# Melanin Transferred to Keratinocytes Resides in Nondegradative Endocytic Compartments

Maria S. Correia[1,2], Hugo Moreiras[1,2], Francisco J.C. Pereira[1,3], Matilde V. Neto[1,3], Tiago C. Festas[1], Abul K. Tarafder[1,4], José S. Ramalho[1], Miguel C. Seabra[1] and Duarte C. Barral[1]

Melanin transfer from melanocytes to keratinocytes and subsequent accumulation in the supranuclear region is a critical process in skin pigmentation and protection against UVR. We have previously proposed that the main mode of transfer between melanocytes and keratinocytes is through exo/endocytosis of the melanosome core, termed melanocore. In this study, we developed an in vitro uptake assay using melanocores secreted by melanocytes. We show that the uptake of melanocores, but not melanosomes, by keratinocytes is protease-activated receptor-2–dependent. Furthermore, we found that the silencing of the early endocytic regulator Rab5b, but not the late endocytic regulators Rab7a or Rab9a, significantly impairs melanocore uptake by keratinocytes. After uptake, we observed that melanin accumulates in compartments that are positive for both early and late endocytic markers. We found that melanin does not localize to either highly degradative or acidic organelles, as assessed by LysoTracker and DQ-BSA staining, despite the abundance of these types of organelles within keratinocytes. Therefore, we propose that melanocore uptake leads to storage of melanin within keratinocytes in hybrid endocytic compartments that are not highly acidic or degradative. By avoiding lysosomal degradation, these specialized endosomes may allow melanin to persist within keratinocytes for long periods.

*Journal of Investigative Dermatology* (2018) **138,** 637–646; doi:10.1016/j.jid.2017.09.042

## INTRODUCTION

Skin pigmentation is achieved through the synthesis of the pigment melanin in specialized membrane-bound organelles termed melanosomes, followed by subsequent transfer of these organelles from melanocytes to surrounding keratinocytes (Marks and Seabra, 2001). After melanin is internalized by keratinocytes, it forms a supranuclear cap, which acts as a natural sunscreen and protects the genetic material by absorbing and scattering UVR (Boissy, 2003; Byers et al., 2003).

The molecular processes involved in the biogenesis and transport of melanin inside melanocytes have been the subject of intense study in recent years. However, the molecular mechanisms underlying intercellular transfer of pigment from donor melanocytes and pigment processing within acceptor keratinocytes remain poorly understood (Van Den Bossche et al., 2006). Several models for the transfer mechanism have been proposed, and two of them have received more attention recently: the shedding of melanosome-loaded vesicles by melanocytes that are subsequently phagocytosed by keratinocytes and the exocytosis by melanocytes of melanosome cores or melanocores, which are then endo/phagocytosed by keratinocytes (Wu and Hammer, 2014). A recent study using chicken embryonic skin found evidence for the shedding/phagocytosis model (Tadokoro et al., 2016). On the other hand, we proposed that coupled exo/endocytosis of melanocores is the prevalent mechanism of melanin transfer present in human skin (Tarafder et al., 2014). Furthermore, several studies suggest that the transfer process is regulated by the protease-activated receptor (PAR)-2, a keratinocyte receptor that controls the uptake of melanin (Seiberg, 2001).

Melanosomes within melanocytes are considered lysosome-related organelles (Raposo and Marks, 2002). Lysosome-related organelles are a group of cell-type-specific subcellular compartments with unique composition, morphology, and structure that share features with endosomes and lysosomes, such as an acidic pH and the presence of late endosome/lysosome markers. Presently, the nature of the compartment where melanin resides within keratinocytes remains elusive (Boissy, 2003; Borovanský and Elleder, 2003; Wolff, 1973). Melanin is thought to be retained in human skin, only disappearing on keratinocyte terminal differentiation, in a process that has not been characterized (Boissy, 2003; Hori et al., 1968). Previous studies reported that melanin within keratinocytes is contained in lysosomal

[1]*CEDOC, Chronic Diseases Research Centre, NOVA Medical School|Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Lisbon, Portugal*

[2]*These authors contributed equally to this work.*

[3]*These authors contributed equally to this work.*

[4]*Present address: Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK*

*Correspondence: Duarte C. Barral or Miguel C. Seabra, CEDOC, NOVA Medical School, Universidade NOVA de Lisboa, Campo dos Mártires da Pátria 130, 1169-056, Lisboa, Portugal. E-mail: duarte.barral@nms.unl.pt (D.C. Barral) or miguel.seabra@nms.unl.pt (M.C. Seabra)*

*Abbreviations: LAMP; lysosomal-associated membrane protein; PAR; protease-activated receptor; PBS; phosphate buffered saline; siRNA; small interfering RNA*

*Received 12 January 2017; revised 11 September 2017; accepted 11 September 2017; accepted manuscript published online 24 October 2017; corrected proof published online 1 February 2018*

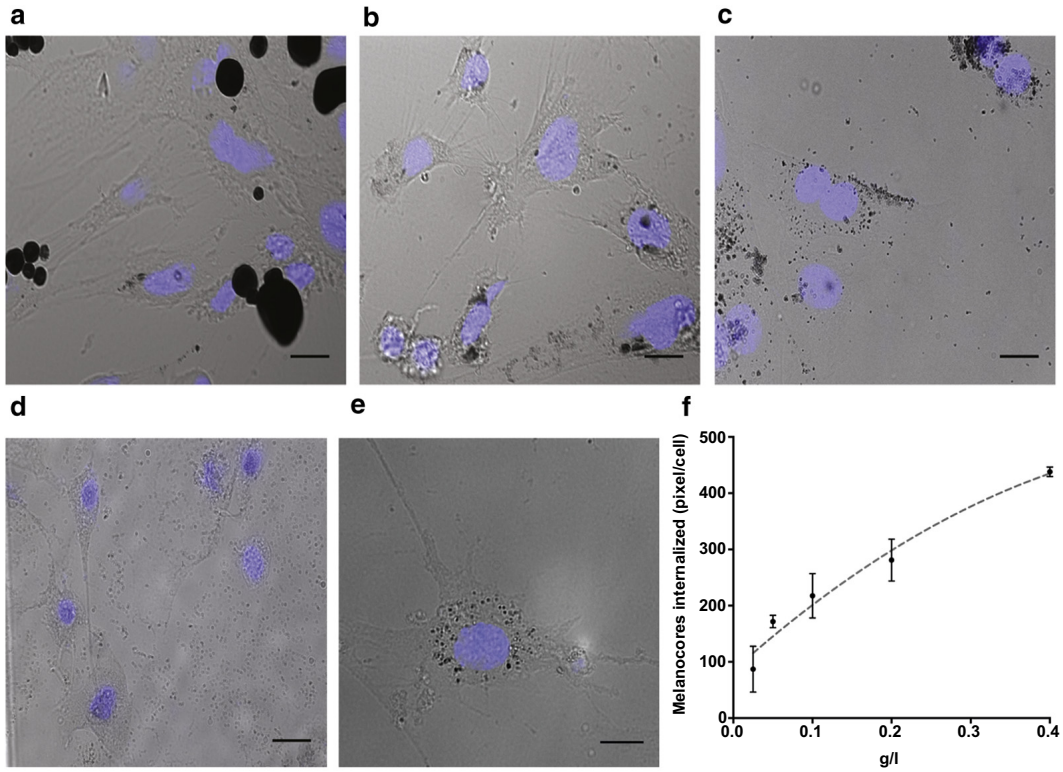


**Figure 1. Melanocores are efficiently internalized by keratinocytes.** XB2 keratinocytes were incubated for 24 hours with various types of melanin: (**a**) natural melanin from *Sepia officinalis*; (**b**) synthetic melanin; (**c**) melanosomes isolated from MNT-1 cells; (**d**) melanocores from MNT-1 conditioned medium centrifuged on a Percoll gradient; or (**e**) melanocores from MNT-1 conditioned medium concentrated by vivaspin. Cells were fixed and analyzed by brightfield/fluorescence microscopy. Nuclei were stained with DAPI. Scale bars = 20 μm. (**f**) XB2 keratinocytes were incubated with increasing concentrations of melanocores (0–0.4 g/l) for 24 hours. Cells were fixed and examined by brightfield microscopy.

compartments (Borovanský and Elleder, 2003; Thong et al., 2003; Wolff, 1973). Recently, autophagy was reported to regulate melanosome degradation in keratinocytes, as melanin levels in human skin cultures are reduced by activators of autophagy and enhanced by its inhibitors (Murase et al., 2013).

Rab small GTPases are involved in multiple regulatory processes in all membrane trafficking pathways and are highly compartmentalized (Hutagalung and Novick, 2011; Seabra et al., 2002), serving as organelle identifiers. The canonical endocytic pathway is regulated by Rab5 isoforms in the early stages and Rab7 in late stages (Cantalupo et al., 2001; Hutagalung and Novick, 2011; Zerial and McBride, 2001). Moreover, Rab9 regulates transport between late endosomes and the *trans*-Golgi network (Soldati et al., 1994). In skin cells, several Rab proteins such as Rab32/38 and Rab27a regulate different steps of melanosome biology, including melanosome biogenesis, transport, and tethering to the cortical actin (Marks et al., 2013; Wasmeier et al., 2006).

Here, we report the development of an in vitro model system to examine the uptake and processing of melanin by keratinocytes. Using this assay, we show that the uptake of melanocores, but not melanosomes, is dependent on PAR-2. We also found that Rab5b silencing impairs melanocore uptake by keratinocytes, whereas the silencing of Rab7a or Rab9a does not. Importantly, melanin rapidly accumulates and is maintained for a long period within keratinocytes, in an endocytic compartment that is neither highly degradative nor acidic.

## RESULTS

### Establishment of an in vitro melanocore uptake assay

To characterize the uptake and processing of melanin by keratinocytes, we first developed a suitable melanin internalization assay. There are several types of melanin commercially available (melanin from *Sepia officinalis* and synthetic melanin) and also several protocols to extract and purify melanosomes from melanocytes. To evaluate the ability of keratinocytes to uptake melanin, XB2 keratinocytes were incubated for 24 hours with each type of melanin (Supplementary Table S1 online), and internalization was examined by brightfield microscopy. We observed that both natural and synthetic melanin form large lumps and are internalized as big aggregates, which do not appear to be physiological (Figure 1a and b). Melanosomes isolated from MNT-1 cells by serial centrifugations and Percoll gradient are also internalized as aggregates for the most part and also appear to be very sticky, as they were not easily washed away from the coverslip (Figure 1c). According to our model, keratinocytes take up melanocores, that is, the core of melanosomes secreted by melanocytes, lacking the limiting membrane. Contrary to wild-type melanocytes, we found that the melanoma cell line MNT-1 constitutively secretes melanocores to the medium. Therefore, we also treated XB2 keratinocytes with melanocores isolated by centrifugation from MNT-1 conditioned medium (Figure 1d). However, these do not seem to be well internalized by keratinocytes and strongly stick to surfaces. Conversely, melanocores isolated from MNT-1 conditioned

medium by vivaspin concentration are efficiently internalized by keratinocytes and do not stick to surfaces, allowing the removal of noninternalized melanocores (Figure 1e).

Using the latter method, we performed titration and kinetic studies of melanocore uptake by XB2 keratinocytes. For this, cells were fed with increasing concentrations of melanocores (0.025−0.4 g/l) and internalization by keratinocytes was measured after 24 hours by brightfield microscopy analysis. We found that XB2 keratinocytes are able to uptake melanocores linearly, up to a concentration of 0.1 g/l (Figure 1f). At higher concentrations, the morphology of the cells is compromised and the cell membranes are disrupted. Therefore, a concentration of 0.1 g/l was used for subsequent assays.

**Silencing of PAR-2 or Rab5b, but not Rab7a or Rab9a, impairs the uptake of melanocores by XB2 keratinocytes**

A crucial role for PAR-2 in mediating melanin uptake by keratinocytes has been established (Marthinuss et al., 1995; Santulli et al., 1995). Known PAR-2 activators include trypsin, mast cell tryptase, and the synthetic peptides SLIGRL and SLIGKV. Moreover, PAR-2 stimulation was shown to increase melanin uptake by keratinocytes, even in the absence of melanocytes, as well as melanosome transfer (Seiberg et al., 2000). Hence, we decided to evaluate the contribution of PAR-2 for the uptake of melanocores. For this, XB2 keratinocytes were either transfected with small interfering RNA (siRNA) targeting PAR-2 or a nontargeting siRNA control, before being incubated for 24 hours with melanocores. Internalization was stopped after 24 hours by extensive washing and fixation, and intracellular melanin was measured by brightfield microscopy using an Image J macro developed for the purpose. As shown in Figure 2a, PAR-2 silencing significantly impairs melanocore uptake (by approximately 82%). The mRNA levels of PAR-2 were analyzed by qRT-PCR 48 hours after transfection, confirming the silencing efficiency (approximately 48%) (Supplementary Figure S1 online). Some transfer models postulate that intact melanosomes are internalized by keratinocytes. Therefore, we probed for the PAR-2 dependence of melanosome uptake. Strikingly, we observed that PAR-2 silencing does not significantly affect the internalization of melanosomes by XB2 keratinocytes (Figure 2b). We also investigated the effect on melanocore uptake of activating PAR-2 with SLIGRL peptide. We observed that keratinocytes internalize significantly more melanocores when treated with SLIGRL (approximately 172%), as compared with a control peptide (SFLLRN) (Figure 2c). Conversely, the activation of PAR-2 with SLIGRL peptide does not affect melanosome internalization (Figure 2d). These results support the involvement of PAR-2 in melanocore uptake by keratinocytes, validating the specificity of our in vitro assay and suggesting that it is physiologically relevant.

Using the assay developed by us to assess the uptake of melanocores by XB2 keratinocytes, we decided to explore whether melanocores traffic through the endocytic pathway on internalization. Therefore, we probed the role of endocytic Rab proteins, namely early endosomal Rab5a, Rab5b, and Rab5c and late endosomal Rab7a and Rab9a. Briefly, siRNA pools targeting Rab5a, Rab5b, Rab5c, Rab7a, or Rab9a were incubated with XB2 keratinocytes for 48 hours, as described above. In every experiment, three controls were performed: a scrambled siRNA as a negative control, PAR-2 silencing to decrease the uptake of melanocores, and treatment with SLIGRL to increase melanocore uptake. The mRNA levels of the Rab GTPases were quantified by qRT-PCR, confirming the efficiency of the silencing (approximately 60%) (Figure S2a). We found that the silencing of Rab5 isoforms significantly impairs the uptake of melanocores by keratinocytes, suggesting that Rab5 is required for melanocore uptake (Figure 2e). To show the specificity of this effect, we silenced Rab5b using micro RNA (miR) and we were able to confirm the impairment of melanocore uptake (Figure 2f), on efficient silencing of Rab5b (Figure S2b). In contrast, silencing of Rab7a or Rab9a has no significant effect on melanocore internalization (Figure 2e) (Mascia et al., 2016).

**Melanocores persist for at least 7 days in XB2 keratinocytes**

The absence of an effect of the silencing of Rab7a or Rab9a in melanocore internalization could be explained if melanocores escape the canonical endocytic pathway and do not fuse with lysosomes. This would imply that melanin persists inside keratinocytes without being degraded. To test this hypothesis, we evaluated the degradation of melanin within XB2 keratinocytes. For this, we fed the cells with melanocores and intracellular melanin was measured every 4 hours, over a time course of 48 hours (Figure 3a) and daily for a period of 7 days (Figure 3b). We observed that melanin within XB2 keratinocytes reaches saturation after 16−20 hours and after that time the amount of intracellular melanin remains stable and the melanin clusters in the perinuclear area (Figure 3c), until at least 7 days. Thus, melanin can persist inside keratinocytes for a long period of time.

**XB2 keratinocytes express significant levels of late endosome/lysosome markers and have abundant acidic and degradative compartments**

The persistence of melanocores within keratinocytes for long periods could also be explained if XB2 keratinocytes have a poor degradative capacity. To explore this possibility, we first evaluated the expression of late endosome and lysosome markers, namely lysosomal-associated membrane protein 2 (LAMP2) and Cathepsin D, in these cells. For this, we quantified the amount of LAMP2 and Cathepsin D mRNA in XB2 keratinocytes and 3T3 mouse fibroblasts by qRT-PCR. We observed that keratinocytes express significant amounts of LAMP2 and Cathepsin D, 40−60% higher than fibroblasts (Figure 4a). Moreover, we used DQ-BSA and LysoTracker to examine the presence of degradative and acidic compartments, respectively, within XB2 keratinocytes. LysoTracker freely permeates cell membranes and selectively accumulates in intracellular compartments with a low internal pH, whereas DQ-BSA is a fluid-phase marker that fluoresces in a degradative environment. Therefore, we incubated XB2 keratinocytes with 10 μg/ml of DQ-BSA or 50 nM LysoTracker for 2 hours. Before incubation of keratinocytes with these markers, the cells were also incubated with 25 mM $NH_4Cl$ in growth medium to inhibit vesicle acidification. The amount of fluorescence was then quantified by microscopy. We observed that XB2 keratinocytes possess degradative and

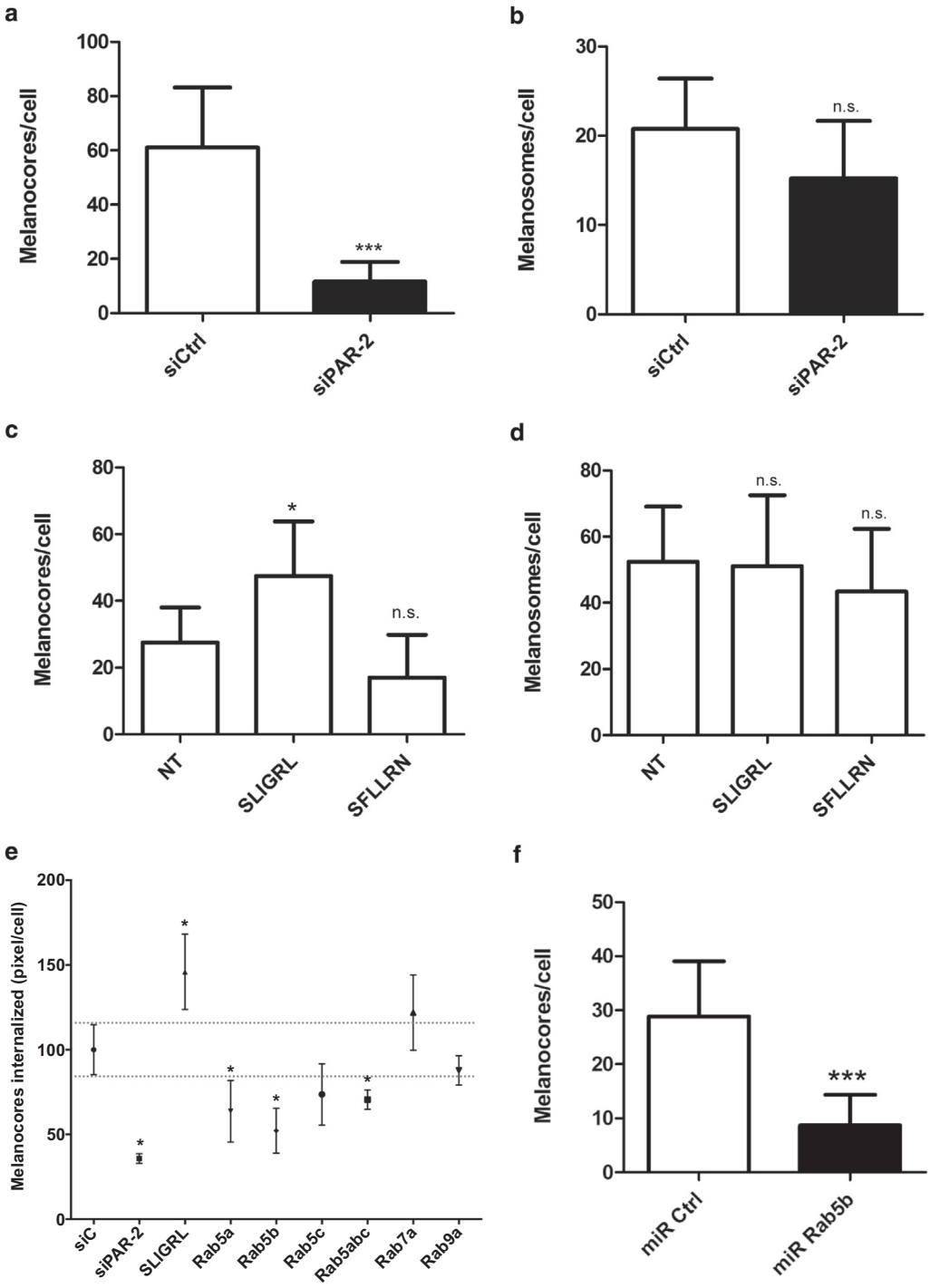


**Figure 2. Silencing of PAR-2 or Rab5b, but not Rab7a or Rab9a, significantly impairs the uptake of melanocores by keratinocytes.** (**a, b**) XB2 keratinocytes were treated with nontargeting siRNA (siCtrl) or siRNA targeting PAR-2 (siPAR-2) for 2 days. Then, XB2 cells were incubated with 0.1 g/l of (**a**) melanocores or (**b**) melanosomes for 24 hours. (**c, d**) XB2 keratinocytes were treated with 30 μM SLIGRL or SFLLRN for 2 days. Then, XB2 cells were incubated with 0.1 g/l of (**c**) melanocores or (**d**) melanosomes for 24 hours. (**e**) The specified Rab proteins were silenced by siRNA in XB2 keratinocytes, and cells were incubated with melanocores for 24 hours. Values were normalized to the value of the mean of nontargeting siControl. All cells were fixed 24 hours later and examined by brightfield microscopy. (**f**) XB2 keratinocytes were transduced with lentiviruses encoding nontargeting miRNA (miR Ctrl) or miRNA targeting Rab5b (miR Rab5b). Then, XB2 cells were incubated with 0.1 g/l of melanocores for 24 hours. *$P < 0.05$; ***$P < 0.0001$. Statistical significance was calculated using unpaired *t*-test; miRNA, micro RNA; n.s., nonsignificant; PAR-2, protease-activated receptor 2; siRNA, small interfering RNA.

acidic compartments in abundance (Figure 4b and c), suggesting that a low degradative capacity of keratinocytes is not likely to explain the persistence of melanin. As expected, incubation with $NH_4Cl$ prevented vesicle acidification and led to a significant reduction of LysoTracker and DQ-BSA staining in both cell types.

### Melanocores reside in compartments that are positive for early and late endosome/lysosome markers

Next, we characterized the compartment where the internalized melanocores are stored within keratinocytes, by performing colocalization studies with markers of the endocytic pathway. Therefore, we stained XB2 keratinocytes with antibodies for early endosome antigen 1 (EEA1) and Rab5, which mark early endosomes; transferrin, which marks early and recycling endosomes; LAMP2, a marker of late endosomes/lysosomes; and CD63, a marker of late endosomes/multivesicular bodies and also lysosome-related organelles (Chen et al., 1985; Huynh et al., 2007; Marks et al., 2013). For this, XB2 cells were fed for 2 hours with melanocores, chased for 4 hours, and then fixed and incubated with the referred antibodies. Interestingly, EEA1 and Rab5 could be observed surrounding melanocores (Figure 5a and b, respectively), suggesting that melanocores follow the early

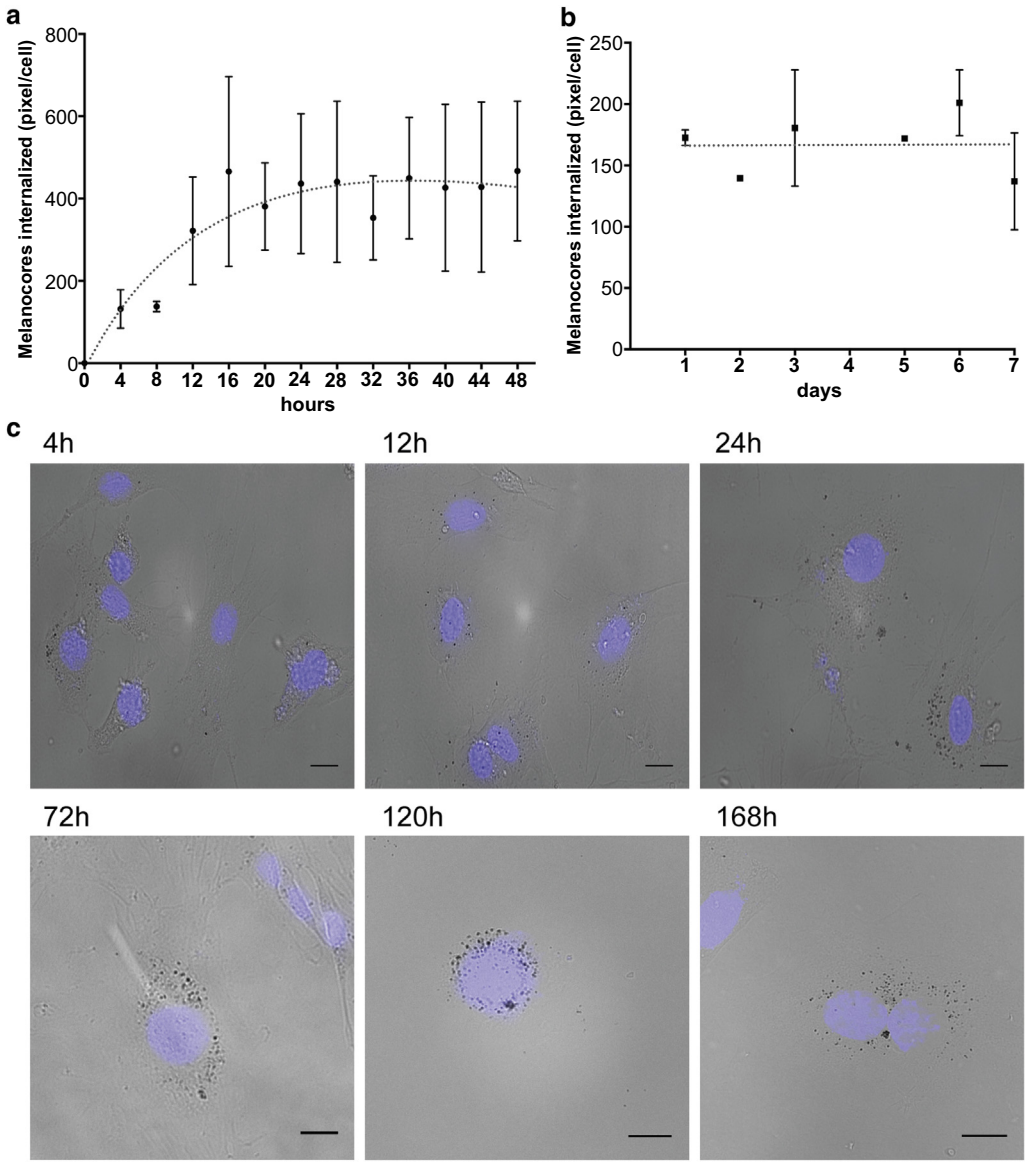


**Figure 3. Melanocores persist within keratinocytes for 7 days.** XB2 keratinocytes were incubated with 0.1 g/l of melanocores for different times. Uptake of melanocores by keratinocytes was assessed (**a**) every 4 hours, over 48 hours (dotted line represents the nonlinear fit), or (**b**) every day for 7 days (dotted line represents linear regression). Cells were fixed and analyzed by brightfield microscopy. (**c**) XB2 keratinocytes were incubated with 0.1 g/l of melanocores for increasing amounts of time, from 4 hours up to 168 hours (7 days). Cells were fixed and analyzed by brightfield microscopy. Nuclei were stained with DAPI. Scale bars = 15 μm.

endocytic pathway. Moreover, transferrin was also found surrounding melanocores, confirming their association with the early endocytic pathway (Figure 5c). These observations are consistent with the impairment of melanocore uptake on Rab5b silencing. Furthermore, melanocores could be often observed surrounded by LAMP2 and CD63 (Figure 5d and e, respectively), suggesting that melanocores accumulate in late endocytic compartments within keratinocytes. The quantification displayed in Figure 5f shows that around 50% of melanocores overlap with markers of late endosomes/lysosomes, whereas around 20–30% are surrounded by markers of early endosomes. Similar results were obtained immediately after the 2-hour pulse with melanocores (data not shown), suggesting that the accumulation in these specialized endosomes is rapid.

### Melanocores do not reside in highly acidic or degradative compartments

To further characterize the compartment where internalized melanocores reside, we stained acidic and degradative compartments with LysoTracker and DQ-BSA, respectively. Late endocytic/lysosomal compartments present these two characteristics: they are acidic and degradative. Therefore, XB2 keratinocytes were fed with melanocores, followed by incubation with 50 nM LysoTracker-Red or 10 μg/ml DQ-BSA-Red, for 2 hours and then washed and chased for 4 hours. Strikingly, we observed that LysoTracker and DQ-BSA exhibit only minor colocalization with melanocores (Figure 6a and b). The percentage of overlap between melanocores and both markers was quantified, and we observed that approximately 20% melanocores are associated with LysoTracker or DQ-BSA-positive vesicles (Figure 6c). Altogether, these data suggest that melanocores reside in a compartment that does not highly acidify or has a high degradative capacity, despite the presence of late endocytic markers.

## DISCUSSION

The transfer of melanin from melanocytes to keratinocytes and its processing within keratinocytes are crucial processes

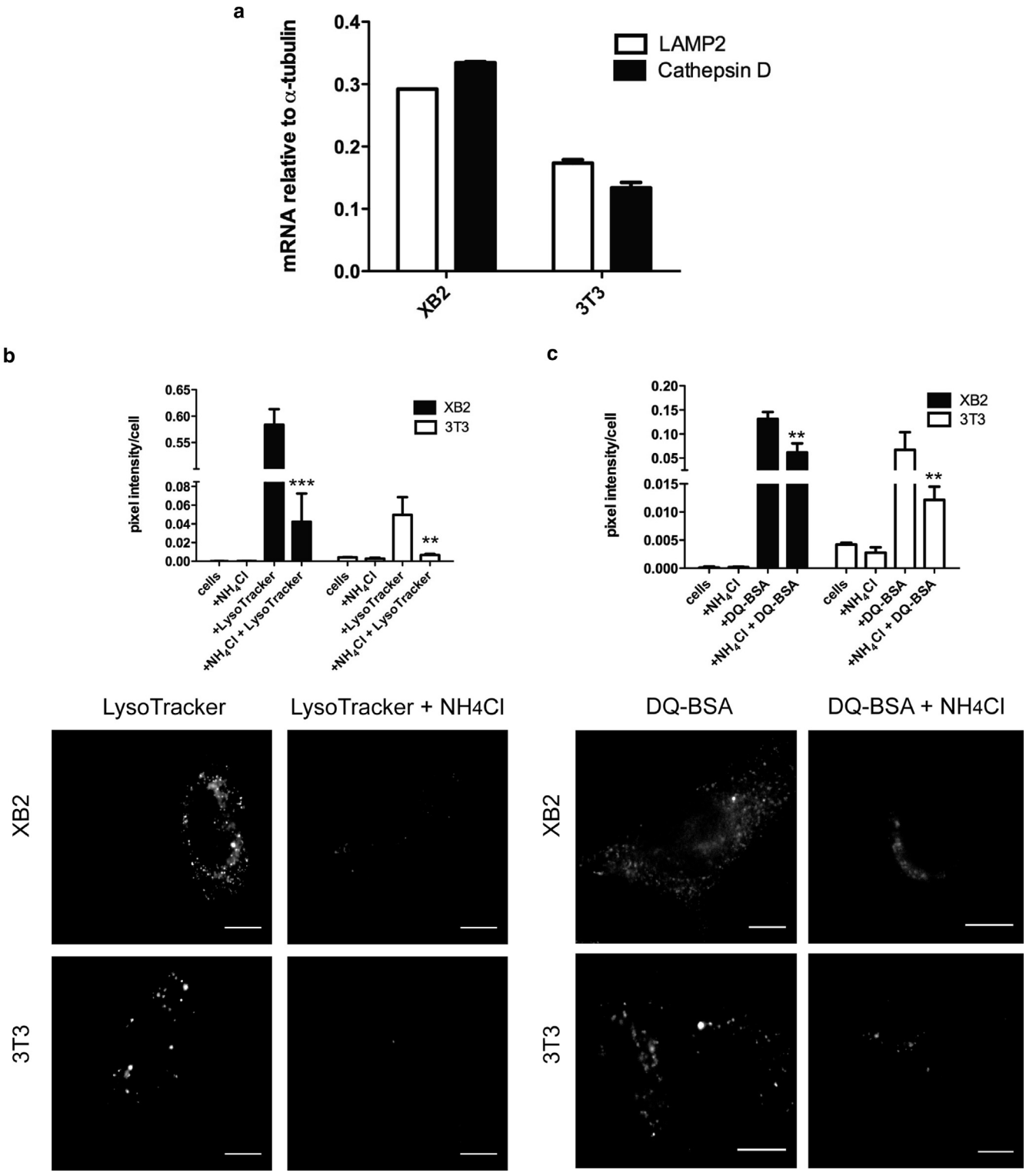


**Figure 4. Keratinocytes express significant levels of late endosome/lysosome markers and acidic and degradative markers.** (**a**) XB2 keratinocytes and 3T3 fibroblasts were cultured for 3 days, and the levels of LAMP2 and Cathepsin D mRNA were measured by qRT-PCR. (**b, c**) XB2 and 3T3 cells were incubated for 2 hours with (**b**) LysoTracker or (**c**) DQ-BSA. Before incubation with the fluorescent markers, controls were incubated with $NH_4Cl$ for 10 minutes. Cells were washed and fixed for immunofluorescence. For the quantification, the Image J threshold command was applied and the corresponding intensity (in pixels) was measured. Nuclei were counted to ensure similar cell confluence in all samples and the amount of fluorescence per cell was calculated. Results are displayed as pixel intensity per cell. Representative images of both conditions are shown. $^{**}P < 0.001$; $^{***}P < 0.0001$. Statistical significance was calculated using unpaired *t*-test. Scale bars = 10 μm. LAMP2, lysosomal-associated membrane protein 2.

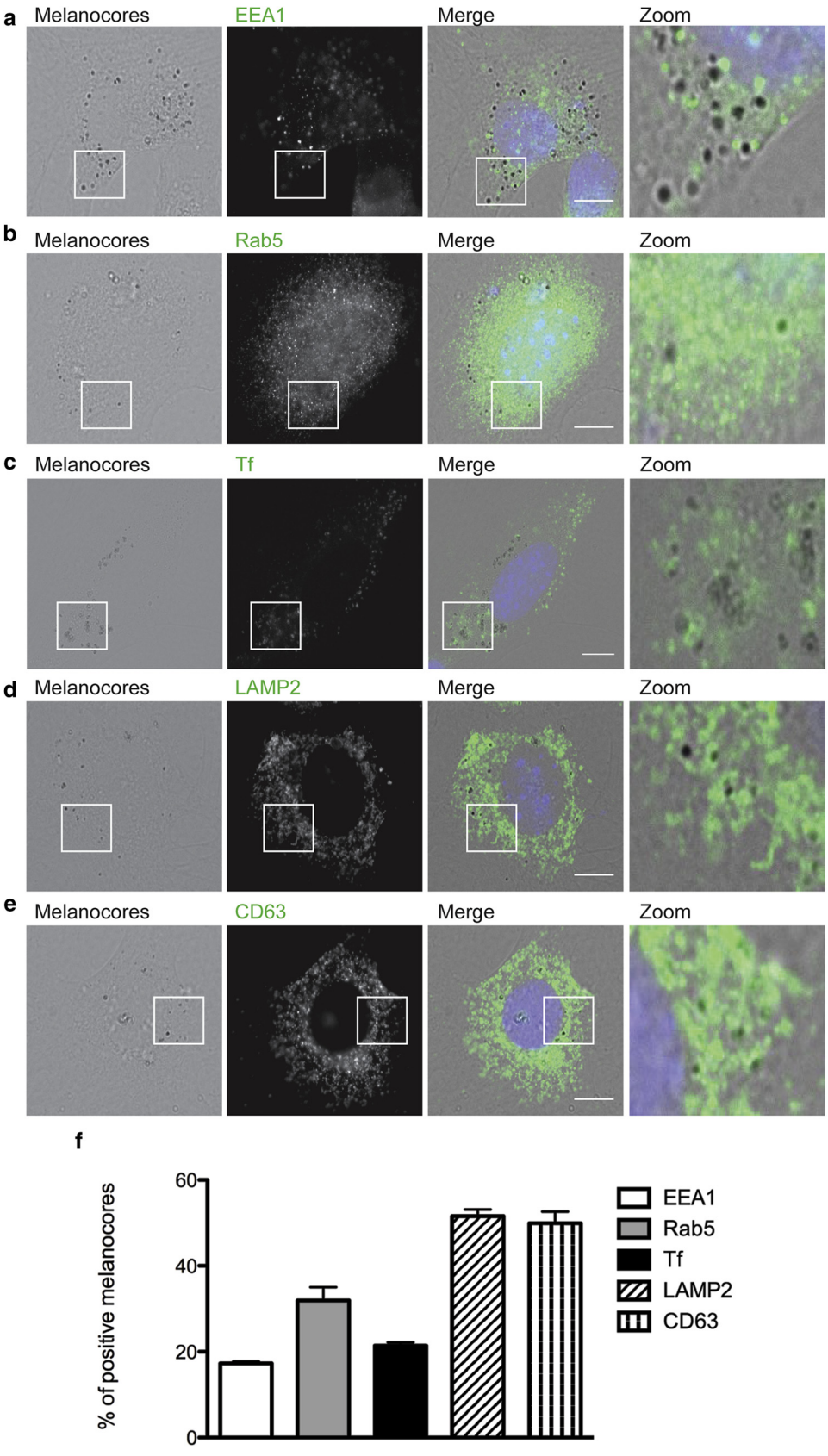


**Figure 5. Melanocores are surrounded by early and late endosomes/lysosomes inside keratinocytes.** XB2 cells were preincubated with melanocores for 2 hours and fixed after a chase of 4 hours. Cells were immunostained with antibodies for (**a**) EEA1, (**b**) Rab5, (**d**) LAMP2, or (**e**) CD63. (**c**) Alexa Fluor 488-conjugated Tf was incubated at the same time of melanocores. Nuclei were visualized by DAPI staining (blue). Scale bars = 10 μm. (**f**) Plot represents the percentage of stained melanocores for each marker. EEA1, early endosome antigen 1; LAMP2, lysosomal-associated membrane protein 2; Tf, transferrin.

underlying skin pigmentation and photoprotection against UVR-induced damage. Despite its importance, the precise molecular mechanisms of melanin transfer and maintenance within recipient keratinocytes remain controversial. Our previous work led us to propose that melanin transfer occurs through coupled exocytosis of the melanosome core by melanocytes

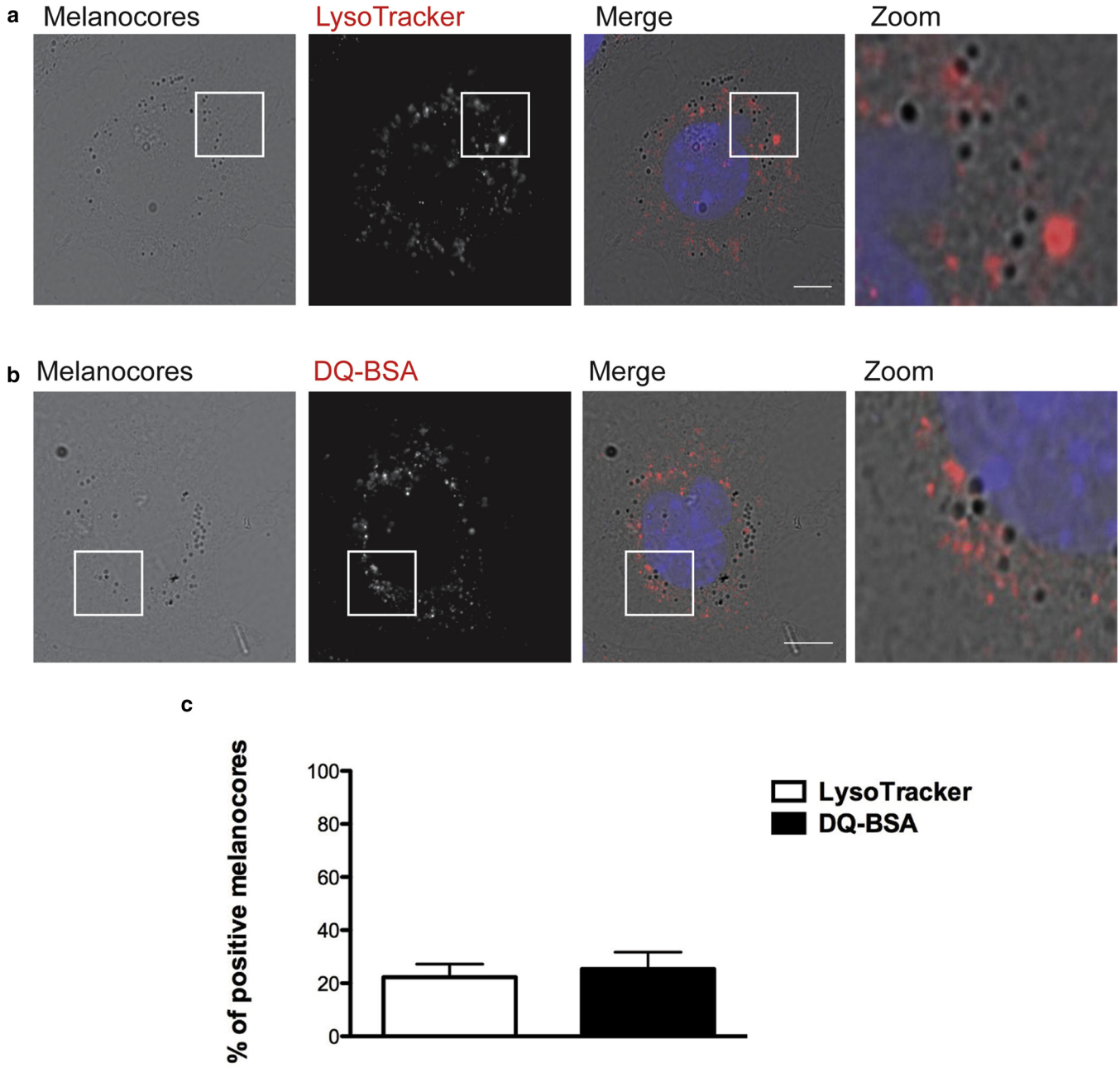


**Figure 6. Melanocores internalized by keratinocytes do not reside in highly acidic or degradative compartments.** XB2 cells were incubated with melanocores and (**a**) LysoTracker or (**b**) DQ-BSA for 2 hours and fixed after a chase of 4 hours. Nuclei were visualized by DAPI staining (blue). Scale bars = 10 μm. (**c**) Plot represents the percentage of stained melanocores for each marker.

and subsequent endo/phagocytosis by keratinocytes (Tarafder et al., 2014). Here, we established an assay that reproduces this mode of transfer in vitro, which also enabled us to study the uptake and processing of pigment within keratinocytes. A key feature of this assay is the use of melanocores, that is, melanin with the proteinaceous core lacking the limiting membrane, obtained from MNT-1 melanocyte conditioned medium. We show that cultured keratinocytes are able to uptake melanocores when these are added exogenously to the medium, reproducing the transfer process in which melanocores are released to the extracellular space and internalized by keratinocytes. Being microscopy-based, the assay allows direct visualization of the internalized melanocores, which cluster in the perinuclear area (see Figures 1e and 3c).

We demonstrate that the keratinocyte receptor PAR-2 is required for the uptake of melanocores by keratinocytes. Indeed, PAR-2 silencing results in a decrease in the amount of melanocores taken up by keratinocytes, whereas PAR-2 activation induces melanocore uptake. Conversely, the uptake of melanosomes was found to be independent of PAR-2, suggesting that the use of melanosomes in this assay does not lead to physiological uptake of melanin by keratinocytes. Although other modes of transfer remain possible in vivo, these results validate our model for melanocore exo/endocytosis as the main form of melanin transfer between melanocytes and keratinocytes.

Given the well-documented involvement of Rabs in the regulation of membrane trafficking, an siRNA screen for

endocytic Rabs was performed to unravel regulatory Rabs in melanocore uptake. We found that Rab5b, but not Rab7a or Rab9a, is required for the uptake of melanocores by epidermal keratinocytes. Rab5b belongs to the Rab5 subfamily of proteins, which is well known to play a key role in the early endocytic pathway, more specifically in the homotypic fusion of early endosomes (Bucci et al., 1995). Therefore, the observation that the silencing of Rab5b decreases the uptake of melanocores by XB2 keratinocytes validates our screen. Furthermore, silencing of Rab5a or Rab5c also impairs the uptake of melanocores by keratinocytes, albeit to a lower extent. We hypothesize that this can be due to the weaker silencing levels of these two isoforms. Melanocores measure 0.5 μm or more, which is the lower size limit of particles that are phagocytosed (Hirota and Ter, 2012). Therefore, it is generally assumed that melanin is internalized by keratinocytes via phagocytosis. Interestingly, it has been shown that Rab5 is required for phagocytosis of heat-inactivated *Pseudomonas aeruginosa* (Mustafi et al., 2013). Thus, our results are consistent with melanocores being internalized by phagocytosis mediated by the PAR-2 receptor and Rab5b. However, it remains possible that Rab5b acts at a later step in endosome maturation, which could affect the efficiency of internalization observed in our assay on depletion of Rab5b. We also found that the silencing of Rab7a or Rab9a does not affect melanocore uptake. Because both these proteins are well-established regulators of the late endocytic pathway (Rink et al., 2005; Soldati et al., 1994), this result suggests that melanin does not follow the canonical endocytic pathway, which leads to cargo degradation in lysosomes after trafficking through late endosomes. Moreover, this result may explain the persistence of melanin within keratinocytes.

To explain how melanin can avoid degradation within keratinocytes, we first assessed the degradative capacity of XB2 keratinocytes. We found that these cells express higher levels of the lysosomal markers LAMP2 and Cathepsin D than 3T3 mouse fibroblasts. Furthermore, the fluorescent markers DQ-BSA and LysoTracker, which label degradative and acidic compartments, respectively, stain more XB2 than 3T3 cells, as assessed by fluorescence microscopy and image analysis. Therefore, this suggests that XB2 keratinocytes do not have an impaired degradative capacity that could explain why melanin persists inside these cells.

To further investigate the pathway followed by melanin on internalization, we assessed the colocalization between melanocores and early and late endosomal markers. We found that EEA1, Rab5, transferrin, LAMP2, and CD63 surround melanocores, albeit to a different extent, suggesting that the latter reside in endocytic compartments. Therefore, our data suggest that the intracellular organelles where melanocores reside within keratinocytes are either hybrid or transitional early-to-late endosomes. These compartments show only moderate acidification and poor hydrolytic capacity, which is expected of a storage organelle optimized to retain melanin during the terminal differentiation program of keratinocytes in the skin. Future studies should focus on a more detailed characterization of this melanin storage compartment. We note that a significant percentage (approximately 50%) of melanocores does not overlap with the markers tested. Furthermore, the presence of early and late endocytic markers in this compartment needs to be further refined to assess whether both types of markers are present simultaneously. Three-dimensional reconstructions should clarify some of these issues. Also, these initial studies were performed using a keratinocyte cell line and the results need to be validated in primary keratinocytes and intact skin.

We conclude that melanocores are stored in non-degradative endocytic compartments within keratinocytes, allowing melanin to be preserved for long periods. The in vitro assay described here should allow for further understanding of the processing of melanin within keratinocytes.

## MATERIALS AND METHODS

### Melanocore preparation

MNT-1 cells were cultured in 150 $cm^2$ flasks (Corning, NY) for 7 days. Conditioned medium was collected and concentrated in a vivaspin Centricon (Sigma, Darmstadt, Germany) with a pore size of 300,000 Da at 2,683*g* for a minimum of 35 minutes or centrifuged at 300*g* for 5 minutes to pellet floating cells. The supernatant was then transferred to a clean tube and centrifuged at 20,000*g* for 1 hour at 4°C. Pelleted melanocores were resuspended in phosphate buffered saline (PBS). Melanocore solution absorbance was measured using a Nanodrop 2000 (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA) at 340 nm, and the concentration calculated according to a calibration curve developed in house (Absorbance = 1.8546 × Concentration [g/l] − 0.0422]. For internalization studies, a concentration of 0.1 g/l of melanocores was used, unless otherwise stated.

### Melanocore uptake assay

On the day after the incubation with siRNA for 3.5 hours or with 30 μM of SLIGRL or SFLLRN (Sigma, Darmstadt, Germany) for 2 days, 0.1 g/l of melanocores or melanosomes were added to the cells, which were immediately centrifuged for 5 minutes at 300*g* and 4°C and incubated for 18 hours at 37°C and 10% $CO_2$. Cells were then washed three times with PBS and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, PA) in PBS for 20 minutes at room temperature. After a second wash, cells were incubated with DAPI (Invitrogen, Carlsbad, CA) for 5 minutes to label nuclei. To measure melanin uptake, the Image J (NIH, Bethesda, MD) threshold command was applied and the corresponding intensity (in pixels) measured, where the units were presented in pixels/cell; or the number of melanocores was counted per cell and the mean calculated, where the units were presented in melanocores/cell. Nuclei were counted to ensure similar cell confluence in all samples and calculate the amount of melanin internalized per cell. Melanin uptake reflects the total amount of melanin internalized by XB2 cells in one coverslip.

### Immunofluorescence microscopy

Cells were grown on coverslips for immunofluorescence and fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature. Excess fixative was removed by extensive washing in PBS and quenched by incubation with 50 mM $NH_4Cl$ (Sigma) for 10 minutes. Cells were blocked and permeabilized with 1% BSA (Sigma) and 0.05% saponin (Sigma) in PBS for 30 minutes. Fixed cells were then incubated with primary antibodies for 30 minutes, washed extensively four times, and incubated for 30 minutes with appropriate secondary antibodies conjugated with a fluorophore (Molecular Probes, Eugene, OR). Alexa Fluor-conjugated secondary antibodies were from Invitrogen and used at 1:400. Coverslips containing fixed cells were mounted in MOWIOL mounting medium (Calbiochem Calbiochem-Merck, Darmstadt, Germany). All antibody incubations

and washes were performed with 1 × PBS, 0.5% BSA, and 0.05% saponin. To visualize the nuclei, cells were incubated with DAPI (Invitrogen, Carlsbad, CA) for 5 minutes. Images were acquired on a Zeiss Observer Z2 widefield microscope, equipped with a Zeiss 506 Mono camera using the ×63, 1.4 NA Oil objective. Images were processed using ImageJ and Adobe Illustrator 6.0 (Adobe, San Jose, CA) software.

### LysoTracker and DQ-BSA

To visualize acidic and degradative compartments, XB2 cells were incubated with 50 nM LysoTracker-Red (Molecular Probes) or 10 μg/ml DQ-BSA Red (Molecular Probes), respectively, for 2 hours and then chased in complete medium for 4 hours. Cells were then fixed for immunofluorescence. As a control, cells without any treatment or cells treated with $NH_4Cl$ at a final concentration of 25 mM in growth medium were used.

For more details on materials and methods, see Supplementary Materials and Methods online.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

## ACKNOWLEDGMENTS

We thank the staff from the Unit of Imaging and Cytometry of the Instituto Gulbenkian de Ciência for assistance in microscopy and flow cytometry protocol adjustments, the CEDOC Microscopy Facility for assistance in image acquisition and analysis, Dot Bennett for the kind gift of cell lines, and Alistair Hume for the kind gift of anti-Rab5 antibody. This project was supported by Fundação para a Ciência e a Tecnologia (FCT), Portugal (PTDC/BIA-BCM/111735/2009, EXPL/BEX-BCM/0379/2013), MSC and HM were supported by FCT PhD studentships (SFRH/BD/65381/2009 and PD/BD/114118/2015, respectively), FJCP was supported by an FCT postdoctoral fellowship (SFRH/BPD/70337/2010), and DCB was supported by the FCT Investigator Program (IF/00501/2014/CP1252/CT0001).

## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2017.09.042.

## REFERENCES

Boissy RE. Melanosome transfer to and translocation in the keratinocyte. Exp Dermatol 2003;12(Suppl 2):5–12.

Borovanský J, Elleder M. Melanosome degradation: fact or fiction. Pigment Cell Res 2003;16:280–6.

Van Den Bossche K, Naeyaert J-M, Lambert J. The quest for the mechanism of melanin transfer. Traffic 2006;7:769–78.

Bucci C, Lütcke A, Steele-Mortimer O, Olkkonen VM, Dupree P, Chiariello M, et al. Co-operative regulation of endocytosis by three Rab5 isoforms. FEBS Lett 1995;366:65–71.

Byers HR, Maheshwary S, Amodeo DM, Dykstra SG. Role of cytoplasmic dynein in perinuclear aggregation of phagocytosed melanosomes and supranuclear melanin cap formation in human keratinocytes. J Invest Dermatol 2003;121:813–20.

Cantalupo G, Alifano P, Roberti V, Bruni CB, Bucci C. Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. EMBO J 2001;20:683–93.

Chen JW, Murphy TL, Willingham MC, Pastan I, August JT. Identification of two lysosomal membrane glycoproteins. J Cell Biol 1985;101:85–95.

Hirota K, Ter H. Endocytosis of particle formulations by macrophages and its application to clinical treatment. In: Ceresa B, editor. Molecular regulation of endocytosis. Rijeka, Croatia: InTech; 2012.

Hori Y, Toda K, Pathak MA, Clark WH, Fitzpatrick TB. A fine-structure study of the human epidermal melanosome complex and its acid phosphatase activity. J Ultrastruct Res 1968;25:109–20.

Hutagalung AH, Novick PJ. Role of Rab GTPases in membrane traffic and cell physiology. Physiol Rev 2011;91:119–49.

Huynh KK, Eskelinen E-L, Scott CC, Malevanets A, Saftig P, Grinstein S. LAMP proteins are required for fusion of lysosomes with phagosomes. EMBO J 2007;26:313–24.

Marks MS, Heijnen HFG, Raposo G. Lysosome-related organelles: unusual compartments become mainstream. Curr Opin Cell Biol 2013;25:495–505.

Marks MS, Seabra MC. The melanosome: membrane dynamics in black and white. Nat Rev Mol Cell Biol 2001;2:738–48.

Marthinuss J, Andrade-Gordon P, Seiberg M. A secreted serine protease can induce apoptosis in Pam212 keratinocytes. Cell Growth Differ 1995;6:807–16.

Mascia A, Gentile F, Izzo A, Mollo N, De Luca M, Bucci C, et al. Rab7 Regulates CDH1 endocytosis, circular dorsal ruffles genesis, and thyroglobulin internalization in a thyroid cell line. J Cell Physiol 2016;231:1695–708.

Murase D, Hachiya A, Takano K, Hicks R, Visscher MO, Kitahara T, et al. Autophagy has a significant role in determining skin color by regulating melanosome degradation in keratinocytes. J Invest Dermatol 2013;133:2416–24.

Mustafi S, Rivero N, Olson JC, Stahl PD, Barbieri MA. Regulation of Rab5 function during phagocytosis of live *Pseudomonas aeruginosa* in macrophages. Infect Immun 2013;81:2426–36.

Raposo G, Marks MS. The dark side of lysosome-related organelles: specialization of the endocytic pathway for melanosome biogenesis. Traffic 2002;3:237–48.

Rink J, Ghigo E, Kalaidzidis Y, Zerial M. Rab conversion as a mechanism of progression from early to late endosomes. Cell 2005;122:735–49.

Santulli RJ, Derian CK, Darrow AL, Tomko KA, Eckardt AJ, Seiberg M, et al. Evidence for the presence of a protease-activated receptor distinct from the thrombin receptor in human keratinocytes. Proc Natl Acad Sci USA 1995;92:9151–5.

Seabra MC, Mules EH, Hume AN. Rab GTPases, intracellular traffic and disease. Trends Mol Med 2002;8:23–30.

Seiberg M. Keratinocyte-melanocyte interactions during melanosome transfer. Pigment Cell Res 2001;14:236–42.

Seiberg M, Paine C, Sharlow E, Andrade-Gordon P, Costanzo M, Eisinger M, et al. The protease-activated receptor 2 regulates pigmentation via keratinocyte-melanocyte interactions. Exp Cell Res 2000;254:25–32.

Soldati T, Shapiro AD, Svejstrup AB, Pfeffer SR. Membrane targeting of the small GTPase Rab9 is accompanied by nucleotide exchange. Nature 1994;369:76–8.

Tadokoro R, Murai H, Sakai K, Okui T, Yokota Y, Takahashi Y. Melanosome transfer to keratinocyte in the chicken embryonic skin is mediated by vesicle release associated with Rho-regulated membrane blebbing. Sci Rep 2016;6:38277.

Tarafder AK, Bolasco G, Correia MS, Pereira FJC, Iannone L, Hume AN, et al. Rab11b mediates melanin transfer between donor melanocytes and acceptor keratinocytes via coupled exo/endocytosis. J Invest Dermatol 2014;134:1056–66.

Thong H-Y, Jee S-H, Sun C-C, Boissy RE. The patterns of melanosome distribution in keratinocytes of human skin as one determining factor of skin colour. Br J Dermatol 2003;149:498–505.

Wasmeier C, Romao M, Plowright L, Bennett DC, Raposo G, Seabra MC. Rab38 and Rab32 control post-Golgi trafficking of melanogenic enzymes. J Cell Biol 2006;175:271–81.

Wolff K. Melanocyte-keratinocyte interactions in vivo: the fate of melanosomes. Yale J Biol Med 1973;46:384–96.

Wu X, Hammer JA. Melanosome transfer: it is best to give and receive. Curr Opin Cell Biol 2014;29:1–7.

Zerial M, McBride H. Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2001;2:107–17.