A melanosomal two-pore sodium channel regulates pigmentation

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Intracellular organelles mediate complex cellular functions that often require ion transport across their membranes. Melanosomes are organelles responsible for the synthesis of the major mammalian pigment melanin. Defects in melanin synthesis result in pigmentation defects, visual deficits, and increased susceptibility to skin and eye cancers. Although genes encoding putative melanosomal ion transporters have been identified as key regulators of melanin synthesis, melanosome ion transport and its contribution to pigmentation remain poorly understood. Here we identify two-pore channel 2 (TPC2) as the first reported melanosomal cation conductance by directly patch-clamping skin and eye melanosomes. TPC2 has been implicated in human pigmentation and melanoma, but the molecular mechanism mediating this function was entirely unknown. We demonstrate that the vesicular signaling lipid phosphatidylinositol bisphosphate PI(3,5)P₂ modulates TPC2 activity to control melanosomal membrane potential, pH, and regulate pigmentation.

Intracellular ion channels are important for various functions in organelles that underlie critical cellular and organismal physiology, but the identity and function of many organelar ion channels is yet to be discovered. The melanosome is a lysosome-related organelle that produces melanin in pigment cells of the skin and eye. Defects in melanin synthesis and storage result in reduced pigmentation in the skin, hair and eyes, leading to decreased protection against ultraviolet radiation, visual deficits, and increased susceptibility to skin and eye cancers. Although a number of genes encoding putative melanosomal ion transport proteins are essential for pigmentation, ionic signaling in melanosomes remains poorly understood.

Phosphatidylinositol 3,5-bisphosphate [PI(3,5)P₂] is a low abundance organelar phospholipid (0.1% or less of total cellular phospholipids) that plays an important role in organelle biogenesis and has been implicated as an endolysosomal cation channel agonist. Defects in PI(3,5)P₂ metabolism result in neurodegenerative disorders and are associated with pigmentation defects. Recently, the direct electrophysiological characterization of several PI(3,5)P₂-regulated endolysosomal cation channels has uncovered their role in organelle biogenesis, cellular metabolism, and endocytosis.

Two-pore channels (TPC1 and TPC2) are endolysosomal cation channels directly regulated by PI(3,5)P₂ and implicated in pigmentation. The primary function of two-pore channels has been disputed: TPCs were first characterized as nicotinic acid adenine dinucleotide phosphate (NAADP)-activated Ca²⁺ channels in acidic organelles, while recent electrophysiological recordings from endolysosomes describe TPCs as Na⁺-selective cation channels. As suggested by the conflicting findings, much remains to be learned about the function and regulation of TPCs. TPC2 has been implicated in pigmentation as a determinant of human hair color, a regulator of pigmentation in Xenopus oocytes, and a factor in the development of melanoma, but the mechanisms underlying TPC2-mediated regulation of pigmentation remain unclear.

Here, using direct patch-clamp recordings from skin and eye melanosomes, we report the first melanosomal cation conductance mediated by two-pore channel 2 (TPC2). We show that the organelar signaling lipid PI(3,5)P₂, an important regulator of pigmentation, activates a TPC2-mediated Na⁺-selective current in melanosomes. We found that TPC2 serves as a negative regulator of pigmentation by increasing melanosomal membrane potential and acidity to decrease cellular melanin content. Thus, in pigment cells, TPC2 mediates a PI(3,5)P₂-activated melanosomal Na⁺ channel to regulate pigmentation by modulating the melanosome’s membrane voltage and pH.

Results

PI(3,5)P₂ activates a large inward current in the melanosome ($I_{pp2}$). To investigate endogenous melanosomal ion channels, we measured native currents by direct patch-clamp recordings of melanosomes from

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a dermal melanocyte cell line derived from mice deficient in ocular albinism 1 (Oa1−/−)18, in which melanosomes are enlarged up to 1.5 μm diameter19. Melanosomes dissected from Oa1−/− melanocytes were patch-clamped using a NaCl-based luminal solution and KGluс-based cytoplasmic solution (scale bar = 10 μm). Application of PI(3,5)P2 activated an inward current (IPIP2) and shifted the Erev in the positive direction (red) (representative of 7 melanosomes). (c) Substituting luminal Cl− for Gluc− reduced the outwardly rectifying basal current amplitude and shifted Erev in the positive direction. Application of PI(3,5)P2 activates an inward current similar to IPIP2 (representative of 5 melanosomes, p < 0.0001 for the difference in current measured at −120 mV before and after PI(3,5)P2). (d) Melanosomes dissected from freshly isolated bullfrog RPE were used for patch-clamp experiments (scale bar = 10 μm). (e) In a representative RPE melanosome PI(3,5)P2 activated an inwardly rectifying current similar to IP2 measured in dermal melanosomes. The PI(3,5)P2-activated component of the current (red) obtained by subtracting the basal current from IPIP2 was inward rectifying, with a positive reversal potential (representative of 3 melanosomes). (f) Substituting luminal Cl− for Gluc− reduced the outwardly rectifying basal current (black) and revealed the inwardly rectifying current activated by PI(3,5)P2 (blue) (representative of 3 melanosomes, p < 0.0001 for the difference in current measured at −120 mV before and after PI(3,5)P2).
We next tested if the PI(3,5)P₂-dependent current could also be measured in melanosomes found in the retinal pigment epithelium (RPE) of the eye. By patch-clamping melanosomes from RPE cells of the American bullfrog, *Lithobates catesbeianus* (Fig. 1d), which are larger than those from mammalian RPE cells, we measured a current with properties similar to dermal melanosomal *I*₁*₉₅⁺. *I*₁*₉₅⁺ activated an inward current that did not affect *I*₁basal (Fig. 1e). When basal currents were subtracted from these recorded in the presence of PI(3,5)P₂, an inward current with a very positive Erev was revealed (*I*₁PI(3,5)P₂) (Fig. 1e). Substituting luminal Cl⁻ with Gluc⁻ also reduced *I*₁basal and isolated *I*₁PI(3,5)P₂ (Fig. 1f).

*I*₁PI(3,5)P₂ has properties similar to endolysosomal TPC2. To further characterize the properties of *I*₁PI(3,5)P₂, we used a Gluc⁻-based luminal solution to block outwardly rectifying Cl⁻-dependent currents while measuring inward *I*₁PI(3,5)P₂. (b) 2 μM PI(3,5)P₂, but not the TRPML1 agonist ML-SA1 (25 μM) activated *I*₁PI(3,5)P₂ in a representative melanosome. Average fold increase in current density measured at −120 mV (± s.e.m., n = 3 melanosomes, p < 0.001 for PI(3,5)P₂- vs. ML-SA1-elicited currents). (c) In a representative melanosome, *I*₁PI(3,5)P₂ was inhibited by the TPC antagonist verapamil (150 μM). Average fold increase in current density measured at −120 mV (± s.e.m., n = 4 melanosomes, p < 0.0001 for *I*₁PI(3,5)P₂ vs. *I*₁PI(3,5)P₂ in the presence of verapamil). (d) In a representative melanosome, PI(3,5)P₂ activated an inward current in response to negative voltage steps; no significant PI(3,5)P₂-dependent currents were elicited by depolarizing voltages (representative of 3 melanosomes, p < 0.001 for basal currents vs *I*₁PI(3,5)P₂). (e) PI(3,5)P₂ activated-currents were selective for Na⁺, but not K⁺, Ca²⁺, or the impermeant cation NMDG⁺. Currents were recorded from the same melanosome with 140 mM luminal NaGluc while substituting symmetrical concentrations of cytoplasmic K⁺, NMDG⁺, or 100 mM Ca²⁺ in a Gluc⁻-based solution. (f) *I*₁PI(3,5)P₂ permeability ratios based on Erev measurements (± s.e.m., n = 4 melanosomes, p < 0.0001 for K⁺, NMDG⁺ or Ca²⁺ vs. Na⁺).

We next tested if the PI(3,5)P₂-dependent current could also be measured in melanosomes found in the retinal pigment epithelium (RPE) of the eye. By patch-clamping melanosomes from RPE cells of the American bullfrog, *Lithobates catesbeianus* (Fig. 1d), which are larger than those from mammalian RPE cells, we measured a current with properties similar to dermal melanosomal *I*₁PI(3,5)P₂: PI(3,5)P₂ activated an inward current that did not affect *I*₁basal (Fig. 1e). When basal currents were subtracted from these recorded in the presence of PI(3,5)P₂, an inward current with a very positive Erev was revealed (*I*₁PI(3,5)P₂) (Fig. 1e). Substituting luminal Cl⁻ with Gluc⁻ also reduced *I*₁basal and isolated *I*₁PI(3,5)P₂ (Fig. 1f).

*I*₁PI(3,5)P₂ has properties similar to endolysosomal TPC2. To further characterize the properties of *I*₁PI(3,5)P₂, we used a Gluc⁻-based luminal solution to block the contribution of the Cl⁻-dependent outwardly rectifying current (*I*₁basal). In endolysosomes PI(3,5)P₂, but not PI(4,5)P₂, has been shown to activate both TRPML and TPCs⁴,⁵. To test if phosphoinositide regulation of melanosomal *I*₁PI(3,5)P₂ is specific to the organellar species of PIP₂, we bath-applied the plasma membrane specific PI(4,5)P₂ while in whole-melanosome configuration. Basal currents were unaffected by PI(4,5)P₂ treatment, but, following a wash period, a large inward current was elicited by application of PI(3,5)P₂ to the same melanosome (Fig. 2a). To test if TRPML channels contribute to the PI(3,5)P₂-dependent inward current, we used the TRPML-specific agonist ML-SA1⁷. ML-SA1 treatment did not elicit a significant increase in basal currents, while subsequent application of PI(3,5)P₂ evoked large inward currents in the same melanosomes (Fig. 2b). Additionally, acidifying luminal pH from 6.8 to 4.6, a known regulator of TRPML channels, had no effect on *I*₁PI(3,5)P₂ (Supplementary Fig. S1c). Treatment with verapamil, however, an antagonist of TPCs⁵,⁹, nearly abolished *I*₁PI(3,5)P₂ in both dermal (Fig. 2c) and RPE melanosomes (Supplementary Fig. S1b), suggesting that TPCs might contribute to *I*₁PI(3,5)P₂.

Since two types of endolysosomal TPCs have been characterized, TPC1 and TPC2, we wondered if a specific subtype mediates *I*₁PI(3,5)P₂. Because TPC1 only is highly sensitive to luminal pH and *I*₁PI(3,5)P₂ is insensitive to changes...
ureable tail currents in melanosomes, but PI(3,5)P2 application activated an inward current (Fig. 2d), suggesting more selective for Na ions in the presence of cytoplasmic PI(3,5)P2. Erev for IPIP2 was near 0 mV under symmetrical Na 
Gluc calculated permeability ratios (Pxo/PNa) show that IPIP2 is with properties similar to TPC2, previously described as a voltage-independent endolysosomal Na 
protein 1 (TYRP1) and melanin-positive compartments (TPC2 overlap with melanin

in melanosome pH (Supplementary Fig. S1c), IP2 is not likely mediated by TPC1. Another major difference between the two types of channels is their voltage dependence: TPC1 mediates a depolarization-activated nonactivating conductance while TPC2 is voltage independent, similar to leak channels11. Because voltage ramps, as used in our study, mask the voltage-dependence of TPC1 activation, we used voltage steps to test for the presence of voltage-activated currents. Voltage steps between −80 and +80 mV did not elicit channel activation or measurable tail currents in melanosomes, but PI(3,5)P2 application activated an inward current (Fig. 2d), suggesting that TPC2 is the most likely melanosomal candidate.

Several reports suggested that TPCs form Ca2+ channels14,15,21, while recent evidence suggests they are Na+-selective12. We determined the cation permeability of IP2 by measuring Erev with 140 mM Na+ in a Gluc–based pipette/luminal solution to reduce Cl–dependent permeation and varying bath/cyttoplasmic cations in the presence of cytoplasmic PI(3,5)P2. Erev for IP2 was near 0 mV under symmetrical Na+ and >+90 mV for cytoplasmic K+, Ca2+, or the impermeant cation NMDG+, suggesting IP2 is Na+-selective (Fig. 2e). The calculated permeability ratios (Pxo/PNa) show that IP2 is >100 times more selective for Na+ than Ca2+ and >50 times more selective for Na+ than K+ (Fig. 2f). Furthermore, currents elicited in symmetrical Na+ exhibited leak-like properties, consistent with the lack of voltage-dependent activation. Thus, IP2 is a highly Na+-selective channel with properties similar to TPC2, previously described as a voltage-independent endolysosomal Na+ channel15,21.

TPC2 localizes to melanosomes to mediate IP2 and regulate pigmentation. Because TPCs are regulated by the organelar PI(3,5)P2 and have been implicated in pigmentation5,12, we tested whether they reside in the melanosomal membrane. Expression of GFP-tagged TPC1 or TPC2 in pigment cells (melan-a mouse melanocytes) revealed that only TPC2 overlapped significantly with the melanosomal marker tyrosinase-related protein 1 (TYRP1) and melanin-positive compartments (TPC2 overlap with melanin = 50.9 ± 3.2%, p < 0.0001; TPC1 overlap with melanin = 16.2 ± 9.9%, p > 0.5) (Fig. 3a,b). This result is consistent with the observation that IP2 has biophysical properties similar to TPC2, which has been implicated in pigmentation. Interestingly, in melanosomes TPC2-GFP did not exhibit significant overlap with the endolysosomal marker LAMP2 (TPC2 overlap with LAMP2 = 7.0 ± 2.7%, p < 0.0001) (Fig. 3c and Supplementary Fig. S2), suggesting that, unlike in other cell types, in pigment cells TPC2 is predominately localized to melanosomes. Although expressed TPC2 preferentially traffics to melanosomes, it is possible that endogenous TPC2 also localizes and functions in the endolysosomes of pigment cells. The GFP-tagged endolysosomal protein LAMP1 colocalized well with LAMP2 (LAMP1 overlap with LAMP2 = 71.4 ± 6.2%, p < 0.0001), but not melanin or TYRP1 (LAMP1 overlap with melanin = 12.1 ± 2.1%, p > 0.5), indicating that not all endolysosomal proteins localize to melanosomes when overexpressed in pigment cells (Fig. 3b,c, Supplementary Fig. S2). Similar to TPC2, the GFP-tagged melanosomal protein OCA222 significantly colocalized with melanin and TYRP1, but not with LAMP2 (OCA2 overlap with

Figure 3. TPC2 localizes to melanosomes in pigment cells. (a) GFP-tagged TPC2, but not GFP-TPC1 (green), localized primarily to tyrosinase related protein 1 (TYRP-1, red) and melanin (bright field) containing compartments in mel-an-a melanocytes. Enlarged images of outlined regions are shown in lower panels. Arrows indicate colocalization of TPC2-GFP and TRYP1 (yellow) and darkly pigmented melanosomes. TPC2-GFP exhibits significant overlap with melanosomes (50.9 ± 3.2%), similar to the melanosomal protein OCA2-GFP (41.3 ± 5.1%). TPC1 does not significantly overlap with melanosomes (16.2 ± 9.9%), similar to the lysosomal protein LAMP-1 (12.1 ± 2.1%) (n = 13 cells per condition from 3 independent experiments, p < 0.0001 for TPC2 or OCA2 vs. LAMP1, p > 0.5 for TPC1 vs. LAMP1). (b) Colocalization analyses of GFP tagged TPC2, TPC1, OCA2 and LAMP1 with melanin-containing melanosomes. TPC2-GFP exhibits significant overlap with melanosomes (50.9 ± 3.2%), similar to the melanosomal protein OCA2-GFP (41.3 ± 5.1%). TPC1 does not significantly overlap with melanosomes (16.2 ± 9.9%), similar to the lysosomal protein LAMP-1 (12.1 ± 2.1%) (n = 13 cells per condition from 3 independent experiments, p < 0.0001 for TPC2 or OCA2 vs. LAMP1, p > 0.5 for TPC1 vs. LAMP1). (c) Colocalization analyses of GFP tagged TPC2, OCA2 and LAMP1 with the lysosomal protein LAMP2. Neither TPC2 (7.0 ± 2.7%, p < 0.0001) nor OCA2 (7.9 ± 2.4%, p < 0.0001) exhibit significant overlap with anti-LAMP2 antibodies, while the lysosomal protein LAMP1 shows substantial colocalization with LAMP2 (71.4 ± 6.2%) (n = 4–7 cells per condition from 3 independent experiments).
RepoRts suggest that TPC2, previously characterized as an endolysosomal protein, is primarily expressed in melanosomes. TPC2 localization is similar to melanosomal rather than endolysosomal proteins (Fig. 3b,c). Thus, our data suggest that TPC2, previously characterized as an endolysosomal protein, is primarily expressed in melanosomes in pigment cells.

To determine if TPC2 is required for $I_{ppp}$, we generated $tpc2$-deficient Oa1−/− mouse melanocytes using the CRISPR-Cas9 system23 (Supplementary Fig. S3a–d). In melanosomes from Oa1−/− melanocytes expressing $tpc2$-targeted CRISPR-Cas9, PI(3,5)P2 failed to activate an inward current (9 out of 13 melanosomes) (Fig. 4a,b). Expression of GFP-tagged human TPC2 in $tpc2$-targeted CRISPR-Cas9-expressing mouse melanocytes was sufficient to rescue $I_{ppp}$, suggesting that $I_{ppp}$ is mediated by TPC2 (Fig. 4a,b). Because TPC2 has been previously implicated in pigment formation22 and we now find that it functions in melanosomes, we tested if TPC2 expression in melanosomes contributes to pigment formation. We found that reducing the levels of TPC2 by either $tpc2$-targeted CRISPR-Cas9 in melano-a or by siRNA in Oa1−/− melanocytes had markedly increased cellular melanin content, compared with control melanocytes (Fig. 4c and Supplementary Fig. S3e,f). Thus, TPC2 localizes to melanosomes in pigment cells, where it mediates $I_{ppp}$ to regulate melanogenesis.

How does TPC2 regulate melanosome function? Because TPC2 forms a Na+ leak current, it is likely to contribute to melanosome membrane potential. To test this, we performed current-clamp recordings of membrane potential in melanosomes from control and $tpc2$-targeted CRISPR-Cas9-expressing Oa1−/− melanocytes in the presence of PI(3,5)P2. In melanosomes from $tpc2$-deficient melanocytes that did not exhibit a PI(3,5)P2-induced current the resting membrane potential ($V_m$, defined as $V_m = V_{cytosol} - V_{lumen}$) was greatly reduced compared with melanosomes from control melanocytes (+7.9 ± 1.7 mV for $tpc2$-deficient and +17.2 ± 0.9 mV for control, Fig. 4d). Thus, TPC2 activity modulates the melanosome’s membrane voltage.

**TPC2 modulates melanosomal pH to regulate pigmentation.** The vacuolar proton pump V-ATPase is modulated by membrane voltage and is present in melanosomes where it regulates luminal pH and melanin production24–26. Because TPC2 significantly contributes to the membrane potential of melanosomes p < 0.01 for $V_m$ of control vs. $tpc2$-deficient melanocytes in the presence of PI(3,5)P2, it is conceivable that TPC2-mediated changes in membrane potential modulate V-ATPase activity and melanosomal pH. To test this hypothesis, we sought to measure melanosomal pH from control and $tpc2$-deficient melanocytes.

Fluorescence-based pH measurements have not been possible in pigmented melanosomes because fluorescence indicator uptake is impaired and melanin interferes with fluorescence emission. To circumvent these difficulties we used B16-F1 mouse melanocytes, which are weakly pigmented in their basal state. To measure pH in the melanosomes of B16-F1 cells we used the albinism-associated V443I-OCA2 mutant as a melanosomal marker (Supplementary Fig. S4) because it lacks Cl− channel activity, does not significantly increase organellar pH and only modestly induces pigmentation20,27. These missing features are present with wild-type OCA2 over-expression, which substantially increases pigmentation in B16-F1 melanocytes (Fig. 5b,c), thereby impairing fluorescence-based pH measurements.

Our pH measurements indicate that melanosomes from control B16-F1 melanocytes have a more acidic pH (5.51 ± 0.02) than melanosomes from melanocytes expressing $tpc2$-targeted CRISPR-Cas9 (5.85 ± 0.03) (Fig. 5a). This result is consistent with TPC2’s ability to increase melanosome membrane potential and provide a cation counterflux to support V-ATPase-mediated melanosomal acidification.

Because our results show that TPC2 acidifies melanosomes and melanogenesis is enhanced at more neutral pH values24–26, we tested if TPC2 serves as a negative regulator of melanin content by examining its interaction with a positive regulator of melanogenesis, OCA2. Consistent with OCA2’s function as a melanosomal anion channel component that raises organellar pH and enhances melanogenesis20, overexpression of OCA2 in B16-F1 melanocytes increased pigmentation by ~75%, as illustrated by the presence of numerous darkly pigmented melanosomes (Fig. 5b,c, top two right panels). We tested if TPC2 negatively regulates OCA2-induced pigmentation by coexpressing mCherry-OCA2 and TPC2-GFP, which colocalized (Fig. 5c, bottom right panels). Interestingly, TPC2-GFP expression reduced OCA2-induced pigmentation by ~30%, indicating that it negatively regulates OCA2-induced melanogenesis (Fig. 5b,c, faint appearance of melanosomes in bottom two right panels). In addition, expression of the albinism-associated mutant V443I-OCA2, which has reduced Cl− transport and pH regulation function25, also colocalized with TPC2, but resulted in only a slight increase in B16-F1 pigmentation (~27%) that was completely abolished by coexpression with TPC2-GFP (Fig. 5d). Thus, the melanosomal cation channel TPC2 acidifies pH to counterbalance the effect of the OCA2-mediated melanosomal anion channel on luminal pH and to serve as a negative regulator of melanogenesis and pigmentation.

**Discussion**

Our results reveal the first cation conductance in melanosomes. We find that in pigment cells the endolysosomal cation channel TPC2 localizes predominantly to melanosomes, where it mediates a Na+-selective current to modulate melanosomal membrane potential, pH, and pigmentation. Our findings are consistent with the role of TPC2 in pigmentation as a determinant of human hair color12, a factor in the development of melanoma16,17, and a regulator of pigmentation in Xenopus oocytes13.

We find that the important organellar signaling molecule PI(3,5)P2 regulates the activity of TPC2 in melanosomes. PI(3,5)P2, is a known modulator of endolysosomal cation channels4,5 and affects pigmentation4, possibly by regulating melanosome biogenesis26. Our results show that PI(3,5)P2 is required for the melanosomal TPC2-mediated Na+ current; our experimental conditions do not allow us to evaluate whether in intact cells basal PI(3,5)P2 is sufficient for TPC2 activity, resulting in a constitutively active Na+ current or if an unknown physiological signal modulates PI(3,5)P2 levels to regulate melanosomal TPC2 activity.
We show that TPC2 functions as a negative regulator of pigmentation by increasing melanosomal membrane potential and melanosomal acidity, which reduces the activity of the key melanogenic enzyme tyrosinase and subsequent melanogenesis. We hypothesize that TPC2 regulates melanosomal pH by providing a cation counterflux to enhance V-ATPase H\(^+\) transport into the melanosomal lumen, consistent with the requirement for an inward cation current in lysosomal acidification\(^{29}\). We have recently shown that a melanosomal anion channel mediated by OCA2 reduces organelle acidity to enhance melanogenesis, possibly by decreasing melanosomal membrane potential to inhibit V-ATPase activity\(^{20}\). We find that the effect of OCA2 on pigmentation is counterbalanced by TPC2, possibly through the regulation of luminal acidity (Fig. 6). It is likely the two conductances regulate one another through voltage-dependent processes and ion homeostasis; it is also possible that the two conductances do not function simultaneously to balance the pH, but rather are differentially regulated by cellular signaling to elicit dynamic changes in melanosomal pH. A more comprehensive model of ionic signaling in melanosomes requires a better understanding of melanosomal membrane potential and luminal ionic concentrations in intact cells; it also entails the molecular identification and characterization of other melanosomal ion channels and transporters. Thus, our studies provide a basis for future work exploring molecular mechanisms underlying ionic signaling in melanosomes and other organelles and could uncover novel therapeutic targets for pigmentation disorders and skin and eye cancers.

**Methods**

**Cells and tissue.** Immortalized mouse melanocytes melan-a\(^{30}\) and melan-Oa1\(^{−/−}\)\(^{18}\) were grown in RPMI 1640, 10% fetal bovine serum (FBS, Atlanta Biologicals), 1% penicillin/streptomycin (P/S), 200 nM phorbol 12-myristate-13-acetate (Sigma) at 37 °C and 10% CO\(_2\). Immortalized melanocytes were obtained from the Wellcome Trust Functional Genomics Cell Bank. B16-F1 melanocytes were grown in DMEM, 10% FBS, and 1% P/S at 37 °C and 5% CO\(_2\). Transfection of cells was carried out using Lipofectamine 2000 or 3000 (Invitrogen/Life Technologies). Reagents were from Invitrogen/Life Technologies, unless stated otherwise.

**RPE tissue.** RPE tissue was dissected American Bullfrog (Lithobates catesbeianus) eyes as previously described and kept at 4 °C for a maximum of 36 hours in a modified Ringer's solution containing (mM): 111 NaCl, 2.5 KCl, 1 CaCl\(_2\), 1.5 MgCl\(_2\), 0.02 EDTA, 3 HEPES, pH 7.6. Bullfrog eyes were obtained as discarded tissue from Dr. Thomas Roberts’ laboratory at Brown University (protocol number 1303990009) and used in agreement with all the ethics rules and regulations.

**Molecular biology.** TPC1\(^{9}\), TPC2\(^{9}\), OCA2 wild type or V443I mutant\(^{20}\), and LAMP1 (Addgene plasmid 34831) were tagged with GFP or mCherry.

**CRISPR-Cas9 and siRNA–mediated reduction in protein expression.** All-in-one vectors including CMV-driven Cas9 and 20 bp target single-guide RNA sequences (gRNA) were from Genescript. As a control for CRISPR-Cas9 experiments melanocytes expressed Cas9-containing vectors without gene-targeted gRNA.
tpc2-targeted gRNA 5'-AGAGCAGCCCCCTTCTGGGC-3' was cloned into the pGS-gRNA-Cas9-Neo vector and expressed using Lipofectamine 2000 (B16-F1 melanocytes) or Lipofectamine 3000 (melan-a and Oa1−/−melanocytes) following manufacturer's instructions. Stable cell lines were created using geneticin selection (B16-F1: 1200 μg/ml, melan-a: 1000 μg/ml, Oa1−/−melanocytes: 1200 μg/ml, melan-a and Oa1−/−were reduced to 800 μg/ml after 14 days).

Analysis of CRISPR-Cas9 edited cell line. Genomic DNA was extracted using a Blood & Cell Culture Mini Kit (Qiagen) using > 100,000 cells per sample.

Single Clone Sequencing. Genomic DNA was extracted from CRISPR-expressing cells and the CRISPR target sites were amplified with the following primers:

\[ \text{tpc2} \quad \text{F: 5'}-\text{AGTCAGTCAGTAAGTCCCTCAATCAATCAGT-3'} \]
\[ \text{R: 5'}-\text{GGATGTTCGGCCACTCACTG-3'} \]

The PCR products were run on a 1.5% agarose gel and single bands of the predicted size were extracted using QIAquick Gel Extraction Kit (Qiagen). The resulting DNA products were cloned using the TOPO PCR kit (Invitrogen/Life Technologies) according to manufacturer's protocol. DNA from twenty single cell colonies was extracted and sequenced to screen for gene editing.

High Resolution Melt Analysis. 50–60 bp amplicons containing CRISPR sites were created using genomic DNA as template and the following primers:

\[ \text{tpc2} \quad \text{F: 5'}-\text{TGGTGAGATGGCGCGAGA-3'} \]
\[ \text{R: 5'}-\text{GCCACTGCGTCCGCCG-3'} \]

Amplicons were screened for random mutations using real-time PCR (Bio-Rad CFX96) with Precision Melt Supermix (Bio-Rad) according to manufacturer's instructions.
Melanosomal electrophysiology.  

**Melanosome dissection.** Enlarged dermal melanosomes were individually dissected from Oa1−/− mouse melanocytes using a borosilicate patch pipette to cut the cell membrane and push out individual organelles. A new pipette was then used for patch clamp experiments. RPE melanosomes from bullfrog RPE cells were released using two patch pipettes, as previously described20. Data were filtered at 2.9 kHz and digitized at 10 kHz using an EPC 10 amplifier (HEKA Instruments) with PatchMaster software (HEKA Instruments). Membrane potentials were corrected for liquid junction potentials. Patch-clamp were filtered at 2.9 kHz and digitized at 10 kHz using an EPC 10 amplifier (HEKA Instruments) with PatchMaster software (HEKA Instruments). Membrane potentials were corrected for liquid junction potentials. Patch-clamp experiments used borosilicate glass pipettes polished to 10–15 MΩ. Standard voltage-clamp pipette/lumen solution contained approximate physiological endolysosomal ion concentrations9 (mM): 70 NaCl, 70 KCl, 2 CaCl 2, 0.39 CaCl 2, 1 EGTA (Ca2+ buffered to 100 nM), 10 HEPES, pH 7.2. Current-clamp bath/cytoplasmic solution contained 2 μM P(3,5)P 2. Cl− was replaced with Gluc− to block endogenous anion permeability. The holding potential was 0 mV and currents were measured in response to 500 ms voltage ramps from −120 to +100 mV, unless otherwise stated. Whole-organelle current density was calculated by normalizing to capacitance (0.14–0.5 pF). To analyze the effects of some treatments, currents recorded following treatments were normalized to basal currents (normalized current = (Itreatment − Ibasal)/Ibasal). Maximal currents were typically recorded ~100 s into treatments and inhibition after ~100 s of treatment. Treatments that did not elicit responses were followed by a wash period and PI(3,5)P2 treatment in the same melanosome.

**Cation permeability.** Relative permeability of I PIP2 was determined by measuring Erev after the substitution of bath/cytoplasmic cations from Na+ to K+, Ca2+, or NMDG+. Pipette/luminal solution contained 140 mM NaGluc solution and the cytoplasmic/bath solution contained 140 mM X-Gluc, where X = Na+, K+, or NMDG+, or 100 mM CaGluc 2. Permeability ratios were estimated using the Goldman–Hodgkin–Katz equation: P X/P Na = ([Na+]luminal/[X]Cytoplasmic)(exp(ErevF/RT)), P Ca/P Na = ([Ca2+]luminal/[Na+]Cytoplasmic)(exp(ErevF/RT)).

**CRISPR-Cas9 Mutation Detection.** PCR product was generated from purified genomic DNA using primers described for single clone sequencing to amplify tpc2 CRISPR sites. Mutation detection was carried out using the Guide-it Mutation Detection Kit (Clontech) according to manufacturer’s protocol. In brief, 800–1000 ng of PCR products were re-annealed to enable heteroduplex formation (95 °C for 5 min, 95 °C to 85 °C ramping at 2 °C/s, 85 °C to 25 °C at 0.1 °C/s, and cooling at 4 °C). Re-annealing products were treated with Guide-it Resolvase for 30 minutes at 37 °C and analyzed on 1.5% agarose gels. The fraction of PCR product cleaved (Fcut) = (a + b + c)/(a + b + c) where a = undigested product, b and c = digested products.

Mouse tpc2-targeted and control (scrambled) siRNAs were designed with Oligoengine 2.0 software and expressed using the pSUPER RNAi Vector system (Oligoengine). Tpc2-targeted oligos were cloned into pSUPER-GFP-NEO, and expressed in Platinum-E cells to produce retroviral particles. Upon viral transduction of Oa1−/− mouse melanocytes, stable melanocytes lines expressing tpc2-targeted or control siRNA were created using fluorescence-activated cell sorting (FACS) and subsequently kept under G418 selection (800 μg/ml). The relative tpc2 mRNA levels in melanocytes expressing tpc2-targeted or control siRNA were calculated by quantitative PCR relative to actin.

**Melanosome pH and melanogenesis.** PCR products were re-annealed to enable heteroduplex formation (95 °C for 5 min, 95 °C to 85 °C ramping at 2 °C/s, 85 °C to 25 °C at 0.1 °C/s, and cooling at 4 °C). Re-annealing products were treated with Guide-it Resolvase for 30 minutes at 37 °C and analyzed on 1.5% agarose gels. The fraction of PCR product cleaved (Fcut) = (a + b + c)/(a + b + c) where a = undigested product, b and c = digested products.

**Model of ion channel-mediated regulation of melanosome pH and melanogenesis.** TPC2 mediates the major melanosomal Na+ conductance to increase melanosome membrane potential (Ψcell, Ψcytosol = Vcytosol – Vlumen) and provide a counter cation to enhance vacuolar ATPase (V-ATPase) activity and increase the acidity of the melanosome lumen, thus reducing the activity of the key melanogenic enzyme tyrosinase (TYR). Additionally, the major melanosomal anion channel mediated by OCA2 expression transports Cl− and provide a counter cation to enhance vacuolar ATPase (V-ATPase) activity and increase the acidity of the melanosome lumen, thus reducing the activity of the key melanogenic enzyme tyrosinase (TYR). Additionally, the major melanosomal anion channel mediated by OCA2 expression transports Cl− into the cytoplasm to make the melanosome membrane voltage more negative, thereby decreasing V-ATPase-mediated H+ transport and luminal acidity, which enhances the activity tyrosinase and melanin production.

Figure 6. Model of ion channel-mediated regulation of melanosome pH and melanogenesis. TPC2 mediates the major melanosomal Na+ conductance to increase melanosome membrane potential (Ψcell, Ψcytosol = Vcytosol – Vlumen) and provide a counter cation to enhance vacuolar ATPase (V-ATPase) activity and increase the acidity of the melanosome lumen, thus reducing the activity of the key melanogenic enzyme tyrosinase (TYR). Additionally, the major melanosomal anion channel mediated by OCA2 expression transports Cl− into the cytoplasm to make the melanosome membrane voltage more negative, thereby decreasing V-ATPase-mediated H+ transport and luminal acidity, which enhances the activity tyrosinase and melanin production.
**Immunofluorescence microscopy.** Melan-a and B16-F1 melanocytes were seeded on coverslips coated with poly-L-lysine (Sigma) and transfected the following day using 4 μg DNA and 3.3 μl of Lipofectamine 2000 for melan-a or 2 μg of DNA and 6 μl of Lipofectamine 2000 for B16-F1. Cells were fixed using 4% paraformaldehyde (Sigma) for 10 min at room temperature, washed with PBS and labeled with primary and secondary antibodies diluted in PBS with 0.2% (w/v) saponin, 0.1% (w/v) bovine serum albumin, 0.02% (w/v) sodium azide. Antibodies used were: mouse anti-TPR1 (TA99/mel-5, 1:50 dilution, BioLegend/Covance Antibody Products), rat anti-LAMP2 (GL2A7, 1:4, deposited by Granger, B.L. to the Developmental Studies Hybridoma Bank). Goat secondary antibodies conjugated to Alexa 568 or 488 were from Invitrogen. Cells were imaged using a laser-scanning confocal microscope (Zeiss LSM 510) with a 63X objective. Final images were generated from single 0.2 μm stacks and insets magnified using Photoshop software (Adobe). Quantification of colocalization of GFP-tagged proteins and melanin-containing melanosomes or LAMP2 immunostaining was carried out using brightfield and fluorescent confocal images analyzed with ImageJ Fiji software, as previously described. Briefly, after setting an initial threshold the images were made binary for each channel and the area corresponding to fluorescence or melanin-containing melanosomes was quantified. The Image Calculator function was used to multiply the binary images for brightfield melanosomes and fluorescence and the resulting image represents the area of overlap between fluorescently-tagged proteins and melanin-containing melanosomes. The area of overlap of the image resulting from multiplication and the binary image for total fluorescence staining was quantified using the Analyze Particles function to count the area of all structures with an area greater than 5 pixels. The ratio of overlap pixels to total fluorescence pixels gives the % overlap.

**pH imaging.** Control or tpc2-targeted CRISPR-Cas9-expressing B16-F1 melanocytes transfected one day prior to imaging experiments with mCherry-V443I-OCA2 to identify melanosomes were incubated with 1 μM LysoSensor-ND160 (Invitrogen/Life Technologies) for 5 min. LysoSensor was excited at 405 nm and its emission detected at 417–483 nm (W1) and 490–530 nm (W2) using an Olympus XL fluorescent microscope. The ratio of emissions (W1/W2) in melanosomes expressing mCherry-V443I-OCA2 was assigned to a pH value based on a calibration curve generated with solutions containing 125 mM KCl, 25 mM NaCl, 24 mM MES to adjust the pH to 3.5, 4.5, 5, 5.5, 6.5, or 7 (three independent experiments). The fluorescence ratio was linear for pH 4.5–7.0.

**Melanin measurements.** Melanin from wild type (control), tpc2-targeted CRISPR-Cas9 or siRNA-expressing Oat1−/− melanocytes was quantified as previously described. The soluble and insoluble fractions of melanin solutions were separated after cell lysis with 1% Triton X-100 (Sigma) in PBS pH 7.4. Total protein was measured in the soluble fraction using a BCA Protein Assay Kit (Pierce). The insoluble fraction was dissolved in 1 N NaOH by incubation for 2 h at 85 °C and used to quantify melanin by measuring the optical density of each sample at 405 nm, then fit with a standard curve generated using synthetic melanin (Sigma). Average cellular melanin values were quantified as the ratio between total melanin and total protein (μg melanin/mg protein) from the same dish. Three independent experiments were performed, each in triplicate.

To increase melanin content in B16-F1 melanocytes, mCherry-tagged wild-type or V443I-OCA2 variants were expressed alone or coexpressed with TPC2-GFP for 24 h prior to fixation and imaging and 48 h prior to melanin analysis. Images from three independent experiments were acquired with a laser-scanning confocal microscope (Zeiss LSM 510) using a 63X objective. The melanin from each B16-F1 condition was quantified as described above.

**Data analysis.** All data are shown as mean ± s.e.m. Data were considered significant if p < 0.05 using paired or unpaired two-tailed Student t-tests or one-way ANOVA.

**Ethical Approval.** Bullfrog eyes used for RPE cells were obtained as discarded tissue from Dr. Thomas Roberts’ laboratory at Brown University under protocol #1303990009, approved by the Institutional Animal Care and Use Committee and used in agreement with the approved guidelines and all the ethics rules and regulations.

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Author Contributions

N.W.B. and I.E.E. acquired and analyzed data. All others contributed to research design and writing of the manuscript.

Additional Information

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Corrigendum: A melanosomal two-pore sodium channel regulates pigmentation

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This Article contains an error in Figure 4a: the upper right "hTPC2 rescue" trace is a duplication of the upper left "control" trace. The correct Figure 4 appears below as Figure 1.

Figure 1.

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