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Rab11b Mediates Melanin Transfer between Donor Melanocytes and Acceptor Keratinocytes via Coupled Exo/Endocytosis

Abul K. Tarafder[1,2,3,8], Giulia Bolasco[3,8], Maria S. Correia[1,2], Francisco J.C. Pereira[1,2], Lucio Iannone[1,2], Alistair N. Hume[3], Niall Kirkpatrick[4], Mauro Picardo[5], Maria R. Torrisi[5,6], Inês P. Rodrigues[1], José S. Ramalho[1], Clare E. Futter[7], Duarte C. Barral[1] and Miguel C. Seabra[1,2,3]

The transfer of melanin from melanocytes to keratinocytes is a crucial process underlying maintenance of skin pigmentation and photoprotection against UV damage. Here, we present evidence supporting coupled exocytosis of the melanin core, or melanocore, by melanocytes and subsequent endocytosis by keratinocytes as a predominant mechanism of melanin transfer. Electron microscopy analysis of human skin samples revealed three lines of evidence supporting this: (1) the presence of melanocores in the extracellular space; (2) within keratinocytes, melanin was surrounded by a single membrane; and (3) this membrane lacked the melanosomal membrane protein tyrosinase-related protein 1 (TYRP1). Moreover, co-culture of melanocytes and keratinocytes suggests that melanin exocytosis is specifically induced by keratinocytes. Furthermore, depletion of Rab11b, but not Rab27a, caused a marked decrease in both keratinocyte-stimulated melanin exocytosis and transfer to keratinocytes. Thus, we propose that the predominant mechanism of melanin transfer is keratinocyte-induced exocytosis, mediated by Rab11b through remodeling of the melanosome membrane, followed by subsequent endocytosis by keratinocytes.

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INTRODUCTION

Melanocytes reside in the basal layer of the epidermis sparsely spread in a 1:40 ratio among keratinocytes (Jimbow *et al.*, 1979), where the two cell types are proposed to interact in a symbiotic manner (Imokawa, 2004; Yamaguchi and Hearing, 2010). The photoprotective pigment, melanin, is synthesized in melanocytes and packaged into lysosome-related organelles termed melanosomes (Marks and Seabra, 2001; Raposo and Marks, 2002; Hearing, 2005). Consistent with their unique morphology and function, melanosomes contain specific integral membrane proteins such as PMEL (gp100), tyrosinase, and tyrosinase-related protein 1 (TYRP1, gp75) whose sorting and localization have been described previously (Raposo *et al.*, 2001). Fully melanized melanosomes are transported from their site of synthesis to the cell periphery before they are transferred to keratinocytes and transported to the apical area of the cell to form a supranuclear cap that prevents DNA photodamage induced by exposure to UV radiation (Scott, 2003; Byers *et al.*, 2007).

Despite its pathophysiological importance, the molecular mechanism underlying melanin transfer remains poorly characterized, although several hypotheses (H1–H4) have been postulated to date including (Yamamoto and Bhawan, 1994; Seiberg, 2001; Van Den Bossche *et al.*, 2006; Singh *et al.*, 2008): (H1) heterophagocytosis of the melanocyte dendrite tip; (H2) release of melanosome-loaded vesicles from the melanocyte followed by phagocytosis by the keratinocyte (Scott *et al.*, 2002; Ando *et al.*, 2011; Wu *et al.*, 2012) (H3) exocytosis of the melanin core from the melanocyte followed by endocytosis by the keratinocyte; and (H4) transfer of melanosomes from the melanocyte filopodia to the keratinocyte via direct membrane fusion (Singh *et al.*, 2010; Beaumont *et al.*, 2011). The first two mechanisms involve melanosome transport within keratinocytes in double membrane compartments derived from both the donor melanocyte and the acceptor keratinocyte. In contrast, the last two hypotheses predict that melanin granules are transported within keratinocytes in a single membrane-bound structure derived either from the keratinocyte (H3) or from the

[1]*CEDOC, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa, Portugal;* [2]*Instituto Gulbenkian de Ciência, Oeiras, Portugal;* [3]*Molecular Medicine, National Heart and Lung Institute, Imperial College London, London, UK;* [4]*Craniofacial and Orbito-Palpebral Surgery Unit at Chelsea and Westminster Hospital, London, UK;* [5]*Laboratorio di Fisiopatologia Cutanea, Istituto Dermatologico San Gallicano, IFO, Rome, Italy;* [6]*Dipartimento di Medicina Clinica e Molecolare, Azienda Ospedaliera Sant'Andrea, Università di Roma 'La Sapienza', Rome, Italy and* [7]*Institute of Ophthalmology, University College London, London, UK*

[8]*The first two authors contributed equally to this work.*

Correspondence: Miguel C. Seabra or Duarte C. Barral, CEDOC, Faculdade de Ciências Médicas, FCM, Universidade Nova de Lisboa, 1169-056 Lisboa, Portugal. E-mail: miguel.seabra@fcm.unl.pt or duarte.barral@fcm.unl.pt

Abbreviations: LAMP1, lysosomal-associated membrane protein 1; PBS, phosphate-buffered saline; siRNA, small interfering RNA; TYRP1, tyrosinase-related protein 1

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melanocyte (H4). Recently, an alternative mechanism has been proposed where melanocytes transfer melanosome-rich packages by a ''shedding'' mechanism that occurs both at the tips of dendrites and the cell body (Wu *et al.*, 2012).

Despite a number of ultrastructural studies on epidermal cell culture (Okazaki *et al.*, 1976), human hair roots (Mottaz and Zelickson, 1967), and skin samples (Yamamoto and Bhawan, 1994), molecular evidence for the mechanism of melanin transfer has not been forthcoming. Moreover, the lack of a suitable system to reproduce melanin transfer *in vitro* has represented a major impediment to unraveling the molecular basis of this process.

Rab proteins are critical regulators of membrane trafficking (Pfeffer, 2001; Zerial and McBride, 2001; Seabra *et al.*, 2002) and have been implicated in a number of processes involved in skin pigmentation (Wasmeier *et al.*, 2006). Rab32 and Rab38 have been implicated in melanosome biogenesis (Wasmeier *et al.*, 2006). Rab27a has a well-characterized role in melanosome transport within melanocytes where it regulates the peripheral localization of melanosomes (Bahadoran *et al.*, 2001; Hume *et al.*, 2001; Wu *et al.*, 2001; Strom *et al.*, 2002). Recently, depletion of Rab17 and Rab11 have been shown to cause accumulation of pigment in melanocytes (Beaumont *et al.*, 2011). Hence, it is likely that Rab proteins have a crucial role in regulating melanin transfer.

Here, we have characterized the predominant molecular mechanism of melanin transfer from melanocytes to keratinocytes based on a combination of *in vivo* morphological analysis coupled with *in vitro* models of melanin transfer. We found that melanin exocytosis is mediated by Rab11b and that melanocores are subsequently endocytosed by keratinocytes.

RESULTS

Ultrastructural analysis of human skin reveals melanocores present in the extracellular space between melanocytes and keratinocytes

Human skin samples were analyzed by transmission electron microscopy and areas with a high concentration of melanin in keratinocytes were selected for analysis of melanin transfer (Figure 1a). Melanocytes in the basal layer of the epidermis can be readily distinguished from keratinocytes by the lack of keratins, absence of desmosomes at cell–cell junctions (Supplementary Figure S1a–c online), and the presence of melanosomes at different stages of maturation (Supplementary Figure S1c online). Analysis of serial ultrathin sections revealed the presence of melanin granules lacking membranes or associated cytoplasm, herein termed melanocores, in the extracellular space between the melanocyte and the basal lamina (Figure 1b1–8). To exclude artifacts or alteration due to chemical fixation with aldehydes, high-pressure freezing followed by freeze substitution and conventional embedding was also performed on a skin sample, showing similar results. (Supplementary Figure S2a and b online).

Along with individual granules, clusters of melanocores were also observed in close proximity to keratinocyte and melanocyte plasma membranes (Figure 2a and b) and were also seen in the process of being taken up by keratinocytes within plasma membrane invaginations (Figure 2a and c). Moreover, a single membrane delimited individual melanin granules within the keratinocyte (Figure 2d–f). Quantitation of 20 images of equivalent magnification revealed that 64 melanosomes out of 66 had a discernable membrane in melanocytes, whereas in keratinocytes 46 single melanin granules and 51 groups of melanin granules out of 113 had a clear single membrane. The remaining 16 melanin granules did not show any clear surrounding membrane.

The single membrane limiting melanin granules after transfer to keratinocytes lacks melanosomal markers

If melanocores are endocytosed, the single membrane enclosing melanin in the keratinocyte would be derived from the keratinocyte, whereas if melanosomes are transferred following fusion of the melanocyte and keratinocyte plasma membranes it would be the melanosomal membrane itself. To determine the source of the single membrane enclosing melanin granules within keratinocytes, we used TYRP1 as a marker of melanosome membranes (Raposo *et al.*, 2001) by confocal microscopy of semithin (5 μm) frozen sections. TYRP1 (Figure 3b) localized to both the cell body and dendrites of melanocytes (Figure 3b, arrowhead). In contrast, within keratinocytes, TYRP1 staining was absent from both the periphery and the supranuclear cap (Figure 3a and b). These results suggest that the melanocyte-derived melanosome membrane is absent from melanin granules within keratinocytes, regardless of its stage of degradation. To better visualize individual melanosomes and the membranes surrounding them, TYRP1 was localized on ultrathin (50 nm) cryo-sections by cryo-immuno electron microscopy, detecting TYRP1 by immunogold. Consistent with the immunofluorescence results, melanosomal membranes within melanocytes were heavily labeled (Figure 3c and d), but melanin granules within keratinocytes had very few gold particles (Figure 3c and e). Moreover, the low level of labeling within keratinocytes was localized only in the lumen of the granules and not in the surrounding membrane, presumably because of the presence of intraluminal vesicles containing the protein (Figure 3c and e). Quantification of the number of gold particles per melanin granule showed 3.5-fold more TYRP1 associated with melanin granules in melanocytes than those in keratinocytes (Figure 3g). Importantly, the masking effect on intraluminal epitopes of densely packed melanin should be decreased after internalization by keratinocytes because of melanin degradation (Wolff, 1973; Borovanský and Elleder, 2003), and hence scarcity of TYRP1 staining within keratinocytes is likely to reflect true lack of this protein (Figure 3b and e).

To further test our hypothesis that the melanosomal membrane is not present in keratinocytes, we utilized a different melanosome membrane marker and established heterologous co-cultures with a murine melanocyte cell line (Melan-Ink4a) and normal human keratinocytes. After 48 hours in culture, the cells were processed for cryo-immunoEM using an antibody specific for mouse lysosomal-associated membrane protein 1 (LAMP1) to label melanocyte- (murine) but not keratinocyte-derived (human) LAMP1 (Supplementary Figure S3a–c online). LAMP1 localized, as expected, to melanosome

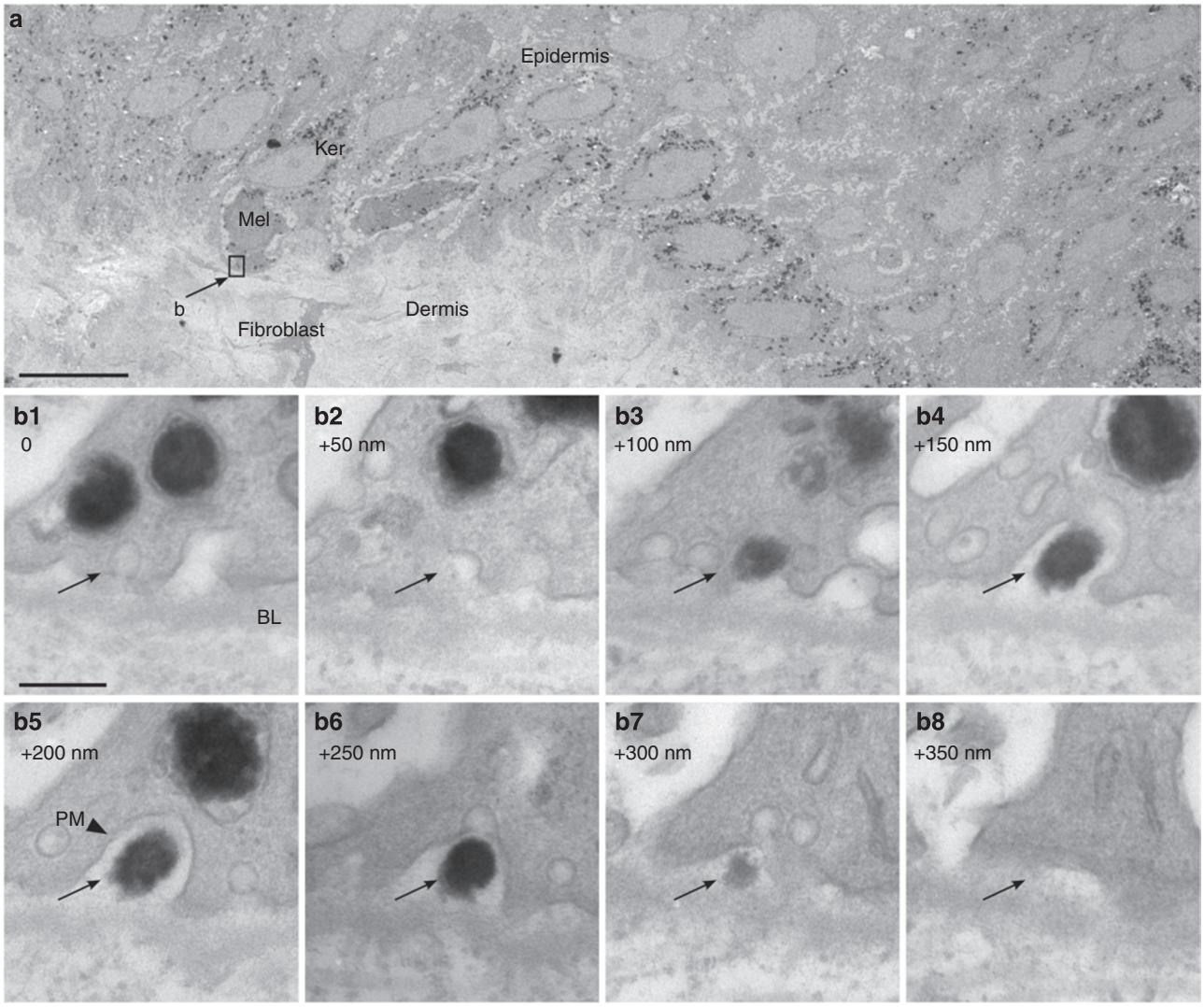

Figure 1. Transmission electron microscopy (TEM) of ultrathin sections of human skin reveals melanocores in the extracellular space. (**a**) Low-magnification TEM image showing the overall structure of the human dermis/epidermis. (**b**1–8) A series of high-magnification TEM images of serial ultrathin (50 nm) sections of the interface between a melanocyte plasma membrane (arrow) and the BL. Inspection of serial sections reveals the presence of a melanocore devoid of membrane in the extracellular space. BL, basal lamina; K, keratin filament; Ker, keratinocyte; Mel, melanocyte; PM, plasma membrane. Bars: (**a**) = 10 μm and (**b**1–8) = 200 nm.

membranes in melanocytes, as previously reported (Supplementary Figure S3b online) (Zhou *et al.*, 1993), but mouse LAMP1 was not detected in melanin granules within keratinocytes (Supplementary Figure S3c online), thus demonstrating the loss of the membrane after melanin transfer.

The presence of melanocores in the extracellular space between cells, together with the observation that melanin taken up by keratinocytes is surrounded by a single membrane lacking melanosomal membrane proteins, suggests that melanin exocytosis followed by endocytosis by keratinocytes is a major mechanism underlying melanin transfer in the skin.

Keratinocytes induce melanin exocytosis by melanocytes in co-culture

Confirmation of this hypothesis required the development of a melanin exocytosis assay. Two melanocyte cell types were utilized, namely Melan-ink4a and primary murine melanocytes derived from C57BL/6 mice. Melanocytes cultured alone displayed low levels of melanin exocytosis into tissue culture medium (Figure 4a). However, when co-cultured with XB2 keratinocytes, both melanocyte types showed a 2–3-fold increase in melanin exocytosis. The increase in melanin exocytosis elicited by keratinocytes was specific as co-culture of melanocytes with both HeLa and NIH-3T3 fibroblasts did not have any significant effect on melanin exocytosis (Figure 4b).

Rab11b modulates keratinocyte-induced melanin exocytosis by melanocytes

To characterize the molecular basis of melanin exocytosis from melanocytes, a small interfering RNA (siRNA) screen of candidate Rab GTPases was employed. Melan-ink4a melanocytes were treated with the appropriate siRNA SMART pools before co-culture with XB2 keratinocytes. After 7 days of co-culture, the quantity of melanin present in tissue culture

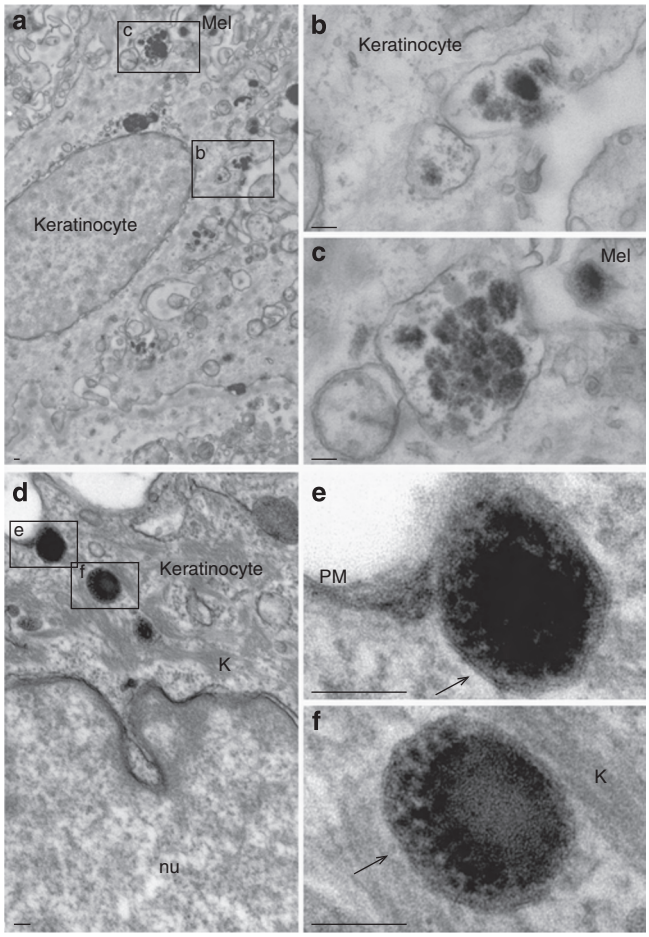

Figure 2. Melanin granules internalized by keratinocytes are present in single membrane-bound compartments. (**a–c**, **d–f**) Transmission electron microscopy (TEM) images of ultrathin section of human skin showing two different areas of melanin-containing keratinocytes in the basal layer. (**a**, **d**) Low-magnification images. (**c**, **b**, **e**, **f**) High-magnification images of upper and lower boxed areas in **a** and **d**, respectively. (**b**, **c**) A cluster of melanin granules is shown: (**b**) being uptaken by a keratinocyte membrane ruffle, and (**c**) in early phase of internalization within a single membrane. (**e, f**) Individual melanin granules are shown that are in the process of being uptaken by a keratinocyte, and are bounded by a single bilayer (arrows) that is continuous with a keratinocyte plasma membrane invagination. K, keratin; Mel, melanocyte; nu, nucleus; PM, plasma membrane. Bars = 100 nm.

media was assayed and normalized to the number of melanocytes present at the end of the assay period. This screen revealed that Rab11b depletion caused a marked decrease in keratinocyte-induced melanin exocytosis (Figure 4c). Surprisingly, Rab27a depletion had no effect on keratinocyte-induced melanin exocytosis. To confirm these findings, single siRNA oligos designed to specifically silence Rab11b and Rab27a were utilized. Quantitative real-time reverse-transcriptase–PCR analysis revealed that these siRNAs gave an ~50% reduction in Rab11b and Rab27a mRNA levels in melanocytes cultured alone as measured 7 days after knockdown was initiated (Supplementary Figure S4a and b online), and no off-target effects on Rab11a expression were observed (Supplementary Figure S4c online). All single oligos specifically designed to deplete Rab11b, but not those targeting Rab27a, significantly reduced keratinocyte-induced melanin exocytosis into tissue culture media to levels seen with Melan-ink4a cultured alone (Figure 4d).

The effect of Rab11b and Rab27a depletion in primary murine melanocytes was also tested using adenovirus encoding miRNA and a green fluorescent protein reporter to infect melanocytes. Consistently, depletion of Rab11b but not Rab27a caused a reduction of keratinocyte-induced melanin exocytosis to the level seen with primary melanocytes alone (Figure 4e). Furthermore, we confirmed that knockdown by miRNA was efficient and off-target effects were not seen (Supplementary Figure S4d–f online).

To further verify the surprising finding that Rab27a had no effect on keratinocyte-induced melanin exocytosis, Melan-*ash* melanocytes (Rab27a-null) were utilized. Consistent with our findings, no significant difference was observed in the keratinocyte-induced increase in melanin exocytosis between Melan-ink4a and Melan-*ash* melanocytes (Figure 4f).

Next, the effect of Rab overexpression on melanin exocytosis was assayed. Primary melanocytes were infected with adenovirus encoding green fluorescent protein–Rab proteins. Overexpression of Rab11a and Rab11b had no effect on melanin exocytosis from melanocytes cultured alone (Figure 4g). However, when co-cultured with XB2, overexpression of Rab11b caused a 2-fold increase in keratinocyte-induced melanin exocytosis (Figure 4h). In contrast, overexpression of Rab11a had little effect on keratinocyte-induced melanin exocytosis.

Disruption of melanin exocytosis causes a reduction in melanin transfer to keratinocytes

We showed that depletion of Rab11b, but not Rab27a, causes a decrease in keratinocyte-induced melanin exocytosis. To determine whether melanin exocytosis is a predominant mechanism of melanin transfer, the amount of melanin transferred to keratinocytes was assayed in conditions where melanin exocytosis was inhibited. Melan-ink4a cells were treated with single siRNA oligos specifically depleting either Rab11b or Rab27a and the amount of melanin transferred to XB2 keratinocytes was assayed by brightfield microscopy. Rab11b depletion resulted in an ~50% decrease in melanin taken up by keratinocytes with all four siRNAs utilized (Figure 5a). Conversely, Rab27a depletion did not lead to a decrease in melanin uptake by keratinocytes with any of the single siRNA oligos (Figure 5a) despite efficient depletion (Supplementary Figure S4b online). These data suggest a correlation between melanin exocytosis and melanin transfer as depletion of Rab11b, but not Rab27a, decreased both melanin exocytosis and melanin transfer to keratinocytes. Furthermore, representative images of the co-culture system clearly show that melanin is taken up by keratinocytes and that these melanin granules lack TYRP1 staining (Figure 5b–d), again confirming that the melanosomal membrane is not present on melanin granules within keratinocytes. Noteworthy, little melanin was transferred when Melan-ink4a were co-cultured with HeLa or NIH-3T3 fibroblasts (Supplementary Figure S5 online).

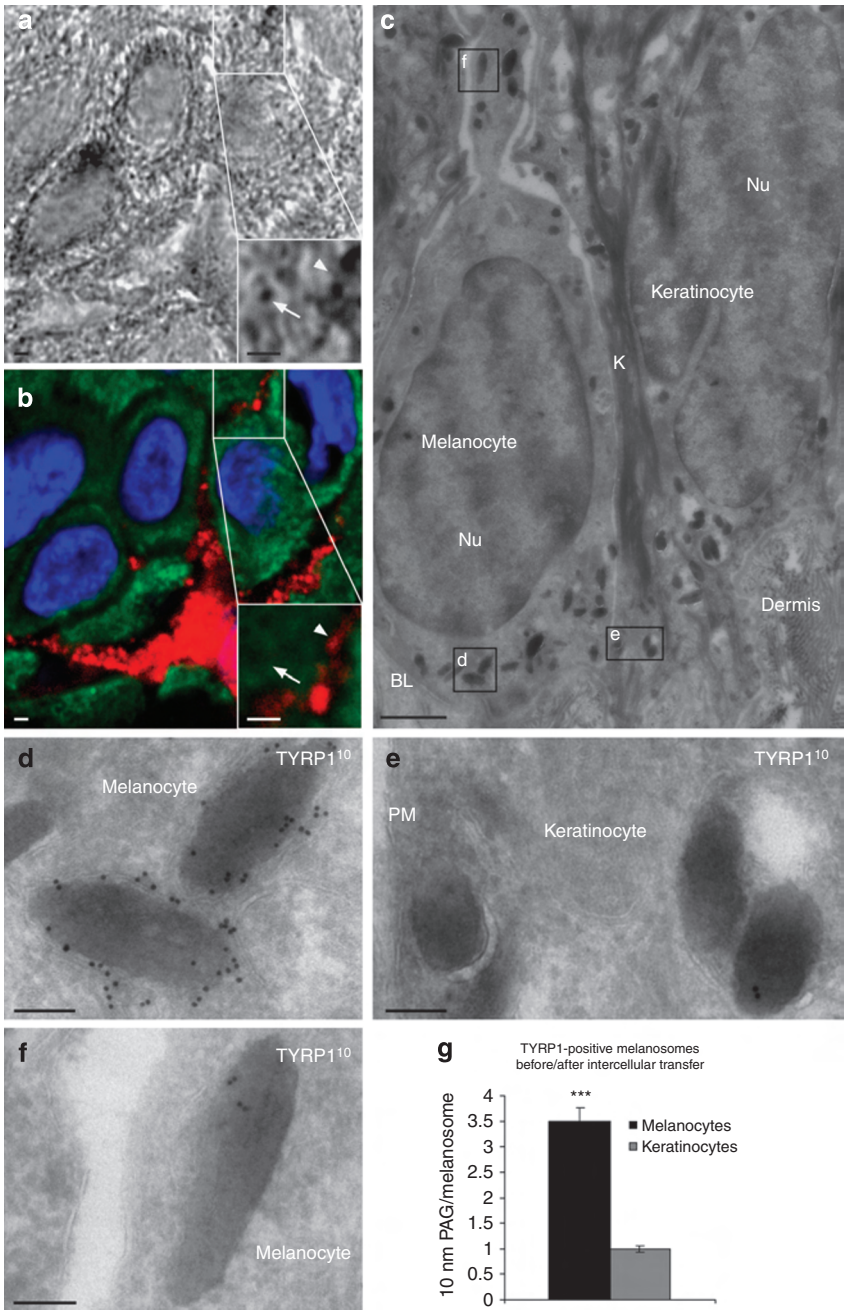

Figure 3. Melanin granules lack tyrosinase-related protein 1 (TYRP1) staining following uptake by keratinocytes. (**a**, **b**) Confocal analysis of immunolabeled semithin frozen sections of human skin revealed absence of TYRP1 staining (**b**, red) in melanin granules within perinuclear melanin cap in keratinocytes (**b**, anti-pankeratin, green; **a**, brightfield). Nuclei are revealed by 4′,6-diamidino-2-phenylindole (DAPI) staining (blue, **b**). High-magnification images of boxed area show strong TYRP1 staining of melanosomes within the melanocyte dendrite tip (arrowhead) compared with those transferred to neighboring keratinocytes (arrow). (**c**) Low magnification of ultrathin cryosection of human skin. (**d**, **e**) Higher magnification of boxed areas in (**c**) (left hand and right hand, respectively) showing immuno-electron microscopy analysis of TYRP1 (TYRP1, 10 nm protein A gold (PAG)) in ultrathin cryosections (50 nm). TYRP1 labeling appears more abundant in both membrane and intraluminal vesicles in melanosomes within melanocytes (**d**, **f**) than in melanin granules after translocation to keratinocytes (**e**). (**g**) Quantification of protein A gold 10 nm particles per melanosome in both melanocytes and keratinocytes. The bar chart shows the decrease in the number of gold particles per melanin granule within keratinocytes (gray) versus melanocytes (black) ($n = 50$ in each case), thus demonstrating the loss of TYRP1 in melanin granules after transfer to keratinocytes. Error bars shown are ± SEM. ***$P < 0.01$. BL, basal lamina; K, keratin; Nu, nucleus; PM, plasma membrane. Bars: **a**, **b** = 1 μm; **c** = 1 μm; **d**, **f** = 100 nm.

Rab11b localizes in vesicles in close proximity to melanosomes in melanocytes

To try and gain insight into the relationship between melanosomes and Rab11b, we colocalized them. Melan-ink4a cells stained with a Rab11b-specific antibody (Lapierre *et al.*, 2003) showed a punctate localization in vesicular structures within the cytoplasm with accumulation in the perinuclear region of the cell (Figure 6). A high degree of colocalization was observed with transferrin receptor in the perinuclear region of the cell as expected for a recycling endosome marker such

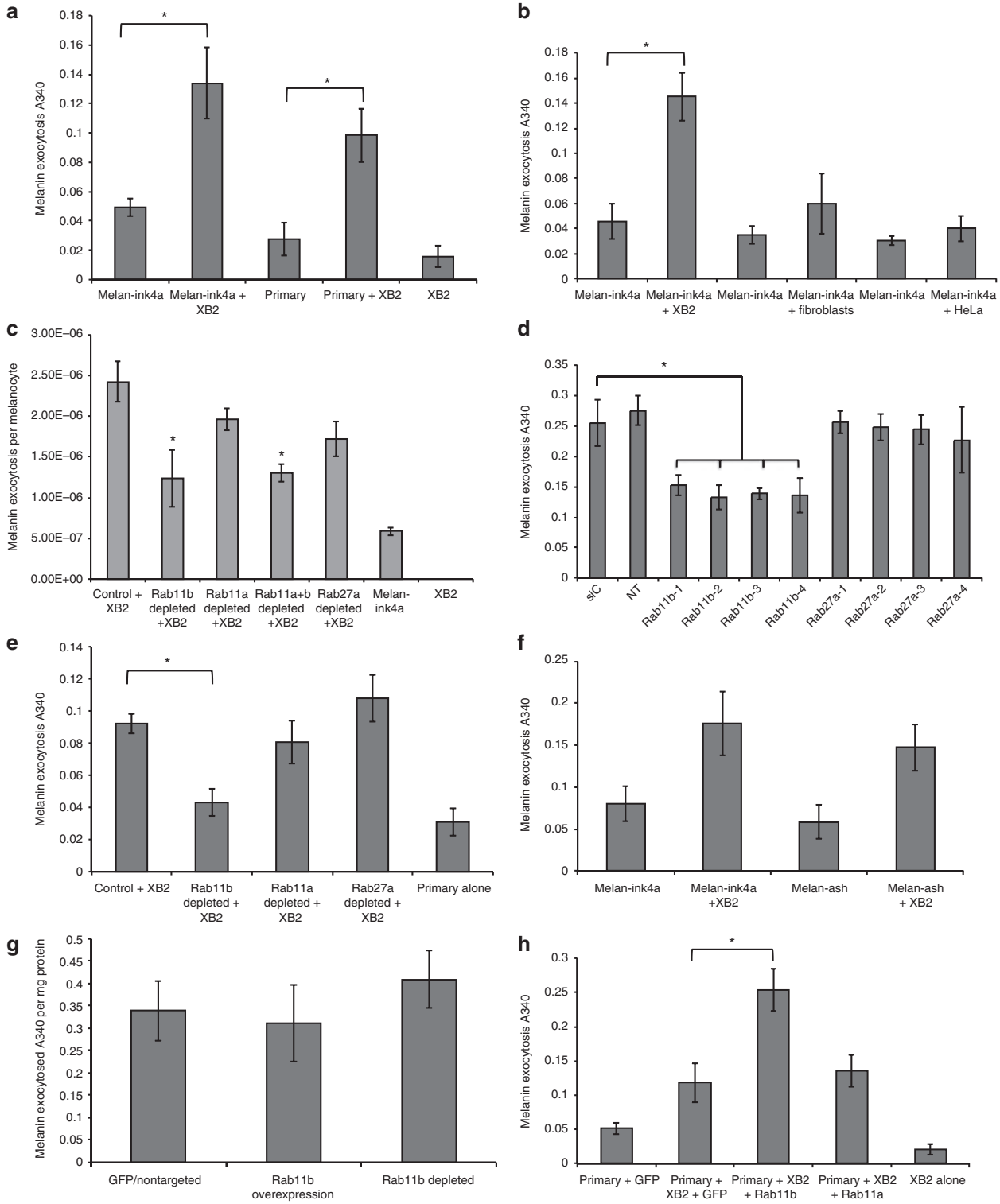

Figure 4. Melanin exocytosis is modulated by the presence of keratinocytes and Rab11b expression levels *in vitro*. Melanocytes were either co-cultured with the indicated cell type (**a**, **b**, **f**) or treated with the indicated siRNA/EGFP-Rab-expressing adenovirus and co-cultured with XB2 keratinocytes (**c**, **d**, **e**, **g**, **h**). After co-culture, media were collected, and melanin isolated and quantified by spectrophotometry. (**a**) Melan-ink4a and primary murine melanocytes (primary) cultured alone or co-cultured with XB2 keratinocytes. (**b**) Melan-ink4a melanocytes alone, co-cultured with XB2 keratinocytes, NIH-3T3 fibroblasts, or HeLa cells. (**c**, **d**) Melan-ink4a were treated with the indicated SMART pool siRNAs (**c**) or single siRNA oligonucleotides (**d**) before co-culture with XB2 keratinocytes. (**e**) Primary melanocytes treated with adenovirus expressing the indicated miRNA before co-culture with XB2 keratinocytes. (**f**) Comparison of melanin exocytosis by Melan-ink4a and Melan-*ash* alone or in co-culture with XB2 keratinocytes. (**g**) Primary murine melanocytes expressing the indicated EGFP-Rab cultured alone. (**h**) Primary murine melanocytes expressing the indicated EGFP-Rab in co-culture with XB2 keratinocytes. Error bars are ± SEM. $^*P<0.05$. EGFP, enhanced green fluorescent protein; siRNA, small interfering RNA.

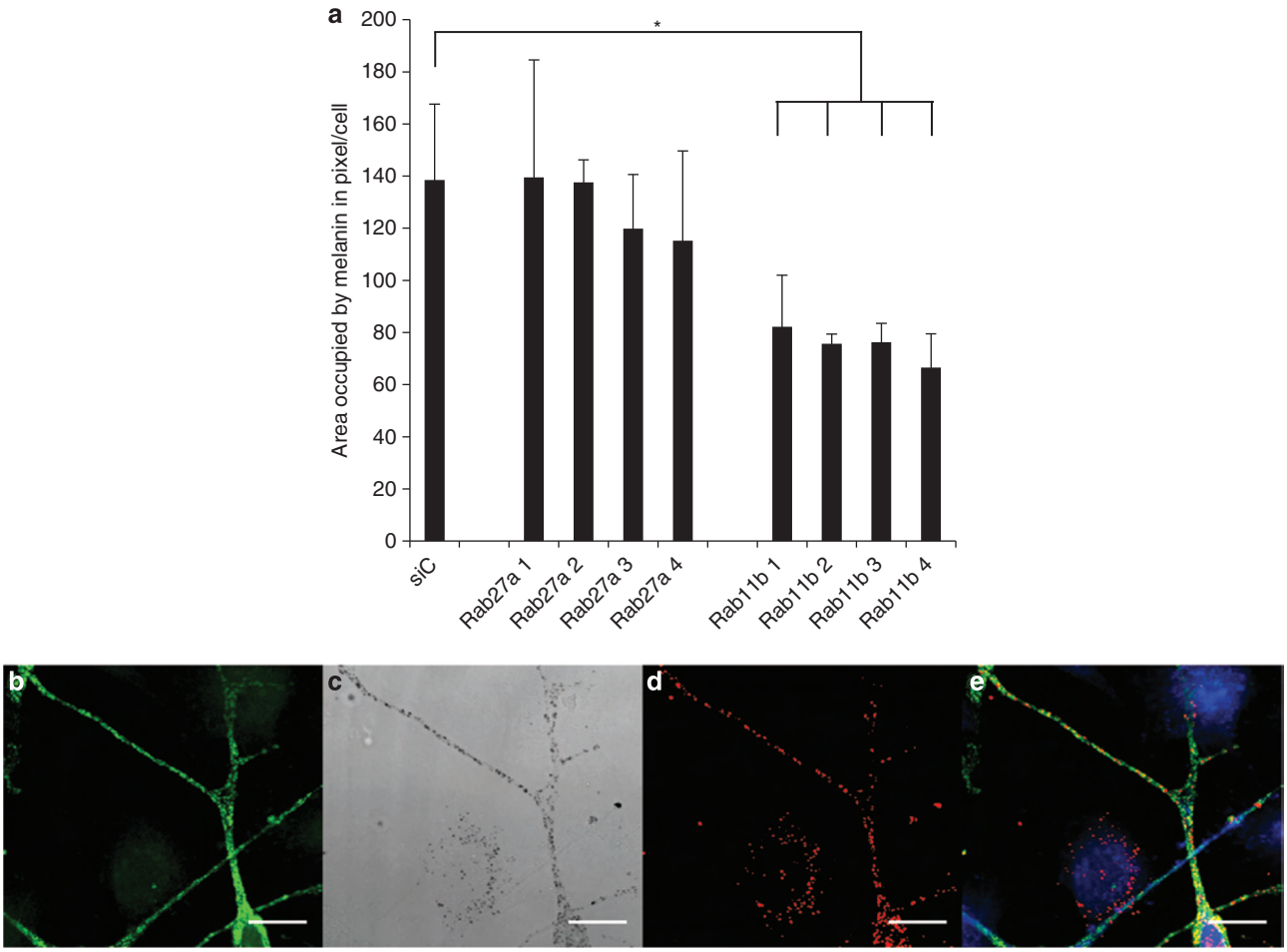

Figure 5. Depletion of Rab11b reduces melanin transfer to keratinocytes. (**a**) Melan-ink4a melanocytes were treated with the indicated small interfering RNA (siRNA) single oligonucleotides for 3.5 hours before co-culture with XB2 melanocytes. After 2 days of culture, cells were fixed and examined by brightfield microscopy. Between 30 and 40 *z*-stacks were taken for each condition and the area of keratinocytes containing melanin pigment was quantified using ImageJ software. (**b**, **c**, **d**, **e**) Melan-ink4a melanocytes were co-cultured with XB2 keratinocytes for 48 hours. Cells were fixed and immunostained for tyrosinase-related protein 1 (TYRP1) (**b**, green) before examination by confocal microscopy. (**b**) TYRP1 staining. (**c**) A brightfield image of the co-culture, where we observe a melanocyte and its dendrites full of melanin granules and an adjacent keratinocyte with internalized melanin surrounding the nucleus. Melanin granules were pseudocolored in red as seen in (**d**). (**e**) Merge with the nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI; blue) is shown. TYRP1 labels melanosomes within melanocytes but not melanin granules present in keratinocytes. Bar = 15 μm.

as Rab11b ($P = 0.806 \pm 0.029$, $n = 15$; Figure 6 i–l). A lower degree of colocalization was observed between Rab11b and pigment granules or TYRP1, although Rab11b-loaded vesicles were often seen in close proximity to melanosomes (Pearson's coefficient (P) $= 0.673 \pm 0.052$, $n = 15$; Figure 6e–h). Little colocalization was observed between Rab11b and PMEL, a marker of immature melanosomes ($P = 0.583 \pm 0.03$, $n = 6$).

DISCUSSION

The transfer of melanin from melanocytes to neighboring keratinocytes is a crucial step in skin pigmentation that forms the basis of skin photoprotection against UV damage and consequently skin cancer. Despite its importance, the precise mechanism of melanin transfer remains enigmatic. Here, using a combination of *ex vivo* and cell culture approaches, we found compelling evidence suggesting that coupled melanin exocytosis followed by endocytosis is the predominant mechanism of melanosome transfer. Furthermore, we identified Rab11b as a key regulator of melanin exocytosis and subsequent transfer to keratinocytes using cell culture models.

Morphological observation of serial ultrathin sections of human skin samples by EM analysis provided three lines of evidence to support a coupled exocytosis/endocytosis mechanism. First, naked melanin without a membrane was observed in the extracellular space between melanocytes and keratinocytes. Second, a single membrane was present at the very early stage of melanin internalization within keratinocytes. Third, loss of the melanosomal membrane after transfer was observed. The observation of a single membrane surrounding the melanin core is in contrast to other reports (Okazaki *et al.*, 1976; Yamamoto and Bhawan, 1994). These differing results can be reconciled because of the fact that in our study we used human skin samples, performed serial sections, and sectioned skin from a saggital

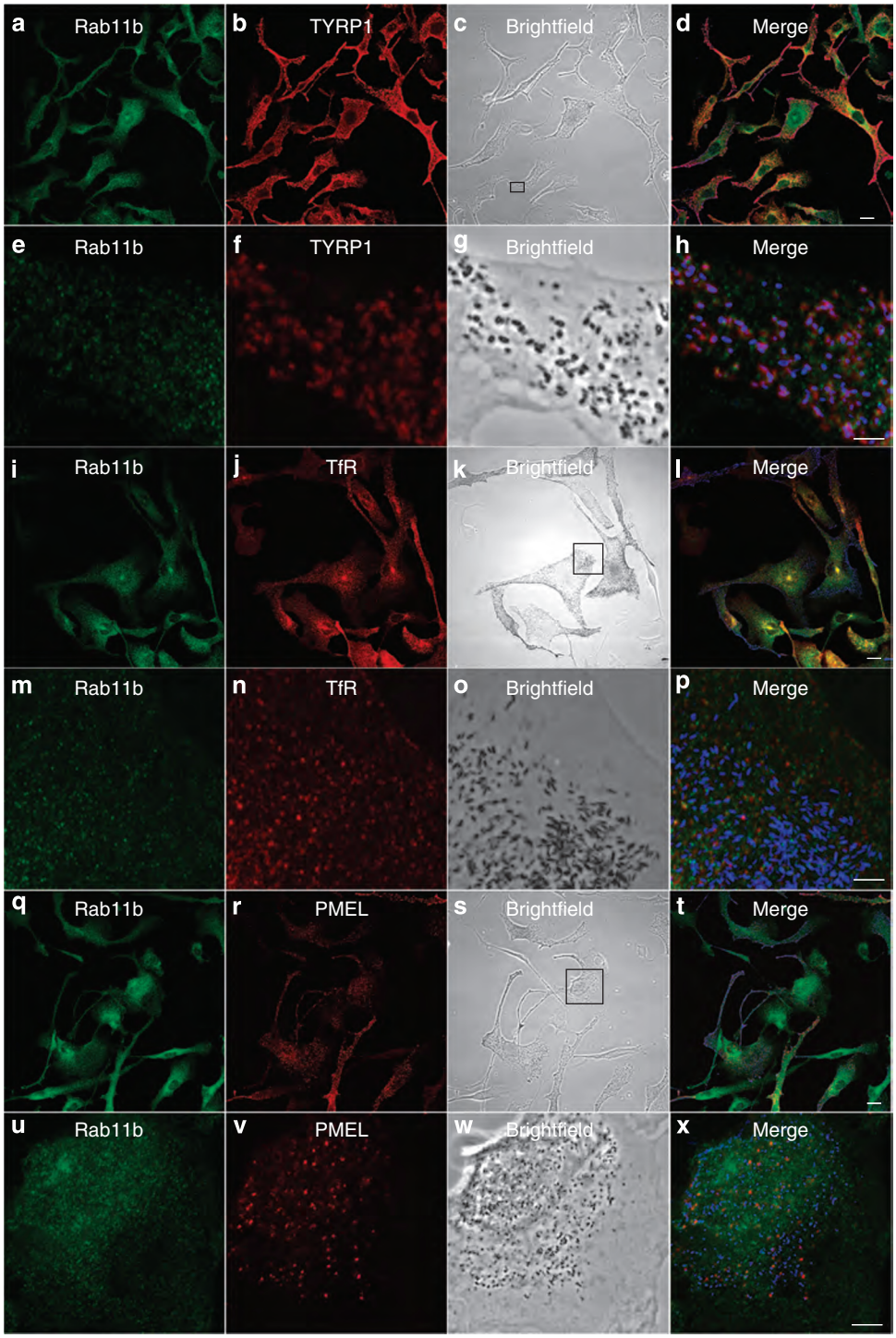

Figure 6. Rab11b does not colocalize to mature melanosomes but localizes to vesicles in close proximity to melanosomes in Melan-ink4a. Melan-ink4a cells were fixed and immunostained with the indicated antibodies before examination by confocal microscopy. (**a**, **e**, **i**, **m**, **q**, **u**) Rab11b staining, (**b**, **f**) tyrosinase-related protein 1 (TYRP1) staining, (**j**, **n**) transferrin receptor (TfR) staining, and (**r**, **v**) PMEL staining. (**c**, **g**, **k**, **o**, **s** and **w**) Brightfield images. (**d**, **h**, **l**, **p**, **t** and **x**) Merged images where melanosomes are pseudocolored in blue; Rab11b antibody staining is in green, and marker antibody staining in red. (**e–h**, **m–p**, **u–x**) Magnifications of the boxed regions indicated in **c**, **k**, and **s**. Bar = 10 μm.

plane (cross-section) that decreases the likelihood of seeing ''indentation'' of melanocytes within keratinocytes.

The presence of a single membrane within keratinocytes excludes both hetero-phagocytosis and phagocytosis of melanin-loaded vesicles as possible mechanisms for melanosome transfer. As for the two other models, endocytosis of naked melanin granules and the incorporation of membrane-bound melanosomes via direct plasma membrane fusion,

although we did not observe a single event of plasma membrane fusion between melanocytes and keratinocytes, our morphological observation did not give any indication on the nature of the membrane surrounding melanin within keratinocytes. However, we found molecular evidence for the loss of the melanosomal membrane during melanin transfer to keratinocytes as we see the absence of the specific melanosomal membrane markers TYRP1 and LAMP1 in melanin granules within keratinocytes. Importantly, this argues against melanosome transfer via a direct plasma membrane fusion mechanism, although we cannot categorically rule out the possibility that the melanosomal membrane is rapidly degraded upon internalization into keratinocytes.

To further investigate the molecular basis of this coupled exocytosis/endocytosis mechanism of melanin transfer, the role of Rab GTPases in the process was probed. Using two melanocyte cell types, melanin exocytosis into tissue culture medium was assayed. Co-culture of melanocytes with XB2 keratinocytes induced melanin exocytosis, suggesting that keratinocyte-derived signals are important for inducing this process. This is consistent with previous research indicating that keratinocyte-derived factors are important for promoting melanogenesis and melanosome transport (Yamaguchi and Hearing, 2010). Interestingly, downregulation of Rab11b, but not Rab27a, in melanocytes caused a marked decrease in both keratinocyte-induced melanin exocytosis and transfer to keratinocytes.

A role for endosomes in transporting melanosomal enzymes to maturing melanosomes has been proposed and transient fusion events between melanosomes and endosomes have been observed (Delevoye *et al.*, 2009). Immunofluorescence analysis of endogenous Rab11b in melanocytes revealed a punctate distribution throughout the cytoplasm with accumulation at the perinuclear region of the cell. Moreover, Rab11b colocalized with transferrin receptor in the perinuclear region of the cell, suggesting it is predominantly localized to recycling endosomes. Interestingly, Rab11b-positive structures were often seen in close proximity to mature melanosomes in the cell periphery, as described previously (Delevoye *et al.*, 2009).

A role for Rab11 and endosomes in exocytosis is not without precedent. Rab11b is present on mature synaptic vesicles in the brain and has been proposed to function as a switch between the constitutive and regulated exocytic pathways (Khvotchev *et al.*, 2003). In cytotoxic T cells, Rab11 has been implicated in the exocytosis of lytic granules, another example of a lysosome-related organelle (Ménager *et al.*, 2007). Given that melanocytes are derived from the neural crest (Weston, 1991) and can be considered relatives of neurons and also because of numerous links between albinism and immunity (i.e., Griscelli syndrome type II) (Stinchcombe *et al.*, 2004), it is plausible that melanocytes would use an exocytic mechanism to transfer melanin in a manner that parallels synaptic and lytic granule release, perhaps by forming a ''dermatological synapse'' with keratinocytes.

Surprisingly, knockdown of Rab27a, which leads to ~60% depletion, did not affect melanin transfer in contrast to a previous report (Yoshida-Amano *et al.*, 2012), suggesting that Rab27a levels are not limiting in this process and possibly that melanin transfer can occur at sites in the cell body as well as at peripheral dendrites, as proposed previously (Wu *et al.*, 2012). As one melanocyte may contact up to 40 different keratinocytes via its dendrites, it is possible that the role of Rab27a is to allow efficient transfer to many keratinocytes concomitantly. Indeed, this could explain the phenotype of the *ashen* mouse that displays pigment dilution, rather than complete loss of pigmentation, suggesting that some melanin transfer to keratinocytes occurs despite loss of Rab27a (Wilson *et al.*, 2000).

Hence, our data suggests the following model for melanin transfer: upon stimulation by keratinocytes, mature melanosomes undergo remodeling by peripheral Rab11b-positive recycling endosomes preparing them for secretion. After remodeling, the melanosome fuses with the melanocyte plasma membrane and exocytosis of the melanocore into the extracellular space between the melanocyte and keratinocyte occurs at sites that could be described as dermatological synapses. Subsequently, keratinocytes uptake the melanocore by endocytosis (Supplementary Figure S6 online). Alternatively, it is possible that cargo that is ultimately required for exocytosis is delivered concomitantly with melanosomal cargoes via the Rab11b-dependent recycling endosome pathway. Future studies should be directed at characterizing Rab11b-mediated melanosome remodeling and the mechanism of melanin endocytosis by keratinocytes.

MATERIALS AND METHODS

Conventional electron microscopy

Samples were fixed with a mixture of 2% (w/v) paraformaldehyde, 2% (w/v) glutaraldehyde (TAAB) in 0.1 M sodium cacodylate buffer (Agar), pH 7.4, post-fixed with 1% (w/v) OsO_4 supplemented with 1.5% (w/v) potassium ferrocyanide, dehydrated in ethanol and infiltrated with propylene oxide (Agar)/epon (Agar) (1:1), followed by Epon embedding. Ultrathin sections were cut with an Ultracut S microtome (Leica, Wetzlar, Germany), counterstained with lead citrate, and observed with a transmission electron microscope Jeol 1010 (Jeol, Tokyo, Japan). Images were obtained using a Gatan (Pleasanton, CA) ORIUS CCD camera.

Ultracryotomy and immunogold labeling

Samples were fixed with 2% (w/v) paraformaldehyde, 0.1% (w/v) in 0.1 M sodium phosphate buffer, pH 7.1. Samples were cut in 0.5 mm^3 squares, embedded in 12% gelatin, and infused in 2.3 M sucrose. Mounted gelatine blocks were frozen in N_2 and ultrathin (50 nm) cryosections were cut at −120 °C with an Ultracryo-microtome (Leica). Sections were retrieved in 1.15 M (w/v) sucrose/2% (v/v) methylcellulose solution and processed for immunolabeling. After blocking step with 0.5% (w/v) BSA, single immunolabeling was performed in a humid chamber with primary antibodies and protein A coupled to 10 nm gold particles (protein A gold, 10 nm).

Melanin exocytosis assay

Melan-ink4a melanocytes (1×10^4) were seeded onto 24-well plates. After 24 hours, cells were either transfected with siRNA or infected with adenovirus. siRNA or adenovirus containing media were removed after 3.5 and 4 hours, respectively, and XB2 media

containing 5×10^4 XB2 keratinocytes added. The following day, 200 pM cholera toxin and 200 nM phorbal myristate acetate were added to the media and co-cultures were incubated for 7 days. Media containing exocytosed melanin was centrifuged at 800 *g* for 5 minutes at 4 °C to pellet cell debris. The supernatant was then centrifuged at 20,000 *g* for 1 hour at 4 °C to pellet melanin. Melanin pellets were washed with ethanol/ether (1:1 v/v) and dissolved in 2 M NaOH/20% DMSO at 60 °C for 1 hour. Melanin content was measured as optical density at 340 nm. For melanocyte counting, a parallel experiment was performed with the same initial cell densities and the same silencing conditions. Total cell number was counted at the end of the assay period and the proportion of melanocytes determined from the cells co-cultured on coverslips by immunofluorescence, using an anti-Tyrp1 (TA99) antibody to specifically stain melanocytes.

Melanin transfer assay

Melan-ink4a melanocytes (2×10^4) were seeded on coverslips on 24-well plates. After 24 hours, siRNA transfection was performed and XB2 cells (1×10^5) in XB2 growth media were added when changing the siRNA-containing media. Then, 200 pM cholera toxin and 200 nM phorbal myristate acetate were added the following day. Cells were co-cultured for 48 hours. Co-cultures were washed 3 × with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Cells were washed 3 × in PBS and the nucleus visualized by incubation with 4′,6-diamidino-2-phenylindole for 5 minutes. Images were taken in a Nikon Eclipse TE2000-S screening microscope (Nikon, Tokyo, Japan) with the same acquisition settings. To measure melanin uptake, the ImageJ (NIH, Bethesda, MD) threshold command was applied and correspondent intensity (in pixel) was measured automatically. Nuclei were counted to ensure similar cell confluency in all samples and to calculate the amount of melanin internalized per cell. Melanin uptake reflects the total amount of melanin internalized by XB2 cells in one coverslip.

Immunoflourescence analysis of cells

Cells grown on coverslips for immunofluorescence were fixed for 15 minutes in 4% paraformaldehyde in PBS for 24 hours. Excess fixative was removed by extensive washing in PBS and quenched by incubation in 50 mM NH4Cl for 10 minutes. Fixed cells were then incubated with diluted primary antibody for 30 minutes, washed extensively, incubated for 30 minutes with appropriate Alexa 568-conjugated secondary antibodies (Molecular Probes, Eugene, OR), washed as before, and mounted in ImmunoFluor medium (ICN, Eschwege, Germany). All antibody incubations and washes used 1 × PBS, 0.5% BSA, and 0.05% saponin. Cells were observed using a Leica SP5 confocal microscope, and images were processed using ImageJ and Adobe Photoshop 5.0 software (Adobe, San Jose, CA). All images presented are single sections in the *z*-plane.

Supplementary methods

For primer sequences and other methods, refer to Supplementary Materials and Methods online.

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 http://www.nature.com/jid

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