1 and Hsp70; unfolded protein response-related chaperone molecules Grp78 and calnexin; unfolded protein response-related autophagy molecules P62 and Lc3B; oxidative stress response-related molecules Gsta4, Hmox1, Nqo1, and Gclc; and mitochondrial oxidative stress-related molecules Pgc1 $\alpha$ , Pink1, Parkin1, and Hif1 $\alpha$  were



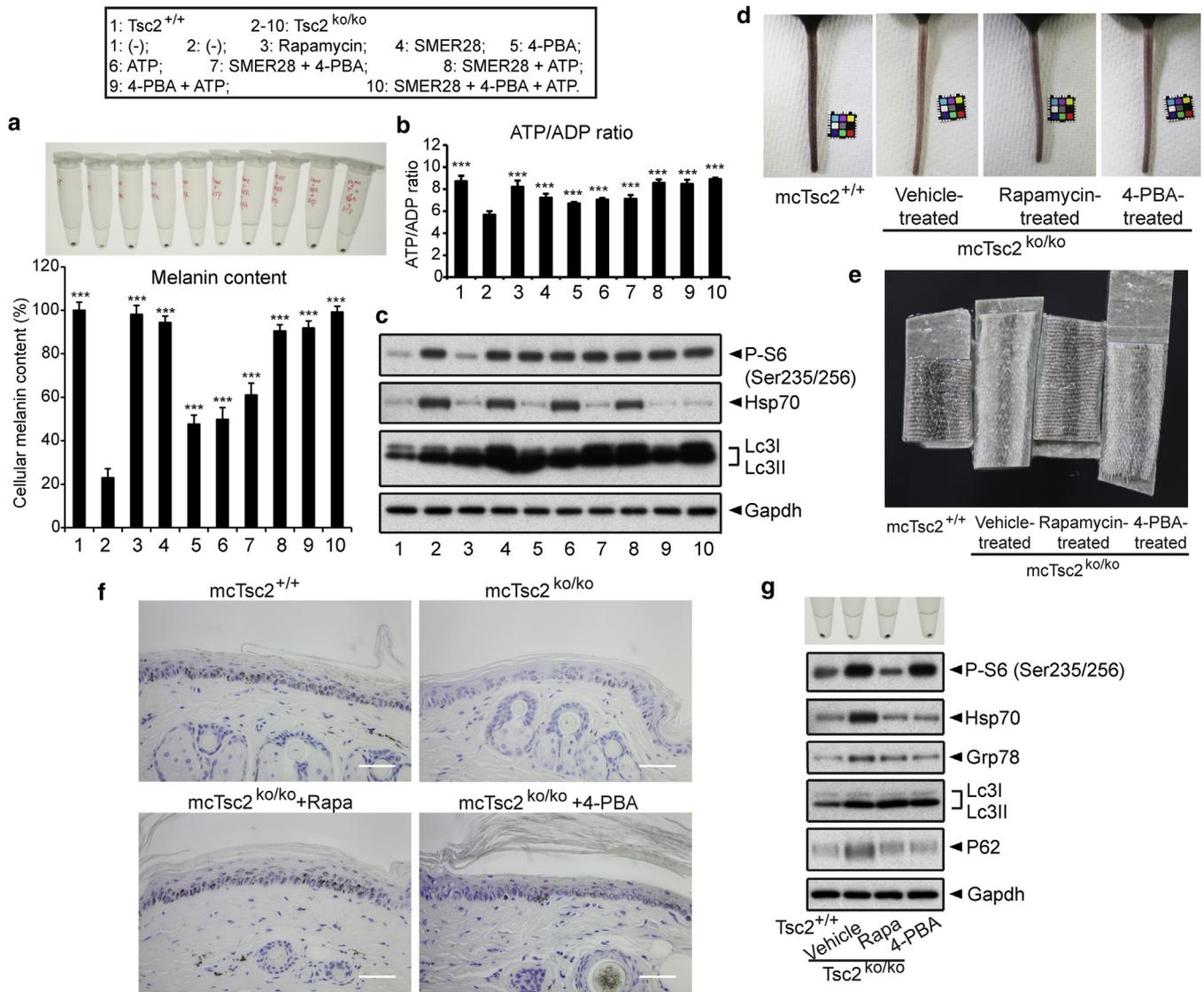
**Figure 3. Melanocyte-specific deletion of Tsc2-induced ultrastructural alterations in mitochondria and endoplasmic reticulum.** Skin specimens from mouse tails were investigated by electron microscopy. Representative higher-magnification images of ultrathin section of melanocytes were observed by electron microscopy. The red rectangle outlines the areas shown in the corresponding right panels. Area 1 shows the representative images for endoplasmic reticulum in melanocytes, and area 2 shows representative images for mitochondria in melanocytes. Endoplasmic reticulum in area 1 and mitochondria in area 2 are indicated by red arrowheads. Scale bars = 500 nm.

significantly up-regulated in  $mcTsc2^{ko/ko}$  mouse melanocytes compared with control littermate mouse melanocytes ( $P < 0.01$ ) (see [Supplementary Figure S2](#) online). Furthermore, immunofluorescence staining showed that Grp78, P62, and parkin were up-regulated. Perinuclear aggresome formation, which indicates misfolded protein accumulation, was also observed in melanocytes from  $mcTsc2^{ko/ko}$  mice (see [Supplementary Figure S3a](#) online). Western blot analysis of cell lysates of isolated tail skin melanocytes from  $mcTsc2^{ko/ko}$  mice showed greatly increased expression of Grp78 and Hsp70 (markers of ER stress), P62 and Lc3 (markers of autophagy), and 4-Hne (marker of mitochondrial oxidative stress), compared with controls (see [Supplementary Figure S3b](#)). Because intracellular adenosine triphosphate (ATP) content is a direct marker of the energy state and lower ATP levels indicate mitochondrial stress, we measured ATP content. The ATP/adenosine diphosphate ratio was significantly lower in melanocytes from  $mcTsc2^{ko/ko}$  mice ( $P < 0.01$ ) (see [Supplementary Figure S3c](#)). Collectively, it suggested that ER stress and mitochondrial oxidative stress were induced in  $mcTsc2^{ko/ko}$  melanocytes and therefore may be involved in the melanocyte dysfunction.

#### mTOR inhibition or ER stress/mitochondrial oxidative stress-related treatment attenuated reduced pigmentation in melanocyte-specific Tsc2-knockout mice

To ascertain whether ER stress/mitochondrial oxidative stress were involved in reduced pigmentation, we treated melanocytes from Tsc2-knockout mice melanocytes in vitro with rapamycin or agents that alleviate ER stress/mitochondrial oxidative stress. The following agents were used in these experiments: the mTOR inhibitor rapamycin (10 nmol/L); the mTOR-independent autophagy inducer SMER28 (50  $\mu$ mol/L); a small chemical chaperone that attenuates ER stress, sodium 4-PBA (20 mmol/L); and the energy supply molecule ATP (10 mmol/L) alone or in the indicated combinations ([Figure 4a–c](#)). Five days after treatment, pharmacological mTORC1 inhibition with rapamycin completely blocked the decrease in ATP and melanin content in melanocytes from  $mcTsc2^{ko/ko}$  mice ( $P < 0.01$ ) ([Figure 4a](#) and [b](#)). mTOR-independent autophagy induction or ER stress/mitochondrial stress alleviation were at last partially effective in abrogating depigmentation in Tsc2-knockout mouse epidermal melanocytes ([Figure 4a–c](#)).

Next, tail skin from  $mcTsc2^{ko/ko}$  mice was treated topically with 0.2% rapamycin (sirolimus gel) once daily for 2 weeks



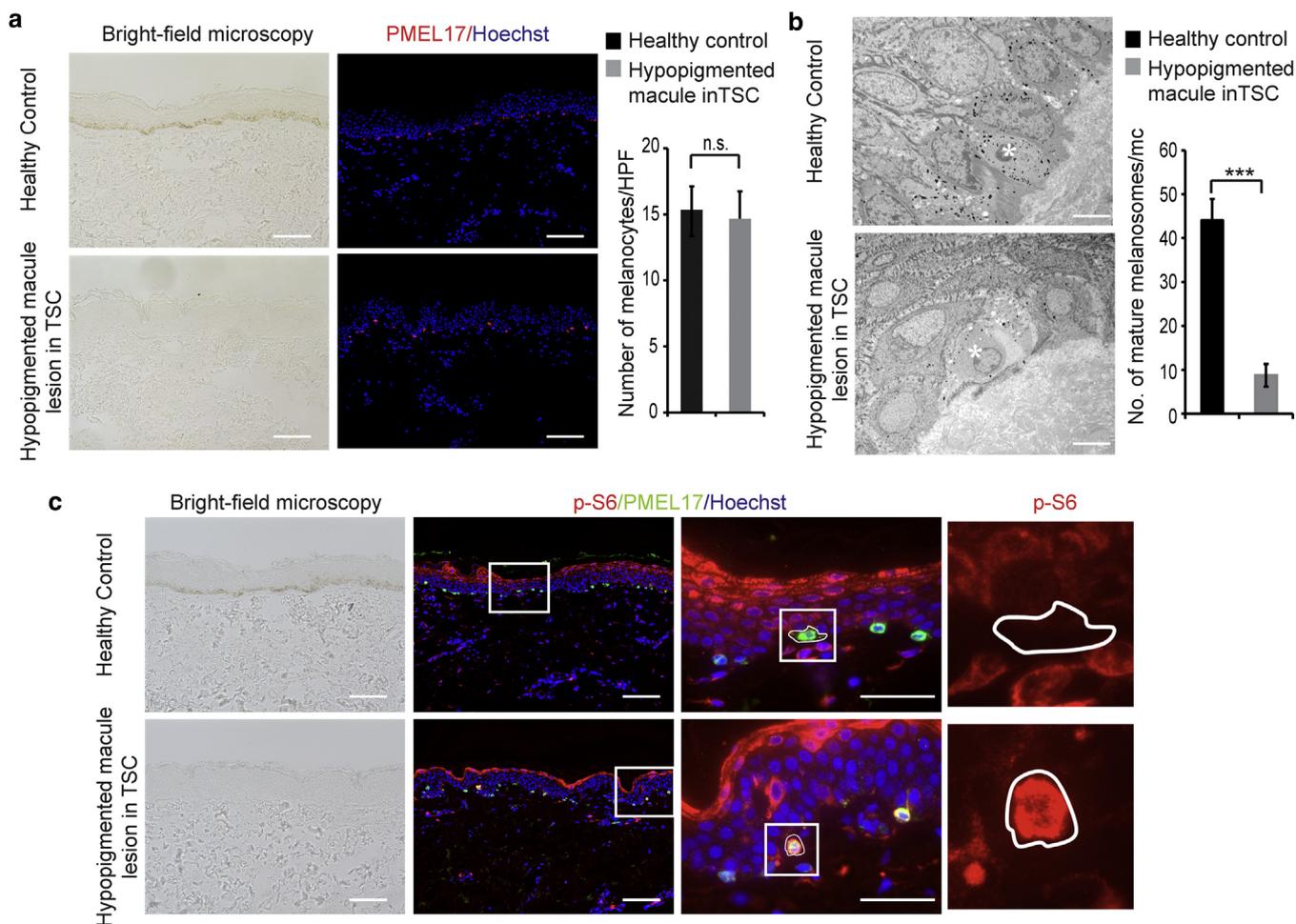
**Figure 4. In vitro and in vivo, mTOR inhibition or endoplasmic reticulum stress/mitochondrial oxidative stress-related treatments attenuated the reduced pigmentation in Tsc2-knockout mouse melanocytes.** (a–c) In vitro, epidermal melanocytes isolated from mouse tail skin were cultured and treated with the indicated agents. (a) Cellular melanin content and the corresponding cell pellet image. (b) Cellular ATP/ADP level. Data in a and b are shown as mean ± standard deviation. \*\*\**P* < 0.01. (c) Cellular proteins were examined by Western blotting. (d–f) In vivo, mice were treated with topical rapamycin gel or with oral 4-PBA solution for 2 weeks. (d) Representative image of tail skin pigmentation. (e) Skin tissue was removed from mouse tail, and then it was flattened on a piece of cardboard for fixation. Representative image of tail skin pigmentation was shown. (f) Skin sections with hematoxylin staining. Scale bar = 100 μm. (g) Epidermal melanocytes were isolated after treatment. Cell pellet images and Western blotting analysis. 4-PBA, 4-phenylbutyrate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

or mice were administrated 4-PBA (1 g/kg/day) orally for 2 weeks. Macroscopically, both rapamycin and 4-PBA treatments resulted in marked improvement of tail skin depigmentation compared with vehicle-treated mcTsc2<sup>ko/ko</sup> mice (Figure 4d). Furthermore, the improvement was also observed on cardboard-fixed flattened tail skin (Figure 4e) and histological examination using sections of tail skin tissue (Figure 4f). In addition, the isolated epidermal melanocytes from animals treated with rapamycin or 4-PBA exhibited increased melanin production (Figure 4g). Topical rapamycin treatment inhibited the activation of mTORC1 signaling and the expression of ER stress-related proteins (Figure 4g). These results indicated that mTORC1 hyperactivation and mTORC1 activation-induced ER stress/mitochondrial

stress were responsible for the reduced pigmentation in mcTsc2<sup>ko/ko</sup> mice.

#### mTORC1 hyperactivation was observed in melanocytes of hypopigmented macule lesions from the skin of patients with TSC

Finally, we investigated whether this mechanism identified in mcTsc2<sup>ko/ko</sup> mice was involved in development of hypopigmented macules in patients with TSC. We examined skin from healthy donors (n = 6) and skin from hypopigmented macule lesions of TSC patients (n = 10). We previously found that melanocytes were present, that melanin granule levels were decreased in TSC-related hypomelanotic macules, and that the volume of melanin granules increased upon topical



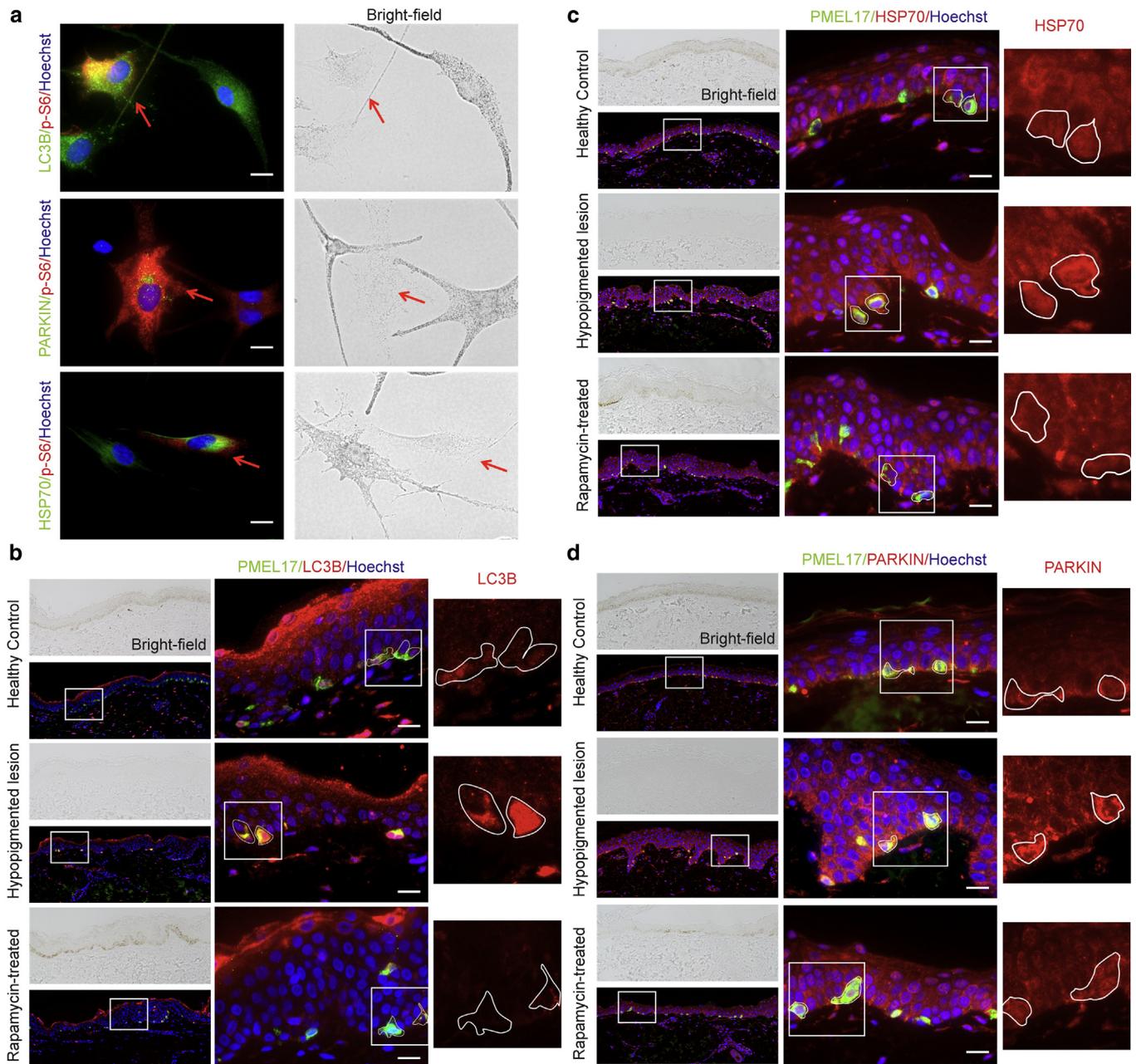
**Figure 5. Loss of melanin and up-regulated mTORC1 signaling in melanocytes of hypopigmented macule lesions from skin of patients with TSC.** (a–c) Skin biopsy specimens from healthy donors and hypopigmented macule lesions from patients with TSC. (a) Representative sections stained with anti-PMEL17 antibody. The number of melanocytes was counted. (b) Representative ultrathin sections image. Scale bar = 7  $\mu$ m. Melanocytes are shown by white asterisks. The number of mature melanosomes in melanocytes was counted. Data in a and b are shown as mean  $\pm$  standard deviation. \*\*\* $P < 0.01$ . (c) Representative sections that were double-stained with anti-p-S6 and anti-PMEL17 antibody. Scale bars in a and c = 100  $\mu$ m. The white rectangle outlines the area shown in the corresponding right panel. The white line-circled area indicates melanocytes. n.s., no significant difference; TSC, tuberous sclerosis complex.

rapamycin treatment (Wataya-Kaneda et al., 2012, 2015). In agreement with our previous findings, the amount of epidermal melanin was markedly decreased in skin from hypopigmented macule lesions of TSC patients; however, immunofluorescence staining for PMEL17 showed no difference in the localization or number of basal layer melanocytes between patient and healthy donor samples (Figure 5a). The lesions from TSC patients were grossly nonpigmented, and visibly mature melanosomes were virtually absent in melanocytes ( $P < 0.01$ ) (Figure 5b). In agreement with our observations in *mcTsc2<sup>ko/ko</sup>* mice, we found that melanocyte numbers were not altered but that the number of mature melanosomes was decreased in skin with hypopigmented macules from patients with TSC.

Furthermore, similar to levels observed in melanocytes from *mcTsc2<sup>ko/ko</sup>* mice, constitutively activated mTORC1/S6 signaling was observed in melanocytes from hypopigmented macule lesions of patients with TSC (Figure 5c), which indicated that mTORC1 hyperactivation also was involved in melanocytes in hypopigmented macules of TSC patients.

#### ER and mitochondrial oxidative stress were observed in melanocytes transfected with *TSC2* small interfering RNA and melanocytes in hypopigmented macule skin lesions from patients with TSC

To further validate these mechanisms in human melanocytes and TSC patients, human normal primary epidermal melanocytes were transfected with *TSC2* small interfering RNA. In *TSC2*-knockdown melanocytes (Figure 6a, red arrow), decreased melanin granules, increased activation of mTOR (Figure 6a, p-S6 in red), and up-regulated expression of the autophagy marker LC3B (Figure 6a, upper panel), mitochondrial stress marker parkin (Figure 6a, middle panel), and ER stress-related protein HSP70 (Figure 6a, lower panel) were observed. Finally, skin tissue from healthy donors (Figure 6b–d, upper panel) and skin tissue from TSC patients before (Figure 6b–d, middle panel) and after (Figure 6b–d, lower panel) treatment with topical rapamycin for 30 days were analyzed. Rapamycin treatment resulted in abrogation of reduced pigmentation in these macules. Increased expression of LC3B (Figure 6b), HSP70 (Figure 6c), and



**Figure 6. Melanin formation, endoplasmic reticulum stress, and mitochondrial oxidative stress by TSC2 knockdown in vitro and topical rapamycin treatment in vivo.** (a) In vitro, cultured human primary skin epidermal melanocytes were transiently transfected with TSC2 small interfering RNA. At 72 hours after transfection, cells were fixed and immunostained with the indicated antibodies. Red arrows indicate TSC2-knockdown cells. Scale bar = 5  $\mu$ m. The corresponding bright field images are presented in the right panels. (b–d) TSC patients with hypopigmented macules were treated with topical rapamycin. Before treatment and 1 month after treatment, skin biopsy samples were analyzed. The white rectangle outlines the area shown in the corresponding right panel. White line-circled areas indicate melanocytes. Scale bar = 20  $\mu$ m. The corresponding bright field images are presented in the upper panels. TSC, tuberous sclerosis complex.

parkin (Figure 6d) were reversed in the skin lesion after rapamycin treatment. These results indicated that constitutive mTOR activation-induced ER stress and mitochondrial dysfunction in melanocytes were also involved in the depigmentation observed in TSC2-knockdown melanocytes and melanocytes of hypopigmented macule lesions.

## DISCUSSION

We used a mouse model to show that deletion of *Tsc2* in melanocytes led to skin depigmentation in vivo. These

experiments showed a mechanism whereby in vivo activation of mTORC1 signaling resulted in ER and mitochondrial oxidative stress and subsequent impaired melanin synthesis.

Using in vitro cell-based assays and clinical samples from TSC patients, mTORC1 activation was implicated in multiple events, including increased ER stress, unfolded protein response activity, and mitochondrial oxidative stress. Moreover, our data showed that inhibition of mTORC1 activity was important for lowering ER/mitochondrial stress and alleviating depigmentation.

To date, the physiological role of the TSC complex/mTORC1 has been investigated in several tissues using a conditional knockout approach (Chen et al., 2008; Mori and Guan, 2012; Mori et al., 2009; Shigeyama et al., 2008; Uhlmann et al., 2002; Wong et al., 2003). However, conditional knockout of *Tsc* in mouse melanocytes has not been investigated. We generated conditional knockout of *Tsc* in melanocytes of mice. Hyperactivation of mTORC1 has been recognized as the mechanism underlying the multisystemic symptoms in TSC (Inoki et al., 2005; Kwiatkowski, 2003a). The exact contribution of hyperactivation of mTORC1 signaling to hypopigmentation, however, is still not fully understood. As is well known, hyperactivation of mTORC1 promotes cell proliferation and tumor formation via overproduction of protein (Saxton and Sabatini, 2017). However, different from highly proliferative cells, melanocytes are a stable cell population, normally living many years without undergoing cell division (Yamaguchi et al., 2007). Thus, in melanocytes, mTOR hyperactivation-induced protein overproduction is difficult to be overcome by cell division. Therefore, subsequently, the balance between energy-consuming protein synthesis and protein degradation may be disrupted, and eventually ER and mitochondrial stress are induced in melanocytes. In addition, energy is critical in regulating melanosomal transporters, such as tyrosine and cysteine transporters, and proton pumps that regulate the pH within melanosomes (Yamaguchi et al., 2007). Thus, mitochondrial dysfunction and bio-energetic collapse might be critical steps in depigmentation. Moreover, Ras-MAPK-ERK, the cell's other key mechanism for controlling growth, cell survival, division, and metabolism, is well known to inhibit melanogenesis. However, some explanations of this mechanism have been controversial (Lin and Fisher, 2007). As a speculative view, we thought that it might arise from a similar mechanism to mTOR hyperactivation-mediated hypopigmentation.

In summary, our model, which shows that mTORC1 hyperactivation in melanocytes results in hypopigmentation, offers an *in vivo* model for depigmentation. This model may also be useful for understanding the pathophysiology of depigmentation, identifying cellular events that are downstream of mTORC1 hyperactivation, and developing therapeutic strategies for the treatment of hypopigmentation. Our current findings provide insight into the mechanisms underlying hypopigmented macules in TSC and the function of mTORC1 in pigmentation regulation. Furthermore, these findings suggest that mTORC1 is a promising target for treating hypopigmentation in TSC and in other forms of depigmentation diseases that result from increased mTORC1 activation.

## MATERIALS AND METHODS

### Generation of melanocyte-specific *Tsc2*-knockout mice and *in vivo* treatments

*Tsc2*<sup>flox/flox</sup> mice and *Mitf*-Cre mice were generated as shown in Figure 1a. Melanocyte-specific *Tsc2*-knockout mice were generated by breeding *Tsc2*<sup>flox/flox</sup> mice with *Mitf*-Cre mice.

For the rapamycin treatment, 0.2% sirolimus gel was prepared as described in our previous reports (Wataya-Kaneda et al., 2012, 2015, 2017). Mice were treated topically with rapamycin gel

every day for 2 weeks. 4-PBA was purchased from Santa Cruz Biotechnology (Paso Robles, CA) and was dissolved in distilled water for oral administration (1 g/kg/day) for 2 weeks. Experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, and the experimental protocol used in this study was approved by the Committee for Animal Experiments at Osaka University (Osaka, Japan).

### Human skin specimens

Paraffin-embedded tissue sections of lesional skin from confirmed TSC patients with hypopigmented macules ( $n = 10$ ) and samples from corresponding sites of healthy donors ( $n = 6$ ) were used in this study. Written informed consent was obtained from all participants before study inclusion. The study was approved by the ethics committee of the Osaka University Faculty of Medicine (no. 10339).

Details on immunohistochemistry, Western blotting analysis, and electron microscopy are provided in Supplementary Materials online.

### Statistics

Experiments were repeated at least three times. Data are presented as mean  $\pm$  standard deviation. Statistical analysis was conducted using two-way analysis of variance for interactions between variables. Unpaired Student *t* test (Microsoft Excel; Microsoft Corp., Redmond, WA) was used for comparison between groups. *P*-values less than 0.05 were considered statistically significant.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at [www.jidonline.org](http://www.jidonline.org), and at <https://doi.org/10.1016/j.jid.2017.10.007>.

### REFERENCES

- Busca R, Bertolotto C, Ortonne JP, Ballotti R. Inhibition of the phosphatidylinositol 3-kinase/p70(S6)-kinase pathway induces B16 melanoma cell differentiation. *J Biol Chem* 1996;271:31824–30.
- Cao J, Tyburczy ME, Moss J, Darling TN, Widlund HR, Kwiatkowski DJ. Tuberous sclerosis complex inactivation disrupts melanogenesis via mTORC1 activation. *J Clin Invest* 2017;127:349–64.
- Chen C, Liu Y, Liu R, Ikenoue T, Guan KL, Zheng P. TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species. *J Exp Med* 2008;205:2397–408.
- Crino PB, Nathanson KL, Henske EP. The tuberous sclerosis complex. *New Engl J Med* 2006;355:1345–56.
- Ess KC. Tuberous sclerosis complex: everything old is new again. *J Neurodev Disord* 2009;1:141–9.
- Gold AP, Freeman JM. Depigmented nevi: the earliest sign of tuberous sclerosis. *Pediatrics* 1965;35:1003–5.
- Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell* 2007;12:9–22.
- Hah YS, Cho HY, Lim TY, Park DH, Kim HM, Yoon J, et al. Induction of melanogenesis by rapamycin in human MNT-1 melanoma cells. *Ann Dermatol* 2012;24:151–7.

- Ho H, Kapadia R, Al-Tahan S, Ahmad S, Ganesan AK. WIP1 coordinates melanogenic gene transcription and melanosome formation via TORC1 inhibition. *J Biol Chem* 2011;286:12509–23.
- Inoki K, Corradetti MN, Guan KL. Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* 2005;37:19–24.
- Inoki K, Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, et al. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* 2006;126:955–68.
- Jeong HS, Lee SH, Yun HY, Baek KJ, Kwon NS, Park KC, et al. Involvement of mTOR signaling in sphingosylphosphorylcholine-induced hypopigmentation effects. *J Biomed Sci* 2011;18:55.
- Jimbow K. Tuberous sclerosis and guttate leukoderms. *Semin Cutan Med Surg* 1997;16:30–5.
- Kenerson HL, Aicher LD, True LD, Yeung RS. Activated mammalian target of rapamycin pathway in the pathogenesis of tuberous sclerosis complex renal tumors. *Cancer Res* 2002;62:5645–50.
- Kobayashi T, Minowa O, Kuno J, Mitani H, Hino O, Noda T. Renal carcinogenesis, hepatic hemangiomas, and embryonic lethality caused by a germ-line Tsc2 mutation in mice. *Cancer Res* 1999;59:1206–11.
- Kwiatkowski DJ. Rhebbing up mTOR: new insights on TSC1 and TSC2, and the pathogenesis of tuberous sclerosis. *Cancer Biol Ther* 2003a;2:471–6.
- Kwiatkowski DJ. Tuberous sclerosis: from tubers to mTOR. *Ann Hum Genet* 2003b;67(Pt. 1):87–96.
- Kwiatkowski DJ, Zhang H, Bandura JL, Heiberger KM, Glogauer M, el-Hashemite N, et al. A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. *Hum Mol Genet* 2002;11:525–34.
- Laplante M, Sabatini DM. mTOR signaling at a glance. *J Cell Sci* 2009;122(Pt. 20):3589–94.
- Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* 2012;149:274–93.
- Lin JY, Fisher DE. Melanocyte biology and skin pigmentation. *Nature* 2007;445(7130):843–50.
- Lindholm D, Korhonen L, Eriksson O, Koks S. Recent Insights into the Role of Unfolded Protein Response in ER Stress in Health and Disease. *Front Cell Dev Biol* 2017;5:48.
- Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci* 2011;36:320–8.
- Mori H, Guan KL. Tissue-specific ablation of Tsc1 in pancreatic beta-cells. Mtor: methods and protocols. *Methods Mol Biol* 2012;821:407–19.
- Mori H, Inoki K, Opland D, Munzberg H, Villanueva EC, Faouzi M, et al. Critical roles for the TSC-mTOR pathway in beta-cell function. *Am J Physiol Endocrinol Metab* 2009;297:E1013–22.
- Ohguchi K, Banno Y, Nakagawa Y, Akao Y, Nozawa Y. Negative regulation of melanogenesis by phospholipase D1 through mTOR/p70 S6 kinase 1 signaling in mouse B16 melanoma cells. *J Cell Physiol* 2005;205:444–51.
- Onda H, Crino PB, Zhang H, Murphey RD, Rastelli L, Gould Rothberg BE, et al. Tsc2 null murine neuroepithelial cells are a model for human tuber giant cells, and show activation of an mTOR pathway. *Mol Cell Neurosci* 2002;21:561–74.
- Raposo G, Tenza D, Murphy DM, Berson JF, Marks MS. Distinct protein sorting and localization to premelanosomes, melanosomes, and lysosomes in pigmented melanocytic cells. *J Cell Biol* 2001;152:809–24.
- Reynolds J. The epidermal melanocytes of mice. *J Anat* 1954;88:45–58.
- Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell* 2017;168:960–76.
- Senft D, Ronai ZA. UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. *Trends Biochem Sci* 2015;40:141–8.
- Shigeyama Y, Kobayashi T, Kido Y, Hashimoto N, Asahara S, Matsuda T, et al. Biphasic response of pancreatic beta-cell mass to ablation of tuberous sclerosis complex 2 in mice. *Mol Cell Biol* 2008;28(9):2971–9.
- Tachibana M. MITF: A stream flowing for pigment cells. *Pigm Cell Res* 2000;13:230–40.
- Tsao YT, Huang YF, Kuo CY, Lin YC, Chiang WC, Wang WK, et al. Hinokitiol Inhibits Melanogenesis via AKT/mTOR Signaling in B16F10 Mouse Melanoma Cells. *Int J Mol Sci* 2016;17:248.
- Uhlmann EJ, Wong M, Baldwin RL, Bajenaru ML, Onda H, Kwiatkowski DJ, et al. Astrocyte-specific TSC1 conditional knockout mice exhibit abnormal neuronal organization and seizures. *Ann Neurol* 2002;52:285–96.
- van Slegtenhorst M, de Hoogt R, Hermans C, Nellist M, Janssen B, Verhoef S, et al. Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. *Science* 1997;277(5327):805–8.
- Wataya-Kaneda M, Nakamura A, Tanaka M, Hayashi M, Matsumoto S, Yamamoto K, et al. Efficacy and safety of topical sirolimus therapy for facial angiofibromas in the tuberous sclerosis complex: a randomized clinical trial. *JAMA Dermatol* 2017;153:39–48.
- Wataya-Kaneda M, Tanaka M, Nakamura A, Matsumoto S, Katayama I. A novel application of topical rapamycin formulation, an inhibitor of mTOR, for patients with hypomelanotic macules in tuberous sclerosis complex. *Arch Dermatol* 2012;148:138–9.
- Wataya-Kaneda M, Tanaka M, Yang L, Yang F, Tsuruta D, Nakamura A, et al. Clinical and histologic analysis of the efficacy of topical rapamycin therapy against hypomelanotic macules in tuberous sclerosis complex. *JAMA Dermatol* 2015;151:722–30.
- Wienecke R, Konig A, DeClue JE. Identification of tuberin, the tuberous sclerosis-2 product. Tuberin possesses specific Rap1GAP activity. *J Biol Chem* 1995;270:16409–14.
- Wong M, Ess KC, Uhlmann EJ, Jansen LA, Li W, Crino PB, et al. Impaired glial glutamate transport in a mouse tuberous sclerosis epilepsy model. *Ann Neurol* 2003;54:251–6.
- Wullschlegel S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell* 2006;124:471–84.
- Yamaguchi Y, Brenner M, Hearing VJ. The regulation of skin pigmentation. *J Biol Chem* 2007;282:27557–61.