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Mammalian pigmentation is regulated by a distinct cAMP-dependent mechanism that controls melanosome pH

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Abstract

The production of melanin increases skin pigmentation and reduces the risk of skin cancer. Melanin production depends on the pH of melanosomes, which are more acidic in lighter-skinned than in darker-skinned people. We showed that inhibition of soluble adenylyl cyclase (sAC) controlled pigmentation by increasing the pH of melanosomes both in cells and in vivo. Distinct from the canonical melanocortin 1 receptor (MC1R)-dependent cAMP pathway that controls pigmentation by altering gene expression, we found that inhibition of sAC increased pigmentation by increasing the activity of tyrosinase, the rate-limiting enzyme in melanin synthesis, which is more active at basic pH. We demonstrated that the effect of sAC activity on pH and melanin production in human melanocytes depended on the skin color of the donor. Last, we identified sAC inhibitors as a new class of drugs that increase melanosome pH and pigmentation in vivo, suggesting that pharmacologic inhibition of this pathway may affect skin cancer risk or pigmentation conditions.

One-sentence summary:

Inhibitors of soluble adenylyl cyclase increase pigmentation and may reduce the risk of skin cancer.

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Competing interests: L.R.L., J.B., and J.H.Z. own equity interest in CEP Biotech, which has licensed commercialization of a panel of monoclonal antibodies directed against sAC. J.H.Z. is a paid consultant and on the medical advisory board of Hoth Therapeutics, is on the medical advisory board of SHADE Inc., and an inventor on an international patent application PCT/US2017/040428 on "Methods of modulating melanosome pH and melanin level in cells." L.R.L., J.B., and J.H.Z. are inventors on a U.S. patent 8,859,213 on the use of antibodies directed against sAC for the diagnosis of melanocytic proliferations.

Data and materials availability: All data needed to evaluate the conclusions are present in the paper or the Supplementary Materials.

Editor's Summary:

A basic way to tan

Darker-skinned individuals have more melanin in their skin and a lower risk for skin cancer than fairer-skinned individuals. The production of melanin occurs in organelles called melanosomes and is regulated by melanosome pH. Zhou *et al.* found that cAMP generated by soluble adenylyl cyclase (sAC) resulted in decreases in melanosome pH and in the activity of tyrosinase, the rate-limiting enzyme in melanin synthesis. sAC deficiency or inhibitors increased melanosome pH and pigmentation in mice. These results define a mechanism of rapidly regulating melanin synthesis that could be exploited to reduce skin cancer risk for fair-skinned individuals.

INTRODUCTION

Human pigmentation has psychosocial implications and affects skin cancer risk (1–5). Differences in pigmentation of the skin, hair, and eyes are the result of variation in the amount and type of melanin produced (5, 6). Melanin is produced in a specialized organelle called the melanosome (7–10). Canonical mechanisms that control melanin production involve changes in the expression of genes encoding synthetic enzymes such as tyrosinase (*TYR*), tyrosinase-related protein 1 (*TYRP1*), and tyrosinase-related protein 2 (*TYRP2*) (11). Genetic analysis of human populations with differences in skin, hair, and eye pigmentation has identified polymorphisms in genes encoding melanosome channels, and functional analysis confirms that these channels influence pigmentation by altering melanosome pH (12–14). Although the discovery of these polymorphisms has identified proteins important for the maintenance of the melanosome pH set point, it remains unclear whether these proteins are dynamically regulated. The melanosome pH set point is not fixed: During early organelle development, the organelle is relatively acidic, progressively becoming more alkaline with maturity (15, 16). Mechanisms that control this active process are poorly understood.

Tyrosinase is the rate-limiting enzyme in melanin synthesis, and its activity substantially affects human pigmentation. Signaling cascades, such as those mediated by activation of the melanocortin 1 receptor (MC1R) by melanocyte-stimulating hormone (MSH), can stimulate transcription factors, such as microphthalmia-associated transcription factor (MITF), to control pigmentation by altering the expression of *TYR*. Because tyrosinase is very pH sensitive and its activity increases as pH is elevated (15, 17), it has been therefore proposed that tyrosinase activity can be differentially regulated without altering its expression (18). However, signaling mechanisms that dynamically regulate melanosome pH to control tyrosinase activity and drugs capable of safely regulating pigmentation by altering melanosome pH have not been described.

Human pigmentation is a reflection of the melanin content in the skin, hair, and eyes. There are two types of melanin: eumelanin and pheomelanin (6). Eumelanin is dark brown to black in color and is effective at blocking ultraviolet radiation (6). Pheomelanin is yellow to red in color and, because of its pro-oxidant chemistry, is carcinogenic (6, 19). Thus, the relative amount of these two types of melanin contributes to skin cancer risk (4). The visual

difference in the skin or hair color of most people correlates with the presence of eumelanin in the tissue (20). The exception are redheads, who make a large amount of pheomelanin due to polymorphisms present in the *MC1R* gene. The pheomelanin content in people with wild-type *MC1R* is variable and is not clearly linked to a genetic polymorphism (21).

Melanosome pH has been reported to be more acidic in lighter-skinned people than in darker-skinned people; therefore, melanosome pH is important for human pigmentation (1, 2, 18). Nonphysiological disruption of vacuolar-type H⁺-ATPase (V-ATPase) activity after treatment with bafilomycin increases melanosome pH and can increase the ratio of eumelanin to pheomelanin (6, 15). However, signaling mechanisms that control melanin synthesis by dynamically regulating melanosome pH have not been described.

Cyclic adenosine monophosphate (cAMP) regulates pigmentation by altering genes important for melanin synthesis (7). Signaling through this second messenger occurs locally in spatially restricted microdomains distributed throughout cells (22–24). cAMP signaling microdomains function independently: The cAMP produced in one microdomain within a cell has independent (and sometimes opposing) effects from cAMP produced in a distinct microdomain. In addition to being defined by their unique effects, cAMP signaling microdomains are also defined by the distinct mechanisms used to control the levels of the second messenger. cAMP is produced by adenylyl cyclases (ACs) and catabolized by phosphodiesterases (PDEs), and the activities of ACs and/or PDEs can regulate cAMP signaling in a microdomain. In mammalian cells, there are two distinct subfamilies of ACs (23). The canonical cAMP cascade is initiated by heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors, leading to G protein-dependent activation of the transmembrane AC (tmAC) located at the plasma membrane (23). There are nine tmAC genes (*ADCY1–9*) in mammalian cells, and the encoded proteins can signal at distinct locations within the plasma membrane or on recycling endosomes (24). The non-canonical cAMP cascade is initiated by soluble AC (sAC; which is encoded by *ADCY10*), which is not G protein stimulated but is instead stimulated by bicarbonate and Ca²⁺ ions (25, 26) and adenosine triphosphate (ATP) (27). Unlike tmACs, sAC is not linked to the plasma membrane and is present at multiple intracellular locations (28). sAC- and tmAC-defined cAMP cascades mediate both similar and disparate effects within a single cell type (27, 29). In melanocytes, tmAC-generated cAMP is responsible for *MC1R*-induced changes in gene expression. We explored whether other cAMP signaling cascades contribute to pigmentation in melanocytes.

sAC regulates pH across the plasma membrane in the kidney and epididymis (30, 31) and the pH set point of lysosomes (32), which share many developmental and regulatory steps with melanosomes. Therefore, we hypothesized that sAC may also control melanosome pH and affect melanin production.

RESULTS

sAC regulates melanosome pH in mouse melanocytes

Using isolated primary melanocytes derived from human donors with varying skin color, we confirmed previous reports of sAC expression in melanocytes in human skin (33, 34) and in isolated mouse melanocytes in culture [Gene Expression Omnibus databases (35) GDS1965

(36), GDS3867(37), and GDS3012 (38); fig. S1, A to C]. To genetically evaluate the role of sAC in controlling melanocyte pH, we used a strain of mice with three exons encoding the second of two catalytic domains of the *Adcy10* gene flanked by loxP sites (*Adcy10^{fl/fl}*) (39, 40) to generate immortalized mouse melanocytes by serial passage (41). *Adcy10^{fl/fl}* melanocytes synthesized melanin, displayed normal cAMP signaling, and expressed the melanocyte markers MITF and tyrosinase (fig. S2, A to C). These parental *Adcy10^{fl/fl}* cells were infected with adenovirus expressing either green fluorescent protein (GFP) fused to Cre recombinase (GFP-Cre) or GFP alone to create paired *Adcy10^{-/-}* (*sAC^{KO}*) and *Adcy10^{fl/fl}* (*sAC^{FF}*) melanocytes, respectively. Genetic deletion of *Adcy10* was confirmed by polymerase chain reaction (PCR) and cAMP accumulation (fig. S2, D and E). *sAC^{FF}* and *sAC^{KO}* melanocytes grew at identical rates regardless of media conditions (fig. S2F).

3-(2,4-Dinitroanilino)-3'-amino-*N*-methyldipropylamine (DAMP) is a weakly basic amine that is taken up by organelles in live cells in a pH-dependent manner [fig. S3A and (42–44)]. We established a large-scale, unbiased microscopy-based technique to both identify organelles and measure their pH using DAMP fluorescence intensity (fig. S3, A to D). We specifically measured melanosome pH by quantitating DAMP fluorescence intensity only at organelles immunocytochemically identified as melanosomes (fig. S3, B to E). We found that the total number and distribution of melanosomes were similar between *sAC^{KO}* and *sAC^{FF}* melanocytes (Fig. 1, A to F, and fig. S3, F to I); hence, loss of sAC did not lead to an overt change in melanosome formation or distribution. The localization of specific proteins to maturing melanosomes through progressive stages of melanogenesis is well established (45). Using TYRP1 as a marker of mature, stage III and IV melanosomes (46), we found that DAMP staining intensity in TYRP1-positive melanosomes was significantly reduced in *sAC^{KO}* relative to *sAC^{FF}* melanocytes (Fig. 1, A and B, and fig. S3J). Thus, *sAC^{KO}* TYRP1-positive melanosomes were more alkaline than *sAC^{FF}* melanosomes. HMB45 is a melanosome marker that is found mainly in stage II, stage III, and a subset of stage IV melanosomes (46–48). Similar to TYRP1-positive melanosomes, HMB45-positive melanosomes were more alkaline in *sAC^{KO}* relative to *sAC^{FF}* melanocytes (Fig. 1, C and D). Similar to genetic loss of sAC, a 4-hour incubation of *sAC^{FF}* cells with the sAC inhibitors KH7 (49) and LRE1 (50) led to an increase in melanosome pH (Fig. 1, E and F). In contrast, sAC inhibitors did not affect the pH of melanosomes in *sAC^{KO}* cells (fig. S3K and table S1). We confirmed the pH increase in organelles after genetic or pharmacologic inhibition of sAC using an independent pH sensor (51) in mouse melanocytes (fig. S4, A to E).

Melanosome pH measurement using our DAMP method was similar regardless of the fluorophore used for antibody detection; thus, our method was unaffected by melanin content (fig. S3, L to N). Loss or inhibition of sAC affected only DAMP staining and did not affect the staining intensity of the melanosome markers TYRP1 and HMB45 (fig. S5, A to C). Last, alkalization of melanosome pH by pharmacologic inhibition of sAC was readily reversible because inhibitor removal allowed for complete recovery of the pH set point in wild-type cells in 8 hours (fig. S3, O and P). Thus, we concluded that loss of sAC leads to a rise in melanosome pH in mouse melanocytes.

sAC regulates melanosome pH in human melanocytes

Because melanosome pH differs depending on the skin tone of the melanocyte donor (18), we investigated the effect of sAC on melanosome pH using human melanocytes from donors with a variety of skin colors. Consistent with previous studies, melanocytes from donors with lighter skin had a more acidic melanosome pH set point, whereas those from a donor with darker skin color had a more basic set point (fig. S6, A and B, and table S1) (1, 2, 18). Pharmacologic sAC inhibition of human melanocytes derived from three different donors with lighter skin tone led to a substantial alkaline shift in melanosome pH (Fig. 1, G to I). In contrast, inhibition of sAC in melanocytes derived from patients with dark skin tone did not substantially alter melanosome pH (fig. S6C and table S1). Hence, we concluded that inhibition of sAC leads to increased melanosome pH in human melanocytes and that sAC inhibition has differential effects on melanosome pH in melanocytes from human donors with different skin colors.

cAMP restores melanosome pH after loss of sAC activity

Because the main function of sAC is to produce cAMP, we next investigated whether the increase in melanosome pH after sAC inhibition was due to the loss of the second messenger cAMP. Four hours after the addition of a nonselective cAMP analog, Sp-8-CPT-cAMPs (cAMP) (52), we observed a significant acidic shift in melanosome pH in *sAC^{KO}* melanocytes (Fig. 2A), but not in *sAC^{FF}* cells (fig. S7A and table S1). Furthermore, co-incubation of *sAC^{FF}* mouse melanocytes (Fig. 2B) or human melanocytes (Fig. 2C) with cAMP mitigated the KH7-induced alkaline shift in melanosome pH. Similar to *sAC^{FF}* melanocytes, cAMP did not alter melanosome pH in human melanocytes in the absence of inhibitor (fig. S7B and table S1). Thus, cAMP rescued the alkalization of melanosomes after sAC inhibition.

We next asked whether MSH-dependent stimulation of tmACs (53) affected melanosome pH after loss of sAC. In contrast to the addition of the cAMP analog (Fig. 2, A to C), MSH-dependent production of cAMP (fig. S2E) over 4 hours did not affect melanosome pH in mouse or human melanocytes after sAC inhibition (fig. S7, C to F, and table S1). Thus, cAMP generated downstream of MSH stimulation did not appear to acutely control melanosome pH.

MSH-dependent signaling has been reported to alter melanosome pH after 48 hours of stimulation by regulating the expression of genes encoding V-ATPases (54). In contrast, sAC-dependent regulation of melanosome pH occurred quickly (within 4 hours), suggesting that new protein synthesis was not required. Consistent with that premise, co-incubation with cycloheximide did not block the ability of cAMP to rescue pH after sAC inhibition (fig. S8, A and B, and table S1). Therefore, we reasoned that loss of sAC-dependent cAMP rapidly increases melanosome pH without requiring new protein synthesis and is thus distinct from the canonical cAMP signaling pathways that increase pigment by altering gene expression.

sAC regulates melanosome pH through EPAC

There are multiple cAMP effector proteins in mammalian cells (55). Because cAMP-dependent regulation of gene expression in melanocytes through activation of protein kinase A (PKA) controls pigmentation (56), we asked whether PKA was required for acute cAMP-dependent regulation of melanosome pH. Treatment of mouse and human melanocytes for 4 hours with the PKA inhibitors H89 or PKI led to a reduction in the phosphorylation of PKA targeted proteins (fig. S9A) but did not affect melanosome pH (fig. S9, B to D, and table S1). Furthermore, addition of H89 or PKI did not block the cAMP-dependent rescue of melanosome pH in *sAC^{KO}* melanocytes (fig. S9, E and F, and table S1). Therefore, PKA did not appear to be the relevant cAMP effector protein for acute melanosome pH regulation.

The exchange proteins activated by cAMP (EPAC) are cAMP effector proteins expressed in melanocytes (57–59) that have not been implicated in controlling pigmentation. To investigate whether EPACs were important for the regulation of melanosome pH, we used ESI-09, a pan-EPAC-specific, cAMP-competitive antagonist (60, 61). In contrast to PKA inhibitors but similar to sAC inhibitors, ESI-09 significantly increased melanosome pH in mouse and human melanocytes (Fig. 3, A to C, fig. S9G, and table S1). As a competitive cAMP analog, ESI-09 should only block EPAC activity in the presence of a physiological source of cAMP. Consistent with that premise, ESI-09 did not affect melanosome pH in *sAC^{KO}* melanocytes (fig. S9H and table S1); thus, sAC-generated cAMP was required for EPAC-dependent regulation of melanosome pH. Furthermore, the EPAC-selective cAMP agonist 8-pHPT-2'-O-Me-cAMP (62, 63) led to melanosome acidification in *sAC^{KO}* melanocytes and mitigated the KH7-induced melanosome alkalization in human and mouse melanocytes (Fig. 3, D to F, fig. S9I, and table S1). Thus, these data identified EPAC as a cAMP effector protein important for sAC-dependent control of melanosome pH.

sAC regulates tyrosinase activity in vivo independently of gene expression

Although the activity of the rate-limiting, melanin-producing enzyme tyrosinase is affected by changes in its expression, the activity of the enzyme is also sensitive to melanosome pH (15). A more alkaline melanosome pH set point can enhance the activity of the enzyme (15); however, an endogenous signaling cascade capable of altering tyrosinase activity by changing melanosome pH has not been described. Because loss of sAC activity led to an increase in melanosome pH, we tested whether tyrosinase activity was higher after sAC inhibition. Tyrosinase activity in live mouse melanocytes was higher in *sAC^{KO}* melanocytes than in *sAC^{FF}* melanocytes (Fig. 4A). In addition, pharmacologic inhibition of sAC increased tyrosinase activity in human melanocytes within a few hours (Fig. 4B). However, tyrosinase abundance was not increased after genetic loss or pharmacologic inhibition of sAC activity (Fig. 4C and fig. S6D). Thus, the sAC signaling cascade was able to rapidly increase tyrosinase activity by altering melanosome pH without affecting *TYR* gene expression.

We next used EM to examine ultrastructural changes in human and murine melanocytes after sAC inhibition. Melanin, the product of tyrosinase, appears as an electron-dense material within the melanosome. Although loss of sAC did not increase the total number of melanosomes (fig. S3, F and G), genetic ablation or pharmacologic inhibition of sAC

increased the number of electron-dense melanosomes (Fig. 4, D and E, and fig. S10, A and B). This increase in the number of electron-dense melanosomes was consistent with the aforementioned data that sAC inhibition leads to tyrosinase activation in vivo. Therefore, these data suggested that sAC regulation of tyrosinase activity can alter melanin synthesis.

sAC regulates melanin synthesis in mouse and human melanocytes

Eumelanin is the major melanin species produced in most melanocytes and is the main contributor to visually apparent color differences in skin, hair, and eyes (6). Because eumelanin levels reflect tyrosinase activity, we next asked whether the increase in in vivo tyrosinase activity after loss of sAC activity affected eumelanin production in melanocytes. Three to five passages after *Adcy10* deletion, *sAC^{KO}* melanocytes grew noticeably darker (Fig. 5A, inset below). Darkening of *sAC^{KO}* melanocytes was due to an increase in eumelanin content (Fig. 5A). This observation repeated in multiple sets of paired *sAC^{FF}* and *sAC^{KO}* melanocytes (fig. S11A) and was not due to altered cell growth (fig. S2F). In addition, treatment of *sAC^{FF}* melanocytes with a sAC inhibitor increased eumelanin levels but had no effect on *sAC^{KO}* cells (fig. S11B). In human melanocytes, pharmacologic inhibition of sAC differentially altered pigmentation depending on the skin color of the human donor. Inhibition of sAC increased eumelanin content in melanocytes derived from donors with light skin tone (Fig. 5B), but not in those from donors with dark skin tone (Fig. 5C). Thus, similar to its effects on melanosome pH (Fig. 1, G to I, and fig. S6, A to C), sAC differentially altered human melanocyte pigmentation depending on the skin tone of the donor.

MSH induces melanin production by stimulating MC1R/tmAC-dependent generation of cAMP (fig. S2B), thereby increasing tyrosinase protein abundance (fig. S2C) and eumelanin synthesis (Fig. 5D) (56). Our data showed that loss of sAC-dependent cAMP (fig. S2E) also stimulated eumelanin synthesis (Fig. 5, A, B, and D), and this effect appeared to have been mediated by an independent cAMP signaling cascade (Fig. 3, A to F, and fig. S7, C to F, and S9, B to F). Loss of sAC-dependent cAMP stimulated eumelanin synthesis through an increase in melanosome pH (Fig. 1, B, D, and F to I), which enhanced tyrosinase activity (Fig. 4, A and B) without changes in tyrosinase protein abundance (Fig. 4C and fig. S6D). Consistent with these cascades acting independently, MSH stimulated eumelanin synthesis to approximately the same extent in both *sAC^{FF}* and *sAC^{KO}* melanocytes (Fig. 5D). Our data suggested that the sAC-dependent effects on melanosome pH were mediated by EPAC (Fig. 3, A to F), and accordingly, ESI-09 treatment increased eumelanin synthesis in human melanocytes (Fig. 5E). These data established the sAC/EPAC signaling cascade as a regulator of eumelanin production in melanocytes and demonstrated that this cAMP signaling cascade was independent of the MC1R/tmAC-dependent cAMP cascade that stimulates *TYR* gene expression.

sAC differentially regulates eumelanin and pheomelanin synthesis

Melanosome pH differentially affects the synthesis of the two main types of melanin, eumelanin and pheomelanin, and the relative ratio of each melanin affects pigmentation, skin cancer risk, and aging (15, 17, 20, 43, 64, 65). The eumelanin-to-pheomelanin ratio is thought to reflect melanosome pH (15, 64), but the signaling pathways directly regulating

melanosome pH to alter this ratio have remained unclear. We reasoned that the sAC-dependent modulation of melanosome pH might affect both eumelanin and pheomelanin synthesis. Loss of sAC activity induced both an increase in eumelanin (Fig. 5A) and a concomitant decrease in pheomelanin content (Fig. 5F), leading to a reduction in the ratio of pheomelanin to eumelanin (Fig. 5G). Thus, sAC regulation of melanosome pH affected the synthesis of both pheomelanin and eumelanin in melanocytes.

sAC regulates pigmentation in mice

We next wanted to confirm the role of sAC in regulating melanocyte pigmentation in an in vivo context. Using a *Tyr::CRE-ERT2;sAC^{fl/fl}* murine model (to enable tamoxifen-dependent ablation of sAC) backcrossed into a C3H/HeJ pigment background, we investigated the role of sAC in melanocytes in mice, which, on the back, lie at the base of the hair follicle and control hair color. The backs of mice were painted with tamoxifen, and we noted that during the first hair cycle, loss of sAC activity in melanocytes led to a darkening of hair color (Fig. 6A and table S2). Histologic examination of the skin did not reveal pathological changes to the hair follicles or epidermis. Hair color in C3H/HeJ mice reflects the relative melanocyte production of eumelanin (black) and pheomelanin (yellow, the main component of the agouti band) in each hair shaft (6). Microscopic examination of hair revealed reduced numbers of hairs with agouti bands (Fig. 6B and table S2), suggesting that loss of sAC led to an increase in eumelanin and a decrease in pheomelanin. This premise was confirmed by analysis of melanin content in the hair, which showed that loss of sAC in melanocytes significantly reduced the ratio of pheomelanin to eumelanin (Fig. 6C and table S2). This change in melanin profile in mouse hair was similar to that observed in melanocytes in vitro after loss of sAC activity (Fig. 5G) and confirmed that loss of sAC activity in melanocytes in vivo led to changes in melanin production.

There is a relative dearth of drugs that can increase pigmentation in vivo. Because sAC inhibitors can increase eumelanin production in mouse and human melanocytes in vitro, we reasoned that these small molecules might increase epidermal pigmentation. In addition to hair follicles, melanocytes also exist within the epidermis at specific areas of mouse skin, for example, the pinnae (66). We used this anatomic location to examine the effects of topical application of sAC inhibitors on epidermal pigmentation. Topical application of KH7 or LRE1 (Fig. 6D, bottom) induced visible darkening of mouse ear epidermis relative to vehicle control (Fig. 6D, top). Histologic examination of the epidermis revealed an increase in epidermal melanin accumulation after pharmacologic sAC inhibition (Fig. 6D, white arrowheads). Ears were examined by a veterinary pathologist, and no inflammation, keratinocyte necrosis, or fibrosis was noted. Pigmentation differences were still visible up to 2 weeks after stopping drug application. These data confirmed that sAC regulation of melanosome pH controls pigmentation in vivo and identified sAC inhibitors as a potential therapeutic strategy for altering pigmentation.

DISCUSSION

Human skin and hair pigmentation has psychosocial and cancer risk importance; thus, understanding how pigmentation is controlled has numerous clinical implications. Much of

our current understanding of human pigmentation is based on the characterization of polymorphisms in genes important for pigmentation such as *MC1R* (20, 56) and those encoding melanosome channels (12, 14, 67). Investigation of the melanocyte proteins containing polymorphisms has helped explain certain disorders of human pigmentation and the red hair phenotype; however, less well understood are the signaling pathways that directly alter melanosome biology to regulate pigment synthesis. Here, we demonstrated that the sAC-dependent cAMP cascade controlled pigmentation by regulating melanosome pH.

Melanosome channels and proton pumps are important for establishing the melanosome pH set point. Biologically similar organelles, such as lysosomes, also dynamically regulate their pH (68). Mechanisms that dynamically regulate the pH of melanosomes are poorly understood. We revealed that melanosome pH rapidly changed in response to alterations in sAC-dependent cAMP signaling. Thus, melanosome pH regulation is not limited to a progressive pH increase as the melanosome matures (15, 16). Furthermore, because sAC modulates lysosomal pH in other cell types (32), sAC signaling cascades may represent a general mechanism of organelle pH control.

Melanosome pH critically affects the activity of the rate-limiting, melanin-synthesizing enzyme tyrosinase (15, 17, 20, 64). In contrast to the mechanisms of tyrosinase regulation by changes in its expression over days, signaling pathways that acutely control tyrosinase activity by directly regulating melanosome pH are not well understood. Our data demonstrated that sAC-dependent cAMP signaling could rapidly (within hours) regulate melanosome pH and tyrosinase activity in cells and revealed a new cAMP-dependent mechanism of tyrosinase regulation that is independent of gene expression (Fig. 6E).

The balance between eumelanin and pheomelanin levels in skin has important implications for melanoma/skin cancer risk and aging (19, 69, 70). pH can differentially influence the synthesis of these two types of melanin (15, 17, 20, 43, 64), although the mechanisms that acutely control eumelanin and pheomelanin synthesis by directly altering melanosome pH have not been described. Here, we showed that sAC control of melanosome pH influenced the eumelanin-to-pheomelanin ratio in melanocytes both in vitro and in vivo. These data suggested that sAC activity may influence skin cancer risk and aging. Notably, sAC expression and subcellular localization change as melanocytes transition from benign to malignant cells (34, 71). We and others have shown that antibodies directed against sAC are an effective adjunct for the diagnosis of pigmented lesions, especially lentiginous growth melanomas (34, 71, 72).

In addition to controlling skin, hair, and eye color, melanin production in multiple tissue types (for example, skin, retina, inner ear, and brain) is important for regulating reactive oxygen species (ROS), protecting tissues from toxins, and ion chelation (73), and these signals have been suggested to influence melanin production. sAC is a metabolic and pH sensor and is stimulated by many intracellular signals, such as HCO_3^-/pH , ATP, calcium, and ROS (Fig. 6E) (26, 27, 74, 75). Therefore, the sAC signaling cascade may provide a mechanism through which melanocytes and other melanin-producing cells can sense and respond to specific stimuli by altering melanin production.

Melanocytes derived from people with different skin and hair color have distinct melanosome pH set points (15, 17, 20, 64). Our data revealed that sAC regulation of melanosome pH, tyrosinase activity, and pigmentation differed depending on the skin color of the melanocyte donor. Therefore, investigation of how sAC-dependent signaling pathways are different in people with varied skin colors may uncover new mechanisms that control pigmentation in humans.

Our data suggested that PKA is not required for sAC-dependent regulation of melanosome pH. Instead, our data supported a role for EPAC in the control of melanosome pH downstream of sAC. The sAC/EPAC pathway and the canonical tmAC/PKA pathway may be completely distinct or may cooperate to control pigmentation (Fig. 6E); therefore, further investigation of the interplay between these two pathways will be important.

Mammalian cells have distinct cAMP microdomains that can have similar or disparate effects on cellular biology (22–24). MSH/MC1R-induced, tmAC-generated cAMP is essential for the expression of pigmentation genes important for melanin production (20). Given the selective expression of the tmAC-encoding *ADCY2* gene in melanocytes (76), *ADCY2* may be the source of MSH-dependent cAMP. In addition, *ADCY6*, which links calcium and cAMP signaling to melanogenesis, is another potential source of tmAC-dependent cAMP (77). sAC-generated cAMP controlled melanosome pH and did not increase *TYR* gene expression (Fig. 6E). We demonstrated here that melanin production was enhanced by increased *TYR* gene expression, resulting from an increase in cAMP generated by the MSH/tmAC pathway, and an alkalinized melanosome pH, resulting from a decrease in cAMP generated by sAC (Fig. 5D). Thus, our data support a model in which tmAC- and sAC-defined microdomains lead to diametrically opposite changes in cAMP that both induce eumelanin synthesis.

Distinct cAMP signaling cascades in melanocytes may help explain why some people with dysfunctional MC1Rs, altered tmAC activity, or aberrant MSH secretion can maintain normal skin and hair color (20, 78–80). In addition, extracutaneous melanocytes and other melanosome-containing cells, such as retinal pigment epithelium and stria vascularis, lack obvious MC1R-dependent signaling cascades (81), and the canonical melanin regulatory pathways do not appear to play a role in these cells. However, melanin synthesis in extracutaneous melanosome-containing cells is affected by melanosome pH (82, 83). Furthermore, diseases such as oculocutaneous albinism type 2 (*OCA2*) are thought to occur when melanosomes cannot alkalinize during organelle development (15). Pharmacologic correction of melanosome pH has been proposed as a method to restore pigmentation in *OCA2* melanocytes to normal levels and reduce skin cancer risk (84). We found that sAC inhibitors increased melanosome pH and epidermal pigmentation in mice. Therefore, drugs targeting the sAC signaling cascade may represent a new therapeutic strategy for treating diseases of melanin synthesis or reducing skin cancer risk.

MATERIALS AND METHODS

Antibody reagents for Western blot analysis

Tyrosinase antibody (used at 1:1000) was a gift from R. Halaban, Yale University School of Medicine (85). Tyrosinase antibody (T311) was purchased from Santa Cruz Biotechnology (1:200; catalog no. sc-20035, lot no. J0616). MITF antibody was purchased from Abcam (1:500; catalog no. ab13703, lot no. GR27425–10). MITF (D5G7V; 1:1000; catalog no. 12590S, lot no. 1), phosphor-(Ser/Thr) PKA substrate (1:1000; catalog no. 9621, lot no. 14), and GAPDH (14C10; 1:1000; catalog no. 2118S, lot no. 8) antibodies were purchased from Cell Signaling Technology. Antibodies directed against (R21) (3.2 mg/ml, 1:2000) were used as previously described (28).

sAC^{KO} mouse melanocyte generation and culture

All experiments involving mice were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee and were performed in accordance with institutional guidelines. Immortalized mouse melanocytes were derived from 1- to 3-day-old *Adcy10^{fl/fl}* agouti newborn mice as previously described (41). Briefly, newborn mice were euthanized, and the skin was removed from the back, placed in a petri dish epidermis side up, and incubated in 2.5 ml of dispase in Eagle's minimal essential medium without calcium and magnesium overnight at 4°C. On the next day, the dermis was discarded, and the epidermis was incubated in trypsin solution until the cells dissociated. Cells were washed to remove the trypsin solution and then cultured in mouse melanocyte medium [Ham's F12 plus glutamine, penicillin-streptomycin, horse serum (7%), fetal bovine serum (FBS; 7%), dibutyryl cAMP (dbcAMP; 500 μM), Na₃VO₄ (1 μM)]. Once the immortalized line was established, the medium was changed to normal mouse melanocytes culture media [Opti-MEM medium supplemented with 10% FBS, 7% horse serum, 1% penicillin-streptomycin, 400 μM dbcAMP, 0.3 nM cholera toxin (CT), and 1.6 μM 12-O-tetradecanoylphorbol-13-acetate (TPA)]. To generate *Adcy10^{-/-}* melanocytes (sAC^{KO}), parental *Adcy10^{fl/fl}* cells were infected with either Ad5-CMV-GFP or Ad5-CMV-CREGFP (Vector BioLabs, Malvern, PA, USA) at 200 multiplicity of infection. Forty-eight hours after infection, cells were fluorescence-activated cell sorted for GFP fluorescence, and only cells that were in the upper 25% of fluorescence were collected and cultured. Independent pairs of *Adcy10^{fl/fl}* (sAC^{FF}) and *Adcy10^{-/-}* (sAC^{KO}) cells were generated. Genetic deletion of sAC was confirmed by PCR and functional assay. All experiments using mouse melanocytes were performed between passages 15 and 28. Before the experiments, melanocytes were cultured in "cAMP starvation media" without dbcAMP and without CT for 48 to 96 hours. The cell growth rate of each cell line under different media conditions was measured using the CyQUANT assay (Thermo Fisher Scientific) at the time point indicated.

Human melanocyte culture

Primary human melanocytes derived from neonatal foreskins were obtained from the Biospecimen Core of the Yale Specialized Programs of Research Excellence (SPORE) in Skin Cancer (New Haven, CT, USA) and grown in Opti-MEM medium supplemented with 5% FBS, 1% penicillin-streptomycin, fibroblast growth factor-2 (10 ng/ml), heparin (1 ng/ml), 0.1 μM dbcAMP, and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX). Designations of

“light,” “medium,” or “dark” are based on the skin color of the donor as per the Biospecimen Core. Before experiments, melanocytes were cultured in cAMP starvation media without dbcAMP and without IBMX for 24 hours.

Immunocytochemistry

Cells were cultured on sterile glass coverslips in 24-mm wells at 5.0×10^4 cells per coverslip in cAMP starvation media for 48 hours. Cells were then fixed with 3% (w/v) paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in Buffer A (125 mM sodium chloride, 10 mM sodium phosphate, 2 mM magnesium chloride) at -20°C for 5 min. Melanosomal labeling was performed using polyclonal antibodies against TRP1/TYRP1 [Abcam, 1:1000, catalog no. ab83774, lot no. GR272706–6; and Novus Biologicals (TA99), 1:200, catalog no. NBP2–32906, lot no. 7306–1P170816] and HMB45 [Melanoma Marker Antibody (HMB45), Santa Cruz Biotechnology, 1:200, catalog no. sc-59305, lot no. E1314]. Melanosome pH was measured using goat anti-dinitrophenol antibody (anti-DNP, Oxford Biomedical Research, 1:200, catalog no. D04, lot no. d4.111212) as described below. Fluorescence was detected after secondary staining with Alexa Fluor at the excitation/emission (Ex/Em) spectra indicated. We surveyed Alexa Fluor secondary antibodies over a range of Ex/Em spectra in melanocytes with a range of melanin contents to identify the best Ex/Em spectra. We found that Ex/Em spectra >500 nm were unaffected by melanin (fig. S3, L to N). Images were acquired using a Zeiss LSM 880 and analyzed using NIS-Elements AR 4.60 (Nikon) as described in the next section.

General immunofluorescence quantitation

Quantitative analyses were performed using the Object Count tool in Nikon AR 4.60. The entire cell was imaged to ensure that no specific region of the cell was favored; however, when analyzing each cell, an equivalent diameter (EqDiameter) was restricted to 1.85 to 15.00 pixels and circularity to 0.20 to 1.00 for optimal identification of individual puncta and exclusion of larger structures such as the Golgi apparatus. The lower intensity threshold limit of each fluorescence channel was defined as the intensity of the dimmest punctum returned using the 3 points circle threshold tool. The upper intensity threshold limit was set to the maximum value. This method assured that while the intensity of fluorescence changed between cell lines and conditions, all melanosome specific data were captured and subjected to analysis.

DAMP synthesis, imaging, and quantification

DAMP was synthesized and verified by nuclear magnetic resonance at our institutional Chemistry Core Facility and was prepared as a stock (1 mg/ml) in 80% ethanol. Cells were cultured on sterile glass coverslips in 24-mm wells at 5.0×10^4 cells per coverslip and treated or not for 4 hours with 30 μM KH7 or LRE1 in the presence or absence of the nonselective cAMP analog Sp-8-CPT-cAMPs (500 μM ; Biolog), the EPAC-selective cAMP analog 8-pHPT-2'-O-Me-cAMP (500 μM ; Biolog), the pan-EPAC inhibitor ESI-09 (10 μM ; EMD Millipore), the NDP-MSH (100 nM; Sigma-Aldrich), the PKA inhibitor PKI (1 μM ; Sigma-Aldrich) or H89 (10 μM ; Sigma-Aldrich), or cycloheximide (10 μM ; Sigma-Aldrich), as indicated. Cells were washed with fresh cAMP starvation media and incubated with 10 μM DAMP for 30 min, fixed with 3% (w/v) paraformaldehyde for 15 min at room

temperature, and washed with 50 mM ammonium chloride. After permeabilization with 0.1% Triton X-100 in Buffer A at -20°C for 5 min, cells were labeled with goat anti-DNP antibody (anti-DNP, Oxford Biomedical Research, 1:200, catalog no. D04, lot no. d4.111212). Melanosomes were identified using a mouse monoclonal antibody against TRP1/TYRP1 [Abcam, 1:1000, catalog no. ab83774, lot no. GR272706–6; and Novus Biologicals (TA99), 1:200, catalog no. NBP2–32906, lot no. 7306–1P170816] or HMB45 [Melanoma Marker Antibody (HMB45), Santa Cruz Biotechnology, 1:200, catalog no. sc-59305, lot no. E1314]. For most of the images, fluorescence was detected after secondary staining with Alexa Fluor 546 donkey anti-goat immunoglobulin G (IgG) antibody (Invitrogen, 1:1000, catalog no. A11056, lot no. 1714714) and Alexa Fluor 647 donkey anti-mouse IgG antibody (Invitrogen, 1:1000, catalog no. A31571, lot no. 1839633). We found minimal effect of melanin on the Ex/Em spectra of these secondary antibodies. Images were acquired using a Zeiss LSM 880 and analyzed using NIS-Elements AR 4.60 (Nikon).

Mean DAMP fluorescence intensity was measured for all DAMP⁺ puncta. Melanosomes were identified as HMB45⁺ or TYRP1⁺ puncta. Melanosome DAMP measurements were only recorded when colocalized with HMB45 or TYRP1, as indicated (fig. S3D). Frequency distributions were generated for each sample from the mean DAMP fluorescence intensity of all DAMP⁺ melanosomes. All analyses were performed on two replicate coverslips ($n = 15$ cells per coverslip), which were then pooled for each condition ($n = 30$ cells per condition) to ensure adequate power when generating frequency distributions and determining statistical significance. We found that to achieve the necessary power to detect a statistically significant pH change (P values; table S1), we needed to measure at least 15 cells, which allowed for the measurement of at least 1500 puncta and the detection of DAMP⁺ melanosomes at various stages.

LysoSensor DND-160 imaging and quantification

Cells were incubated with 1 μM LysoSensor DND-160 (Invitrogen, catalog no. L7545, lot no. 846175) for 5 min at 37°C . LysoSensor was excited at 405 nm, and its emission was detected at 417 to 483 nm (W1) and 490 to 530 nm (W2). The ratio of emissions (W1/W2) in LysoSensor-stained puncta was assigned to a pH value based on a calibration curve generated for each experiment using solutions containing 125 mM KCl, 25 mM NaCl, and 24 μM monensin and using varying concentrations of MES to adjust the pH to 4, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5. The fluorescence ratio was linear for pH 5.0 to 7.0.

Mean W1 and W2 fluorescence intensities at each punctum were measured and used to calculate the W1/W2 ratio. The W1/W2 ratio for each LysoSensor⁺ puncta was compared to the pH standard curve to generate a pH value, as described above. Frequency distributions were generated for each sample from the predicted pH values of LysoSensor⁺ organelles. All analyses were performed on two replicate coverslips ($n = 10$ cells per coverslip).

Transmission EM

As previously published (52), cell monolayers were fixed with a modified Karnovsky's fix (86) and a secondary fixation in reduced osmium tetroxide (87). After dehydration, the monolayers were embedded in an epon analog resin. En face ultrathin sections (65 nm) were

contrasted with lead citrate (88) and viewed on a JEM 1400 electron microscope (JEOL) operated at 100 kV. Digital images were captured on a Veleta 2K × 2K charge-coupled device camera (Olympus Soft Imaging Solutions).

Tritium in vivo tyrosinase assay

Tyrosinase activity of melanocytes in vivo was determined by measuring the amount of radioactive H₂O produced from L-[Ring-3,5-³H]-Tyrosine, as previously described (15). Mouse melanocytes were incubated in six-well plates with cAMP starvation media containing L-[Ring-3,5-³H]-Tyrosine (5 µCi/ml; PerkinElmer) for 4 or 8 hours. Media (1.5 ml) from each well were removed and centrifuged at 1200 rpm in a microfuge (Eppendorf 5154 D) for 5 min. Supernatant (1 ml) was combined with 1 ml of 0.1 M citric acid containing 10% (w/v) activated charcoal to remove excess tyrosine and then centrifuged at 12,000 rpm for 5 min. ³H activity of the supernatant was determined using a scintillation counter. In human cells, tyrosinase activity with and without pharmacologic inhibition of sAC was performed by incubating cells in six-well plates with media containing L-[Ring-3,5-³H]-Tyrosine (5 µCi/ml) and 30 µM KH7, 30 µM LRE1, or DMSO (vehicle control) for 8 hours. Media (1.5 ml) from each well were put through the same process as above. In all experiments, media incubated in parallel wells containing no cells were used as a negative control for tyrosinase activity.

Genetic deletion of melanocyte sAC in vivo

Tyr::CRE-ERT2 (89) mice were mated with *Adcy10/sAC^{fl/fl}* mice (39, 40). These mice were then backcrossed to the C3H/HeJ agouti (brown hair color) strain to generate progeny with brown hair color containing both eumelanin and pheomelanin. *Tyr::CRE-ERT2^{+/-}; sAC^{fl/fl}* mice were then mated with C3H/HeJ *sAC^{fl/fl}* or *sAC^{fl/+}* mice lacking the *Tyr::CRE-ERT2* cassette. The inducible knockout sAC allele was activated by painting the dorsum of all mice in the litter with 20 mM 4-hydroxytamoxifen on postnatal days 2, 3, and 4. Tamoxifen binds to the ERT2 protein fused to the Cre recombinase, thereby revealing a nuclear localization domain and allowing for Cre entry into the nucleus and gene recombination. Hair color during the first hair cycle was blindly evaluated using a (+) “light brown,” (++) “medium brown,” and (+++) “dark brown” scoring system. At 21 days, hair from the dorsum of each mouse was shaved for melanin quantitation (see below) or plucked for microscopic evaluation by a blinded observer, and then (after euthanasia), the epidermis was sampled for genotyping. As expected, roughly 25% of the mice were Cre⁺, and a subset had recombination of both sAC alleles (*n* = 7 mice). Although they had received tamoxifen, none of the Cre⁻ mice showed sAC allele rearrangement (*n* = 13 mice). The percentage of hairs with and without an agouti band was measured in a blinded fashion under a stereo microscope. The treated skin was submitted to an animal pathologist who performed histological evaluation of the epidermis in a blinded fashion.

Melanin analysis

All melanin quantitation was performed in a blinded fashion. Cell samples (0.2 to 1.15 million) were ultrasonicated in 400 µl of Milli-Q H₂O, or mouse hair was homogenized at a concentration of H₂O (10 mg/ml), and 100 µl of water suspensions of samples was subjected to alkaline hydrogen peroxide oxidation (90) and hydroiodic acid hydrolysis (91).

Eumelanin was analyzed as a specific degradation product pyrrole-2,3,5-tricarboxylic acid (PTCA) produced by the alkaline hydrogen peroxide oxidation, whereas pheomelanin was analyzed as the degradation product 4-amino-3-hydroxyphenylalanine (4-AHP) produced by the hydroiodic acid hydrolysis. Eumelanin and pheomelanin were calculated by multiplying the PTCA and 4-AHP contents by factors of 25 and 7, respectively (73).

Pharmacologic inhibition of SAC in vivo

Animal experiments were performed in accordance with the approved Institutional Animal Care and Use Committee protocol at Weill Cornell Medicine. Age- and gender-matched C3H/HeJ mice (female, 7 weeks old) were purchased from the Jackson laboratory. For the analysis of epidermal pigmentation, C3H/HeJ mouse ears were topically treated with 20 μ l of KH7 (42 mg/ml) or LRE1 (28 mg/ml) on the right ear, and DMSO (vehicle control) was topically applied on the left ear twice a day for 2 weeks. In parallel, a different group of mice was treated with DMSO on both ears. Ear skin was monitored daily for irritation and changes in pigmentation. After the final treatment, mice were euthanized, and the treated skin was submitted to an animal pathologist for blinded histological evaluation of the epidermis and specific staining (Fontana-Masson). This experiment was performed twice with three mice per cohort (for a total of six mice).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software). Proliferation rate was assessed by linear regression. LysoSensor calibration curves were generated using a third-order polynomial regression. Comparisons of median DAMP frequency distributions between conditions were analyzed using a Mann-Whitney *U* test. For all other data, comparison of means was performed using an unpaired, two-tailed *t* test (for two groups) or a one-way ANOVA with Tukey correction (for groups of three or more).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES AND NOTES

- Schallreuter KU, Kothari S, Chavan B, Spencer JD, Regulation of melanogenesis—Controversies and new concepts. *Exp. Dermatol.* 17, 395–404 (2008). [PubMed: 18177348]
- Schallreuter KU, Advances in melanocyte basic science research. *Dermatol. Clin.* 25, 283–291 (2007). [PubMed: 17662894]
- Law MH, Medland SE, Zhu G, Yazar S, Vinuela A, Wallace L, Shekar SN, Duffy DL, Bataille V, Glass D, Spector TD, Wood D; MuTHER Consortium, Gordon SD, Barbour JM, Henders AK, Hewitt AW, Montgomery GW, Sturm RA, Mackey DA, Green AC, Martin NG, MacGregor S, Genome-wide association shows that pigmentation genes play a role in skin aging. *J. Invest. Dermatol.* 137, 1887–1894 (2017). [PubMed: 28502801]
- Scherer D, Kumar R, Genetics of pigmentation in skin cancer—A review. *Mutat. Res.* 705, 141–153 (2010). [PubMed: 20601102]
- Beaumont KA, Liu YY, Sturm RA, The melanocortin-1 receptor gene polymorphism and association with human skin cancer. *Prog. Mol. Biol. Transl. Sci.* 88, 85–153 (2009). [PubMed: 20374726]
- Ito S, Wakamatsu K, Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: A comparative review. *Pigment Cell Res.* 16, 523–531 (2003). [PubMed: 12950732]
- Fistarol SK, Itin PH, Disorders of pigmentation. *J. Dtsch. Dermatol. Ges.* 8, 187–202 (2010). [PubMed: 19788584]
- Bellono NW, Oancea EV, Ion transport in pigmentation. *Arch. Biochem. Biophys.* 563, 35–41 (2014). [PubMed: 25034214]
- Schiaffino MV, Signaling pathways in melanosome biogenesis and pathology. *Int. J. Biochem. Cell Biol.* 42, 1094–1104 (2010). [PubMed: 20381640]
- Sitaram A, Marks MS, Mechanisms of protein delivery to melanosomes in pigment cells. *Physiology (Bethesda)* 27, 85–99 (2012). [PubMed: 22505665]
- Slominski A, Tobin DJ, Shibahara S, Wortsman J, Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol. Rev.* 84, 1155–1228 (2004). [PubMed: 15383650]
- Chao Y-K, Schludi V, Chen C-C, Butz E, Phuong Nguyen ON, Müller M, Krüger J, Kammerbauer C, Ben-Johny M, Vollmar AM, Berking C, Biel M, Wahl-Schott CA, Grimm C, TPC2 polymorphisms associated with a hair pigmentation phenotype in humans result in gain of channel function by independent mechanisms. *Proc. Natl. Acad. Sci. U.S.A.* 114, E8595–E8602 (2017). [PubMed: 28923947]
- Bellono NW, Escobar IE, Lefkovith AJ, Marks MS, Oancea E, An intracellular anion channel critical for pigmentation. *eLife* 3, e04543 (2014).
- Wilson S, Ginger RS, Dadd T, Gunn D, Lim F-L, Sawicka M, Sandel M, Schnetkamp PPM, Green MR, NCKX5, a natural regulator of human skin colour variation, regulates the expression of key pigment genes MC1R and alpha-MSH and alters cholesterol homeostasis in normal human melanocytes. *Adv. Exp. Med. Biol.* 961, 95–107 (2013). [PubMed: 23224873]
- Ancans J, Tobin DJ, Hoogduijn MJ, Smit NP, Wakamatsu K, Thody AJ, Melanosomal pH controls rate of melanogenesis, eumelanin/phaeomelanin ratio and melanosome maturation in melanocytes and melanoma cells. *Exp. Cell Res.* 268, 26–35 (2001). [PubMed: 11461115]
- Puri N, Gardner JM, Brilliant MH, Aberrant pH of melanosomes in pink-eyed dilution (p) mutant melanocytes. *J. Invest. Dermatol.* 115, 607–613 (2000). [PubMed: 10998131]
- Ito S, Suzuki N, Takebayashi S, Commo S, Wakamatsu K, Neutral pH and copper ions promote eumelanogenesis after the dopachrome stage. *Pigment Cell Melanoma Res.* 26, 817–825 (2013). [PubMed: 23844795]
- Fuller BB, Spaulding DT, Smith DR, Regulation of the catalytic activity of preexisting tyrosinase in black and Caucasian human melanocyte cell cultures. *Exp. Cell Res.* 262, 197–208 (2001). [PubMed: 11139343]
- Mitra D, Morgan A, Wang J, Hoang MP, Lo J, Guerrero CR, Lennerz JK, Mihm MC, Wargo JA, Robinson KC, Devi SP, Vanover JC, D'Orazio JA, McMahon M, Bosenberg MW, Haigis KM, Haber DA, Wang Y, Fisher DE, An ultraviolet-radiation-independent pathway to melanoma

- carcinogenesis in the red hair/fair skin background. *Nature* 491, 449–453 (2012). [PubMed: 23123854]
20. Ito S, Wakamatsu K, Human hair melanins: What we have learned and have not learned from mouse coat color pigmentation. *Pigment Cell Melanoma Res.* 24, 63–74 (2011). [PubMed: 20726950]
 21. Wakamatsu K, Kavanagh R, Kadekaro AL, Terzieva S, Sturm RA, Leachman S, Abdel-Malek Z, Ito S, Diversity of pigmentation in cultured human melanocytes is due to differences in the type as well as quantity of melanin. *Pigment Cell Res.* 19, 154–162 (2006). [PubMed: 16524431]
 22. Torres-Quesada O, Mayrhofer JE, Stefan E, The many faces of compartmentalized PKA signalosomes. *Cell. Signal.* 37, 1–11 (2017). [PubMed: 28528970]
 23. Tresguerres M, Levin LR, Buck J, Intracellular cAMP signaling by soluble adenylyl cyclase. *Kidney Int.* 79, 1277–1288 (2011). [PubMed: 21490586]
 24. Lefkimmatis K, Zaccolo M, cAMP signaling in subcellular compartments. *Pharmacol. Ther.* 143, 295–304 (2014). [PubMed: 24704321]
 25. Chen Y, Cann MJ, Litvin TN, Iourgenko V, Sinclair ML, Levin LR, Buck J, Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science* 289, 625–628 (2000). [PubMed: 10915626]
 26. Litvin TN, Kamenetsky M, Zarifyan A, Buck J, Levin LR, Kinetic properties of “soluble” adenylyl cyclase. Synergism between calcium and bicarbonate. *J. Biol. Chem.* 278, 15922–15926 (2003). [PubMed: 12609998]
 27. Zippin JH, Chen Y, Straub SG, Hess KC, Diaz A, Lee D, Tso P, Holz GG, Sharp GWG, Levin LR, Buck J, CO₂/HCO₃[−] and calcium-regulated soluble adenylyl cyclase as a physiological ATP sensor. *J. Biol. Chem.* 288, 33283–33291 (2013). [PubMed: 24100033]
 28. Zippin JH, Chen Y, Nahirney P, Kamenetsky M, Wuttke MS, Fischman DA, Levin LR, Buck J, Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains. *FASEB J.* 17, 82–84 (2003). [PubMed: 12475901]
 29. Wu KY, Zippin JH, Huron DR, Kamenetsky M, Hengst U, Buck J, Levin LR, Jaffrey SR, Soluble adenylyl cyclase is required for netrin-1 signaling in nerve growth cones. *Nat. Neurosci.* 9, 1257–1264 (2006). [PubMed: 16964251]
 30. Pastor-Soler N, Beaulieu V, Litvin TN, Silva ND, Chen Y, Brown D, Buck J, Levin LR, Breton S, Bicarbonate-regulated adenylyl cyclase (sAC) is a sensor that regulates pH-dependent V-ATPase recycling. *J. Biol. Chem.* 278, 49523–49529 (2003). [PubMed: 14512417]
 31. P unescu TG, Da Silva N, Russo LM, McKee M, Lu HAJ, Breton S, Brown D, Association of soluble adenylyl cyclase with the V-ATPase in renal epithelial cells. *Am. J. Physiol. Renal. Physiol.* 294, F130–F138 (2008). [PubMed: 17959750]
 32. Rahman N, Ramos-Espiritu L, Milner TA, Buck J, Levin LR, Soluble adenylyl cyclase is essential for proper lysosomal acidification. *J. Gen. Physiol.* 148, 325–339 (2016). [PubMed: 27670898]
 33. Zippin JH, Chadwick PA, Levin LR, Buck J, Magro CM, Soluble adenylyl cyclase defines a nuclear cAMP microdomain in keratinocyte hyperproliferative skin diseases. *J. Invest. Dermatol.* 130, 1279–1287 (2010). [PubMed: 20130594]
 34. Magro CM, Crowson AN, Desman G, Zippin JH, Soluble adenylyl cyclase antibody profile as a diagnostic adjunct in the assessment of pigmented lesions. *Arch. Dermatol.* 148, 335–344 (2012). [PubMed: 22105816]
 35. Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M, Yefanov A, Lee H, Zhang N, Robertson CL, Serova N, Davis S, Soboleva A, NCBI GEO: Archive for functional genomics data sets—Update. *Nucleic Acids Res.* 41, D991–D995 (2013). [PubMed: 23193258]
 36. Hoek K, Rimm DL, Williams KR, Zhao H, Ariyan S, Lin A, Kluger HM, Berger AJ, Cheng E, Trombetta ES, Wu T, Niinobe M, Yoshikawa K, Hannigan GE, Halaban R, Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. *Cancer Res.* 64, 5270–5282 (2004). [PubMed: 15289333]
 37. Ohi Y, Qin H, Hong C, Blouin L, Polo JM, Guo T, Qi Z, Downey SL, Manos PD, Rossi DJ, Yu J, Hebrok M, Hochedlinger K, Costello JF, Song JS, Ramalho-Santos M, Incomplete DNA

- methylation underlies a transcriptional memory of somatic cells in human iPS cells. *Nat. Cell Biol.* 13, 541–549 (2011). [PubMed: 21499256]
38. Muthusamy V, Duraisamy S, Matthew Bradbury C, Hobbs C, Curley DP, Nelson B, Bosenberg M, Epigenetic silencing of novel tumor suppressors in malignant melanoma. *Cancer Res.* 66, 11187–11193 (2006). [PubMed: 17145863]
 39. Chen J, Martinez J, Milner TA, Buck J, Levin LR, Neuronal expression of soluble adenylyl cyclase in the mammalian brain. *Brain Res.* 1518, 1–8 (2013). [PubMed: 23611875]
 40. Watson RL, Buck J, Levin LR, Winger RC, Wang J, Arase H, Muller WA, Endothelial CD99 signals through soluble adenylyl cyclase and PKA to regulate leukocyte transendothelial migration. *J. Exp. Med.* 212, 1021–1041 (2015). [PubMed: 26101266]
 41. Tamura A, Halaban R, Moellmann G, Cowan JM, Lerner MR, Lerner AB, Normal murine melanocytes in culture. *In Vitro Cell. Dev. Biol.* 23, 519–522 (1987). [PubMed: 3610949]
 42. Bin B-H, Bhin J, Yang SH, Shin M, Nam Y-J, Choi D-H, Shin DW, Lee A-Y, Hwang D, Cho E-G, Lee TR, Membrane-associated transporter protein (MATP) regulates melanosomal pH and influences tyrosinase activity. *PLOS ONE* 10, e0129273 (2015).
 43. Tabata H, Kawamura N, Sun-Wada G-H, Wada Y, Vacuolar-type H^+ -ATPase with the $\alpha 3$ isoform is the proton pump on premature melanosomes. *Cell Tissue Res.* 332, 447–460 (2008). [PubMed: 18408955]
 44. Anderson RG, Falck JR, Goldstein JL, Brown MS, Visualization of acidic organelles in intact cells by electron microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 81, 4838–4842 (1984). [PubMed: 6146980]
 45. Jimbow K, Park JS, Kato F, Hirotsaki K, Toyofuku K, Hua C, Yamashita T, Assembly, target-signaling and intracellular transport of tyrosinase gene family proteins in the initial stage of melanosome biogenesis. *Pigment Cell Res.* 13, 222–229 (2000). [PubMed: 10952389]
 46. 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.* 152, 809–824 (2001). [PubMed: 11266471]
 47. Taatjes DJ, Arendash-Durand B, von Turkovich M, Trainer TD, HMB-45 antibody demonstrates melanosome specificity by immunoelectron microscopy. *Arch. Pathol. Lab. Med.* 117, 264–268 (1993). [PubMed: 8442672]
 48. Kushimoto T, Basrur V, Valencia J, Matsunaga J, Vieira WD, Ferrans VJ, Muller J, Appella E, Hearing VJ, A model for melanosome biogenesis based on the purification and analysis of early melanosomes. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10698–10703 (2001). [PubMed: 11526213]
 49. Bitterman JL, Ramos-Espiritu L, Diaz A, Levin LR, Buck J, Pharmacological distinction between soluble and transmembrane adenylyl cyclases. *J. Pharmacol. Exp. Ther.* 347, 589–598 (2013). [PubMed: 24091307]
 50. Ramos-Espiritu L, Kleinboelting S, Navarrete FA, Alvau A, Visconti PE, Valsecchi F, Starkov A, Manfredi G, Buck H, Adura C, Zippin JH, van den Heuvel J, Glickman JF, Steegborn C, Levin LR, Buck J, Discovery of LRE1 as a specific and allosteric inhibitor of soluble adenylyl cyclase. *Nat. Chem. Biol.* 12, 838–844 (2016). [PubMed: 27547922]
 51. Bellono NW, Escobar IE, Oancea E, A melanosomal two-pore sodium channel regulates pigmentation. *Sci. Rep.* 6, 26570 (2016). [PubMed: 27231233]
 52. Christensen AE, Selheim F, de Rooij J, Dremier S, Schwede F, Dao KK, Martinez A, Maenhaut C, Bos JL, Genieser H-G, Døskeland SO, cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *J. Biol. Chem.* 278, 35394–35402 (2003). [PubMed: 12819211]
 53. Newton RA, Smit SE, Barnes CC, Pedley J, Parsons PG, Sturm RA, Activation of the cAMP pathway by variant human MC1R alleles expressed in HEK and in melanoma cells. *Peptides* 26, 1818–1824 (2005). [PubMed: 15992961]
 54. Cheli Y, Luciani F, Khaled M, Beuret L, Bille K, Gounon P, Ortonne J-P, Bertolotto C, Ballotti R, α MSH and cyclic AMP elevating agents control melanosome pH through a protein kinase A-independent mechanism. *J. Biol. Chem.* 284, 18699–18706 (2009). [PubMed: 19389708]
 55. Dremier S, Kopperud R, Døskeland SO, Dumont JE, Maenhaut C, Search for new cyclic AMP-binding proteins. *FEBS Lett.* 546, 103–107 (2003). [PubMed: 12829244]

56. Herraiz C, Garcia-Borrón JC, Jiménez-Cervantes C, Olivares C, MC1R signaling. Intracellular partners and pathophysiological implications. *Biochim. Biophys. Acta Mol. Basis Dis.* 1863, 2448–2461 (2017). [PubMed: 28259754]
57. Baljinnyam E, Umemura M, Chuang C, De Lorenzo MS, Iwatsubo M, Chen S, Goydos JS, Ishikawa Y, Whitelock JM, Iwatsubo K, Epac1 increases migration of endothelial cells and melanoma cells via FGF2-mediated paracrine signaling. *Pigment Cell Melanoma Res.* 27, 611–620 (2014). [PubMed: 24725364]
58. Baljinnyam E, De Lorenzo MS, Xie L-H, Iwatsubo M, Chen S, Goydos JS, Nowycky MC, Iwatsubo K, Exchange protein directly activated by cyclic AMP increases melanoma cell migration by a Ca^{2+} -dependent mechanism. *Cancer Res.* 70, 5607–5617 (2010). [PubMed: 20551063]
59. Baljinnyam E, Iwatsubo K, Kurotani R, Wang X, Ulucan C, Iwatsubo M, Lagunoff D, Ishikawa Y, Epac increases melanoma cell migration by a heparan sulfate-related mechanism. *Am. J. Physiol. Cell Physiol.* 297, C802–C813 (2009). [PubMed: 19657062]
60. Zhu Y, Chen H, Boulton S, Mei F, Ye N, Melacini G, Zhou J, Cheng X, Biochemical and pharmacological characterizations of ESI-09 based EPAC inhibitors: Defining the ESI-09 “therapeutic window”. *Sci. Rep.* 5, 9344 (2015). [PubMed: 25791905]
61. Chen H, Ding C, Wild C, Liu H, Wang T, White MA, Cheng X, Zhou J, Efficient synthesis of ESI-09, a novel non-cyclic nucleotide EPAC antagonist. *Tetrahedron Lett.* 54, 1546–1549 (2013). [PubMed: 23459418]
62. Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG, Døskeland SO, Blank JL, Bos JL, A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat. Cell Biol.* 4, 901–906 (2002). [PubMed: 12402047]
63. Yano N, Suzuki D, Endoh M, Zhao TC, Padbury JF, Tseng Y-T, A novel phosphoinositide 3-kinase-dependent pathway for angiotensin II/AT-1 receptor-mediated induction of collagen synthesis in MES-13 mesangial cells. *J. Biol. Chem.* 282, 18819–18830 (2007). [PubMed: 17493931]
64. Wakamatsu K, Nagao A, Watanabe M, Nakao K, Ito S, Pheomelanogenesis is promoted at a weakly acidic pH. *Pigment Cell Melanoma Res.* 30, 372–377 (2017). [PubMed: 28271633]
65. Ambrosio AL, Boyle JA, Aradi AE, Christian KA, Di Pietro SM, TPC2 controls pigmentation by regulating melanosome pH and size. *Proc. Natl. Acad. Sci. U.S.A.* 113, 5622–5627 (2016). [PubMed: 27140606]
66. Nordlund JJ, Collins CE, Rheins LA, Prostaglandin E2 and D2 but not MSH stimulate the proliferation of pigment cells in the pinna epidermis of the DBA/2 mouse. *J. Invest. Dermatol.* 86, 433–437 (1986). [PubMed: 3528310]
67. Donnelly MP, Paschou P, Grigorenko E, Gurwitz D, Barta C, Lu R-B, Zhukova OV, Kim J-J, Siniscalco M, New M, Li H, Kajuna SLB, Manolopoulos VG, Speed WC, Pakstis AJ, Kidd JR, Kidd KK, A global view of the *OCA2-HERC2* region and pigmentation. *Hum. Genet.* 131, 683–696 (2012). [PubMed: 22065085]
68. Boya P, Lysosomal function and dysfunction: Mechanism and disease. *Antioxid. Redox Signal.* 17, 766–774 (2012). [PubMed: 22098160]
69. Elder DE, Dysplastic naevi: An update. *Histopathology* 56, 112–120 (2010). [PubMed: 20055909]
70. Morgan AM, Lo J, Fisher DE, How does pheomelanin synthesis contribute to melanomagenesis?: Two distinct mechanisms could explain the carcinogenicity of pheomelanin synthesis. *Bioessays* 35, 672–676 (2013). [PubMed: 23650156]
71. Magro CM, Yang S-E, Zippin JH, Zembowicz A, Expression of soluble adenylyl cyclase in lentigo maligna: Use of immunohistochemistry with anti-soluble adenylyl cyclase antibody (R21) in diagnosis of lentigo maligna and assessment of margins. *Arch. Pathol. Lab. Med.* 136, 1558–1564 (2012). [PubMed: 23194049]
72. Juhász MLW, Marmur ES, Reviewing challenges in the diagnosis and treatment of lentigo maligna and lentigo-maligna melanoma. *Rare Cancers Ther.* 3, 133–145 (2015). [PubMed: 27182482]
73. D’Ischia M, Wakamatsu K, Cicoira F, Di Mauro E, Garcia-Borrón JC, Commo S, Galván I, Ghanem G, Kenzo K, Meredith P, Pezzella A, Santato C, Sarna T, Simon JD, Zecca L, Zucca FA, Napolitano A, Ito S, Melanins and melanogenesis: From pigment cells to human health and

technological applications. *Pigment Cell Melanoma Res.* 28, 520–544 (2015). [PubMed: 26176788]

74. Ivonnet P, Salathe M, Conner GE, Hydrogen peroxide stimulation of CFTR reveals an Epac-mediated, soluble AC-dependent cAMP amplification pathway common to GPCR signalling. *Br. J. Pharmacol.* 172, 173–184 (2015). [PubMed: 25220136]
75. Middelhaufe S, Leipelt M, Levin LR, Buck J, Steegborn C, Identification of a haem domain in human soluble adenylyl cyclase. *Biosci. Rep.* 32, 491–499 (2012). [PubMed: 22775536]
76. Reemann P, Reimann E, Ilmjärvi S, Porosaar O, Silm H, Jaks V, Vasar E, Kingo K, Kõks S, Melanocytes in the skin—Comparative whole transcriptome analysis of main skin cell types. *PLOS ONE* 9, e115717 (2014).
77. Motiani RK, Tanwar J, Raja DA, Vashisht A, Khanna S, Sharma S, Srivastava S, Sivasubbu S, Natarajan VT, Gokhale RS, STIM1 activation of adenylyl cyclase 6 connects Ca^{2+} and cAMP signaling during melanogenesis. *EMBO J.* 37, e97597 (2018).
78. Cirillo G, Marini R, Ito S, Wakamatsu K, Scianguetta S, Bizzarri C, Romano A, Grandone A, Perrone L, Cappa M, Miraglia del Giudice E, Lack of red hair phenotype in a North-African obese child homozygous for a novel *POMC* null mutation: Nonsense-mediated decay RNA evaluation and hair pigment chemical analysis. *Br. J. Dermatol.* 167, 1393–1395 (2012). [PubMed: 22612534]
79. Clément K, Dubern B, Mencarelli M, Czernichow P, Ito S, Wakamatsu K, Barsh GS, Vaisse C, Leger J, Unexpected endocrine features and normal pigmentation in a young adult patient carrying a novel homozygous mutation in the *POMC* gene. *J. Clin. Endocrinol. Metab.* 93, 4955–4962 (2008). [PubMed: 18765507]
80. Slominski A, Plonka PM, Pisarchik A, Smart JL, Tolle V, Wortsman J, Low MJ, Preservation of eumelanin hair pigmentation in proopiomelanocortin-deficient mice on a nonagouti (a/a) genetic background. *Endocrinology* 146, 1245–1253 (2005). [PubMed: 15564334]
81. Li L, Hu D-N, Zhao H, McCormick SA, Nordlund JJ, Boissy RE, Uveal melanocytes do not respond to or express receptors for α -melanocyte-stimulating hormone. *Invest. Ophthalmol. Vis. Sci.* 47, 4507–4512 (2006). [PubMed: 17003446]
82. Liu F, Wen B, Kayser M, Colorful DNA polymorphisms in humans. *Semin. Cell Dev. Biol.* 24, 562–575 (2013). [PubMed: 23587773]
83. Sturm RA, Larsson M, Genetics of human iris colour and patterns. *Pigment Cell Melanoma Res.* 22, 544–562 (2009). [PubMed: 19619260]
84. Chen K, Minwalla L, Ni L, Orlow SJ, Correction of defective early tyrosinase processing by bafilomycin A1 and monensin in pink-eyed dilution melanocytes. *Pigment Cell Res.* 17, 36–42 (2004). [PubMed: 14717843]
85. Halaban R, Pomerantz SH, Marshall S, Lambert DT, Lerner AB, Regulation of tyrosinase in human melanocytes grown in culture. *J. Cell Biol.* 97, 480–488 (1983). [PubMed: 6411733]
86. Ito S, Karnovsky MJ, Formaldehyde-glutaraldehyde fixatives containing trinitro compounds. *J. Cell Biol.* 39, 168A–169A (1968).
87. de Bruijn WC, Glycogen, its chemistry and morphologic appearance in the electron microscope: I. A modified OsO₄ fixative which selectively contrasts glycogen. *J. Ultrastruct. Res.* 42, 29–50 (1973). [PubMed: 4567407]
88. Venable JH, Coggeshall R, A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* 25, 407–408 (1965). [PubMed: 14287192]
89. Bosenberg M, Muthusamy V, Curley DP, Wang Z, Hobbs C, Nelson B, Nogueira C, Horner II JW, DePinho R, Chin L, Characterization of melanocyte-specific inducible Cre recombinase transgenic mice. *Genesis* 44, 262–267 (2006). [PubMed: 16676322]
90. Ito S, Nakanishi Y, Valenzuela RK, Brilliant MH, Kolbe L, Wakamatsu K, Usefulness of alkaline hydrogen peroxide oxidation to analyze eumelanin and pheomelanin in various tissue samples: Application to chemical analysis of human hair melanins. *Pigment Cell Melanoma Res.* 24, 605–613 (2011). [PubMed: 21535429]
91. Wakamatsu K, Ito S, Rees JL, The usefulness of 4-amino-3-hydroxyphenylalanine as a specific marker of pheomelanin. *Pigment Cell Res.* 15, 225–232 (2002). [PubMed: 12028587]

Fig. 1. sAC regulates melanosome pH in mouse and human melanocytes.

(A) Confocal microscopic images of DAMP (green) and TYRP1 (red) immunofluorescence in *sAC^{FF}* [floxed/floxed (FF), top] and *sAC^{KO}* [knockout (KO), bottom] mouse melanocytes.

(B) Frequency distribution (left) and median (right) DAMP fluorescence intensity at TYRP1⁺ (as detected by the TA99 antibody) melanosomes in *sAC^{FF}* (FF, black squares or bar) and *sAC^{KO}* (KO, red circles or bar) melanocytes. arb. units, arbitrary units. (C)

Confocal microscopic images of DAMP (green) and HMB45 (red) immunofluorescence in *sAC^{FF}* (FF, top) and *sAC^{KO}* (KO, bottom) mouse melanocytes. (D) Frequency distribution (left) and median (right) DAMP fluorescence intensity at HMB45⁺ melanosomes (left) in

sAC^{FF} (FF, black squares or bar) and *sAC^{KO}* (KO, red circles or bar) melanocytes. (E) Confocal microscopic images of DAMP (green) and HMB45 (red) immunofluorescence in *sAC^{FF}* cells treated with vehicle control (FF, top) or 30 μ M KH7 (FF + KH7, bottom).

(F) Frequency distribution (left) and median (right) DAMP fluorescence intensity at HMB45⁺ melanosomes (left) in *sAC^{FF}* cells treated with vehicle control (FF, black squares or bar) or with 30 μ M KH7 (FF + KH7, blue triangles or bar) or LRE1 (FF + LRE1, gray Xs or bar).

(G to I) Frequency distribution (left) and median (right) DAMP fluorescence intensity at

HMB45⁺ melanosomes in human melanocytes derived from patients with lighter skin tone treated with vehicle control [C38 (G), C226 (H), C532 (I); black squares or bar] or with 30 μ M KH7 [C38 + KH7 (G), C226 + KH7 (H), C532 + KH7 (I); blue triangles or bar]. (B, D, and F to I) Data are from the experiments performed on cells on four distinct coverslips per condition, where at least 15 cells and at least 1000 melanosomes were analyzed per coverslip. (B, D, F, and G to I) Mann-Whitney *U* test, *****P* < 0.001. Scale bars, 10 μ m.

Fig. 2. cAMP rescues the increase in melanosome pH after loss of sAC activity in mouse and human melanocytes.

(A) Frequency distribution (left) and median (right) of DAMP fluorescence intensity at HMB45⁺ melanosomes in *sAC*^{FF} (FF, black squares or bar), *sAC*^{KO} (KO, red circles or bars), and cAMP-treated *sAC*^{KO} (KO + cAMP, green triangles or bars) mouse melanocytes. (B) Frequency distribution (left) and median (right) of DAMP fluorescence intensity at HMB45⁺ melanosomes in *sAC*^{FF} (FF, black squares or bar), KH7-treated *sAC*^{FF} (FF + KH7, blue triangles or bar), and KH7 + cAMP-treated *sAC*^{FF} (FF + KH7 + cAMP, green diamonds or bar) mouse melanocytes. (C) Frequency distribution (left) and median (right) of DAMP fluorescence at HMB45⁺ melanosomes in “light” human melanocytes (C38) after treatment with vehicle control (black squares or bar), KH7 (blue triangles or bar), or KH7 + cAMP (green diamonds or bar). (A to C) Data are from the experiments performed on cells on four distinct coverslips per condition, where at least 15 cells and at least 1000 melanosomes were analyzed per coverslip. “+KH7” consisted of incubation with 30 μ M KH7 for 4 hours. “+KH7+cAMP” treatment consisted of incubation with 30 μ M KH7 and 500 μ M nonselective cAMP analog [Sp-8-CPT-cAMPs] for 4 hours. One-way analysis of variance (ANOVA) with Tukey post hoc analysis, *** P < 0.005, **** P < 0.001.

Fig. 3. Regulation of melanosome pH by EPAC.

(A) Frequency distribution (left) and median (right) of DAMP fluorescence intensity at HMB45⁺ melanosomes in *sAC*^{FF} (FF, black squares or bars), *sAC*^{KO} (KO, red circles or bars), and ESI-09–treated *sAC*^{FF} (FF + ESI-09, purple diamonds or bars) mouse melanocytes. (B and C) Frequency distribution (left) and median (right) of DAMP fluorescence intensity at HMB45⁺ melanosomes in “light” human melanocytes after treatment with vehicle control [dimethyl sulfoxide (DMSO), C226 (B), C537 (C); black squares or bars], KH7 [C226 + KH7 (B), C537 + KH7 (C); blue triangles or bars], or ESI-09 [C226 + ESI-09 (B), C537 + ESI-09 (C); purple Xs or bars]. (D) Frequency distribution (left) and median (right) of DAMP fluorescence intensity at HMB45⁺ melanosomes in *sAC*^{FF} (FF, black squares and bars) and *sAC*^{KO} melanocytes in the absence (KO, red circles or bars) or presence of the EPAC-selective cAMP analog (KO + cAMP^{EPAC}, pink Xs or bars). (E) Frequency distribution (left) and median (right) of DAMP fluorescence intensity at HMB45⁺ melanosomes of *sAC*^{FF} mouse melanocytes after a 4-hour treatment with vehicle (DMSO; FF, black squares or bars), KH7 alone (FF + KH7, blue triangles or bars), or KH7 + the EPAC-selective cAMP analog (FF + KH7 + cAMP^{EPAC}, pink Xs or bars). (F) Frequency distribution (left) or median (right) of DAMP fluorescence intensity at HMB45 melanosomes in “light” human melanocytes after a 4-hour treatment with vehicle control (C226, black squares or bars), KH7 alone (C226 + KH7, blue triangles or bars), or KH7 + the EPAC-selective cAMP analog (C226 + KH7 + cAMP^{EPAC}, pink Xs or bars). (A to F) Data are from the experiments performed on cells on four distinct coverslips per condition, where at least 15 cells and at least 1000 melanosomes were analyzed per coverslip. KH7 treatment consisted of incubation with 30 μ M KH7 for 4 hours. ESI-09 treatment consisted of incubation with 10 μ M ESI-09 for 4 hours. cAMP treatment consisted of incubation with 500 μ M EPAC-selective cAMP analog (8-phPT-2'-O-Me-cAMP) for 4 hours. Mann-Whitney *U* test, ***P* < 0.01, *****P* < 0.001. Scale bars, 10 μ m.

Fig. 4. sAC regulates tyrosinase activity in cells independently of gene expression.

(A) Tyrosinase activity of sAC^{FF} (FF) and sAC^{KO} (KO) mouse melanocytes as measured by production of $^3\text{H}_2\text{O}$ from ^3H -tyrosine per cell over 4 hours ($n = 3$ independent experiments; each point is the average of duplicate determinations). DPM, disintegrations per minute. (B) Tyrosinase activity of “light” human melanocytes (C226, C38, and C611) treated with KH7 (30 μM), LRE1 (50 μM), or vehicle (DMSO) for 8 hours as measured by $^3\text{H}_2\text{O}$ production per cell ($n = 3$ distinct human cell lines; each point is the average of duplicate determinations). (C) Western blot for tyrosinase and MITF in sAC^{FF} (FF) and sAC^{KO} (KO) mouse melanocytes (left). Average quantitation of total tyrosinase immunoreactivity in sAC^{FF} (FF) and sAC^{KO} (KO) mouse melanocytes was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; right) ($n = 3$ independent experiments; each experiment is the average of duplicate determinations). (D and E) Electron microscopy (EM) evaluation of melanosome morphology in sAC^{FF} (FF) and sAC^{KO} (KO) mouse melanocytes (D) and quantitation of stage IV melanosomes (organelles circled in red) per field expressed as percentage of total melanosomes (E) in sAC^{FF} (FF, black bars) and sAC^{KO} (KO, red bars) mouse melanocytes ($n = 3$ experiments, with 15 cells examined per experiment). (A and E) Student’s t test, (B) one-way ANOVA with Tukey post hoc analysis; $*P < 0.05$. Scale bars, 1 μm .

Fig. 5. sAC regulates melanin synthesis in mouse and human melanocytes.

(A) Cellular eumelanin content in sAC^{FF} (FF, black bar) and sAC^{KO} (KO, red bar) mouse melanocytes. Average of triplicate determinations per experiment ($n = 3$ experiments). Below are representative cellular pellets for each condition. (B and C) Cellular eumelanin level of “light” (B, $n = 8$) and “dark” (C, $n = 5$) human melanocytes treated with KH7 (30 μ M, blue bars) or vehicle (DMSO, black and gray bars) for 48 hours expressed as fold over vehicle control. Triplicate determinations were performed for each cell line. n is number of distinct human primary cell lines. Representative cell pellets pictured below. (D) Cellular eumelanin content in sAC^{FF} (FF, black bars) and sAC^{KO} (KO, red bars) mouse melanocytes in the absence (–) or presence (+) of NDP-MSH (MSH, 10 nM) for 72 hours. Average of triplicate determinations per experiment ($n = 3$ experiments). Asterisks above the bars correspond to comparison of – to + MSH conditions in each genotype. Below are representative cellular pellets for each condition. (E) Cellular eumelanin level of “light” human melanocytes ($n = 3$) treated with ESI-09 (10 μ M, purple bar) or vehicle (DMSO, black bar) for 48 hours expressed as fold over vehicle control. Triplicate determinations were performed for each line. n is number of distinct human primary cell lines. Representative cell pellets pictured below. (F) Cellular pheomelanin content in sAC^{FF} (FF, black bar) and sAC^{KO} (KO, red bar) mouse melanocytes. Average of triplicate determinations per experiment ($n = 3$ experiments). (G) Ratio of pheomelanin to eumelanin in sAC^{FF} (FF, black bar) and sAC^{KO} (KO, red bar) mouse melanocytes. Average of triplicate determinations per experiment ($n = 3$ experiments). (A to G) Student’s t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$.

Fig. 6. sAC regulates pigmentation in mice.

(A) Representative photographs of C3H/HeJ *Tyr::CRE-ERT2⁺;sAC^{fl/fl}* (wild type, *sAC^{fl/fl}* left pair) and C3H/HeJ *Tyr::CRE-ERT2⁺;sAC^{-/-}* (*sAC^{KO}*, right pair) mice showing a darkening of hair after loss of sAC in melanocytes. (B) Average percentage of total AWL hairs with agouti banding in *sAC^{fl/fl}* (FF, black bar; *n* = 13 mice) and *sAC^{-/-}* (KO, red bar; *n* = 7 mice). (C) Average ratio of pheomelanin to eumelanin content in hair from *Tyr::CRE-ERT2⁺;sAC^{fl/fl}* (FF, black bar; *n* = 13 mice) and *Tyr::CRE-ERT2⁺;sAC^{-/-}* (KO, red bar; *n* = 7 mice) mice. Student's *t* test; **P* < 0.05, ***P* < 0.01. (D) Representative photographs of mouse ears (top inset) and accompanying Fontana-Masson stain (bottom inset) after treatment with vehicle (DMSO) on both ears (left; *n* = 6 mice), vehicle on the left ear and LRE1 on the right ear (middle; *n* = 6 mice), or vehicle on the left ear and KH7 on the right ear (right; *n* = 6 mice) twice daily for 2 weeks. Arrowheads indicate positive Fontana-Masson staining of melanin content in epidermis. Topical treatments were performed with 20 μ l of KH7 (42 mg/ml), LRE1 (28 mg/ml), or vehicle alone (DMSO). Scale bar, 50 μ m. (E) Schematic of sAC- and tmAC-dependent cAMP signaling domains in melanocytes. MC1R (gray box at plasma membrane) binds to MSH (blue circle), leading to activation of tmACs (brown box at plasma membrane). tmAC-dependent cAMP activates PKA, leading to CREB (cAMP response element-binding protein)-dependent *MITF* expression and, ultimately, increased abundance of TYR (gray transmembrane melanosome protein),

tyrosinase). sAC (blue oval), which responds to changes in HCO_3^- , ATP, and Ca^{2+} (gray circles), stimulates EPAC, leading to altered melanosome pH, likely by regulating melanosome ion channels, and changes in the activity of pH-sensitive tyrosinase. pH regulation of tyrosinase differentially affects eumelanin and pheomelanin synthesis.

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