SLC24A5 Encodes a trans-Golgi Network Protein with Potassium-dependent Sodium-Calcium Exchange Activity That Regulates Human Epidermal Melanogenesis*

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For many years the genetic determinants of human skin color have attracted interest from both pigmentation biologists and anthropologists (1–5). Using high density whole genome array technology, we have defined the key genetic variants associated with natural skin color variation in a population of South Asian ancestry (6). We identified a non-synonymous single nucleotide polymorphism (nsSNP)† in the SLC24A5 gene (rs1426654) that was previously unknown to skin pigmentation biologists. Independently, a group of zebrafish researchers discovered that a different mutation in the zebrafish orthologue of this gene was responsible for the hypo-pigmented phenotype of the zebrafish (7). Using data from the human HapMap project (8), they observed that the alternate alleles of the nsSNP (rs1426654) in human SLC24A5 were present at very different frequencies in populations of African and European ancestry. Furthermore, they demonstrated that the allele encoding threonine 111 of the protein encoded by SLC24A5 was associated with lighter skin in an admixed African-American population. Subsequent analyses have pinpointed this SNP and another in the SLC45A2 gene as participants in the evolution of light skin in Europeans but not East Asians (9–11).

Amino acid sequence alignments predict that SLC24A5 encodes a member of the potassium-dependent sodium calcium exchanger protein family, designated NCKX5. The NCKX family currently consists of five members (NCKX 1–5). NCKX1–4 are located in the plasma membrane. NCKX proteins have been shown to operate using both the inward sodium gradient and the outward potassium gradient to extrude calcium across the plasma membrane. NCKX proteins have been shown to operate in retinal rod and cone photoreceptors as well as in several types of neurons in the brain (for review, see Ref. 12). A sixth protein, designated NCKX6, is now thought to belong to a distinct, but related protein family (CCX) as it shows substantial divergence in phylogenetic analyses (13) and lacks many of the residues conserved between NCKX1–5 that are key for transport activity. Although SLC24A5 is predicted to encode an NCKX protein, the exchanger activity of NCKX5 has yet to be experimentally demonstrated. The residue altered by the nsSNP in SLC24A5 lies in a region of the protein that is highly conserved across all NCKX family members. This region contains a number of residues that are critical for exchange function (14–16), and the experimentally determined topology for NCKX2 suggests that the polymorphic residue of NCKX5 sits in a transmembrane domain (17). NCKX1–4 are located in the plasma membrane, yet surprisingly, overexpressed hemagglutinin-tagged recombinant zebrafish NCKX5 could not be detected in the plasma membrane of MNT1 cells (7), and NCKX5 has been detected in fractionated melanocytes enriched for a subcellular organelle, the melanosome, using sucrose gradient fractionation and proteomic analysis (18).

Here we describe a full analysis of the transcript expression profile of all known sodium-calcium exchangers in cultured human and murine melanocytes together with an immunocytochemical analysis of native NCKX5 expression. We show that this protein has a novel trans-Golgi network intracellular local-
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In normal human epidermal melanocytes (NHM), and using siRNA-mediated knockdown we demonstrate conclusively that SLC24A5 expression is required for melanin synthesis in both cultured mouse and human melanocytes. We also demonstrate that, as predicted, SLC24A5 encodes a protein with potassium-dependent sodium-calcium exchanger activity. Through site-directed mutagenesis in a heterologous expression system, we demonstrate that the residue switch encoded by the nsSNP in SLC24A5 markedly alters exchanger activity when introduced into NCKX2 or NCKX5. Together, these data support the hypothesis that a nsSNP in the SLC24A5 gene directly alters human skin color through its effect on the sodium-calcium exchanger activity of NCKX5.

EXPERIMENTAL PROCEDURES

Cell Culture—NHM from neonatal foreskin were cultured in MGM (M254 medium supplemented with human melanocyte growth supplement) (Cascade Biologics) at 37 °C, 9% CO₂. Melanocytes were dedifferentiated as previously described (19) using M254 and 50 nM EDN3 for 7 days before knockdown (cells denoted MCCMB). B16 F10 mouse melanoma cells (ATCC) were cultured in minimum essential medium with Earle’s salts supplemented with 10% fetal calf serum and 2 mM L-glutamine at 37 °C, 5% CO₂.

Real-time PCR—Total RNA was extracted using the RNeasy mini kit (Qiagen) and quantified using Ribo Green (Invitrogen). 1 μg of total RNA was reverse-transcribed using the Roche Applied Science first strand synthesis kit. Real-time PCR was performed on a Bio-Rad iCycler using human or murine SLC24 or SLC8, human vSNARE, or murine TBP QuantiTect real-time PCR primers (Qiagen) and SYBR Green PCR master mix (Bio-Rad). All measurements from each cell type were made on a single cDNA sample. Data analysis was performed using the comparative cycle threshold method (ΔCT).

Anti-NCKX5 Antibodies—Peptides anti-NCKX5-(I) (DEG-QPFIIRRQRSRTDSSG) and anti-NCKX5-(C) (GNNKIRGCGG) were coupled to keyhole limpet hemocyanin, and rabbits were immunized in a 90-day protocol. Bleeds were affinity-purified using individual peptides conjugated to bovine serum albumin and 1 g/ml goat anti-Trp1 (G17), mouse anti-melanocytome (NMClab), T311 anti-tyrosinase (AbCam), Mel-5 anti-TYRP1 (Signet), and T311 anti-tyrosinase (AbCam). Other antibodies tested for co-localization were Lamp 1, flotillin 2 and syntaxin 8 (BD Biosciences), EEA1, Sar1, β-COP, PEX-19 (AbCam), Lamp 2 (RDI), C423 (anti-clathrin L) (Covance), and syntaxin 6 (Sigma-Aldrich). Secondary antibodies were Alexa Fluor® 488 donkey anti-rabbit, 633 donkey anti-sheep, or 633 goat anti-mouse (Invitrogen). Confocal microscopy was performed using a Leica TCSSp1 confocal S laser microscope with Leica LCS software. Images were acquired using 20× (dry) or 60× (oil immersion 1.4a) objectives. 488- and 633-nm excitation was via argon or HeNe lasers respectively. Optical series were collected at 0.5 μm z-steps. For co-localizations with GM130, the topological separation of the signal from each channel was determined by plotting the intensity profile of each signal in three separate cross-sections of the Golgi stacks.

RNA-mediated Interference Transfections—B16 or NHM were seeded at 2 × 10⁴ cells/cm² for melanogenesis experiments or 1 × 10⁴ cells/cm² for immunofluorescence microscopy experiments; MCCMB cells were seeded at 1.5 × 10⁴ cells/cm². Cells were adhered for 24 h and transfected with 2 μg/ml Lipofectamine 2000 and 50 nM or 100 nM Stealth™ RNA-mediated interference duplex. All dilutions were performed with Opti-MEM. Murine siRNA targeted nucleotides 762–787, with a corresponding scrambled control. Human siRNA targeted nucleotides 185–210 and 492–517 with scrambled control corresponding to 260–285. Cells were incubated with transfection reagents for 6–8 h. NHM were returned to MGM and grown for 5–6 days before analysis. B16 cells were grown in phenol red-free Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 4 mM L-glutamine, after 3 days the media was analyzed for melanin content, and the cells incubated for 1 h in WST-1 reagent (Roche Applied Science) to determine viability.

Sucrose Density Gradient Fractionation—Light and Dark NHM were extracted to obtain a total granule fraction and fractionated essentially as described by Chen et al. (23). Briefly, cells were washed, tryspinized, and resuspended in 0.25 M homogenization buffer. After 60 strokes in a Dounce tissue-grinder, they were centrifuged at 100 × g to remove debris, and the
supernatant was centrifuged at 100,000 × g (4 °C) before loading onto a stepped sucrose gradient (0.8–2 m in 0.2 m increments). Gradients were centrifuged at 25,000 rpm (4 °C) in a Beckman SW28 rotor. Interface fractions were collected, and 7.5 μl of each fraction was loaded per well in SDS reducing buffer.

Melanin Assay—NHM were lysed in 1% Triton X-100, PBS and centrifuged at 15,000 g for 10 min, and protein was quantified by BCA assay. Melanin pellets washed in 1:1 ethanol/diethyl ether were dissolved in 1M NaOH, 10% Me2SO, 30 min, 60 °C. Melanin content of the supernatants or culture media was measured at 450 nm.

Clustal Alignments—Protein sequences were aligned using ClustalW program using the default settings. The resultant alignment files were visualized using Jalview multiple alignment editor (20), and manual adjustments made as necessary.

Functional Analysis of Mutant NCKX2 and NCKX5—Codons for the indicated residues were mutated using cDNA of the Myc-tagged short splice variant of human NCKX2 (AAF25881) or human NCKX5 and cloned into the pEIA vector as described before (14). The Myc tag (EQKLISEEDL) was inserted between Ser-52 and Glu-53 of human NCKX5. The mutant and wild-type NCKX2 and NCKX5 proteins were expressed in insect High Five cells, and protein expression was monitored by Western blotting with the Myc mAb (New England Biolabs). NCKX function was assayed by measuring 45Ca2+ uptake via reverse exchange in Na+-loaded cells as described in detail previously (21, 14). External 45Ca2+ was removed by a rapid filtration/washing procedure using borosilicate glass fiber filters (21). NaCl, KCl, LiCl, and choline chloride were all Sigma Ultra grade. Protein content of cell samples was determined with the Bio-Rad protein assay.

RESULTS

SLC24A5 mRNA Is the Predominant Sodium-Calcium Exchanger Transcript in Human and Mouse Melanocytes—In mammalian cells sodium-calcium exchange is mediated both through NCKX proteins, encoded by the SLC24 gene family, and potassium-independent sodium-calcium exchanger (NCX) proteins encoded by the closely related, but distinct SLC8 gene family. This gene family comprises three members, NCX1–3, transcripts of which are found in many tissues, in particular in the heart and brain (for review, see Ref. 22). The transcript profile of all known sodium-calcium (potassium) exchangers was determined in monolayer cultures of NHM and mouse melanocytes using real-time PCR (Fig. 1). In both cultures the predominant isoform was SLC24A5, which was >100-fold more highly expressed than any of the other SLC24A transcripts, and transcripts of the SLC8 family were not detected in either cell line. In NMH low levels of SLC24A1 and very low levels of SLC24A3 and SLC24A4 were detected, and in the mouse melanocytes, low levels of SLC24A4 and very low levels of SLC24A1 and SLC24A3 were found.

NCKX5 Protein Is Located in an Intracellular Membrane and Partially Co-localizes with the Trans-Golgi Network—The specificity of affinity-purified antisera for native NCKX5 protein was assessed through siRNA-mediated knockdown in NHM. Transfection with two different siRNA duplexes (siRNA 185 and siRNA 492) resulted in >90% knockdown of SLC24A5 transcript levels compared with control cultures after 48 h.3 Western blots of cell extracts harvested 5 days after transfection and probed with affinity-purified anti-NCKX5-(C) sera showed that a triplet of bands of ~43 kDa were clearly detected in extracts from control or untreated cells but were virtually absent in extracts from SLC24A5 siRNA-treated cells (Fig. 2A). The apparent size of these bands is surprisingly low compared with the predicted molecular mass in the absence of glycosylation of 54.9 kDa. However, the lower molecular mass observed here is consistent with that observed for heterologously expressed recombinant hNCKX5 detected through amino and carboxyl-terminal tags, which also runs as a triplet of bands ~43 kDa (Fig. 2B). De-glycosylation of the recombinant protein (Fig. 2B) removes the upper band, suggesting that the lower bands reflect signal peptide cleavage of unglycosylated protein. Non-transfected HEK cells do not show any reactivity with the anti-Myc antibody. These results show that the affinity-purified polyclonal antibody preparation detects NCKX5 protein in human melanocyte extracts, with some nonspecific reactivity, suggested by the detection of other higher and lower molecular weight bands that are not affected by SLC24A5 knockdown.

Cultured dark NHM probed by immunofluorescence with either of our anti-NCKX5 antisera, raised against different pep-

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NCKX5 (green) and TGN46 (red). However no marked effect on the punctate staining was observed (Fig. 3C).³ This confirms that the perinuclear staining we have observed with both antibody preparations is specific to NCKX5 and not due to the nonspecific reactivity detected by Western blot. However the punctate staining is either not specific to this protein, or the protein localized to these structures is not removed by siRNA knockdown even after 10 days.³ Co-localization studies with multiple markers of melanosomes, lysosomes, or endosomes were unable to determine the identity of this punctuate staining (Fig. 3D).³ NCKX5 Co-fractionates with TGN46 in NHM Extracts—Subcellular fractionation provides an alternative approach to determine the localization of proteins. Sucrose density gradient centrifugation has previously been used to enrich for melanosomal proteins (23). Using the same approach, fractionated NHM extracts were probed with anti-NCKX5 (C) and the TGN46 antibody (Fig. 4). Fractions 4–6 (corresponding to the 1.2/1.4 M, 1.4/1.6 M, and 1.6/1.8 M sucrose interfaces, respectively) contained pigment and were similarly enriched for both NCKX5 and TGN46, supporting the co-localization observed between these two antigens using immunofluorescence and indicating that these fractions contain trans-Golgi membranes as well as melanosomes.

SLC24A5 Knockdown Reduces Melanin Production in Mouse B16 Melanocytes—In melanocyte monolayer cultures, where transfer is not possible, melanosome turnover is limited to some degree by cellular turnover, making it difficult to measure the inhibition of melanin production over short time-courses. Mouse B16 cells are unusual in this respect because they secrete melanin directly into the surrounding culture media, and melanogenesis is only triggered when cells reach the stationary phase of growth (24). This affords the opportunity to assess the effect of SLC24A5 knockdown against a low background of pre-existing melanin. Therefore, the effect of SLC24A5 siRNA knockdown on melanin production was first assessed in mouse B16 melanocytes. Treatment with siRNA 762 elicited a marked and reproducible reduction in melanin production 72 h post-knockdown (87.7% reduction \( p < 0.0001 \)) compared with the control siRNA (Fig. 5, A and B) without any detectable toxicity. This demonstrates that this gene and its products play a controlling role in melanogenesis in this system.
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SLC24A5 Knockdown Reduces Melanin Production in NHM—The effect of SLC24A5 knockdown on melanogenesis in cultured NHM was investigated by comparing the melanin content of cell pellets 5 days after siRNA transfection. NHM transfected with either of the SLC24A5 knockdown siRNAs (Fig. 6A) resulted in significant percentage reductions of 22 and 30.5% (siRNA 185 and 492, respectively; p < 0.0001) of total cellular melanin compared with the control cells, demonstrating that SLC24A5 expression plays an important role in melanin synthesis in these cells. The effect of SLC24A5 knockdown on melanogenesis was even clearer when assessed in depigmented melanocytes (Fig. 6B). In these cultures the difficulty of measuring a reduction in de novo melanin synthesis against a large background of pre-existing melanin was substantially lessened. The pigment content of NHM cultured in melanoma blast media for 1 week was greatly reduced (Fig. 6B, MB), and switching these cells back into normal melanocyte media restored pigment production after 5 days (Fig. 6B, MGM). Transfection of de-differentiated cells with SLC24A5 siRNA knockdown duplexes (185 and 492) almost completely blocked pigment synthesis after switching back into melanocyte media compared with the control transfection (Fig. 6B, SC) with a normalized percentage difference in melanin content of 80–90% (p < 0.0001). These results demonstrate for the first time that SLC24A5 expression is necessary for melanin production in human epidermal melanoblasts stimulated to differentiate.

Melanosomal Markers Are Down-regulated after SLC24A5 Knockdown—Protein extracts prepared from re-pigmented melanoblasts treated with control or SLC24A5 knockdown siRNAs were probed by Western blotting for known melanosomal markers (Fig. 7). The expression of early melanosomal markers pmel17 (85-kDa form) and MART1 and late melanosomal markers (Fig. 7). The expression of early melanosomal markers pmel17 (85-kDa form) and MART1 and late melanosomal markers (Fig. 7). The expression of early melanosomal markers pmel17 (85-kDa form) and MART1 and late melanosomal markers pmel17 (85-kDa form) and MART1 and late melanosomal markers pmel17 (85-kDa form) and MART1 and late melanosome biogenesis and that this process is intimately linked with lysosome biogenesis. Switching Threonine for Alanine at Residue 111 of NCKX5 or Residue 177 of NCKX2 Reduces Exchange Activity—The NCKX proteins have a high degree of amino acid sequence similarity in two regions that have been identified as essential for exchange activity (denoted α1 and α2). The non-synonymous SNP (rs1426654) that is associated with natural skin color variation lies within the first of these highly conserved regions. All NCKX proteins and all cross-species orthologues of NCKX5 have an alanine at this position, except the NCKX5 variant identified in light-skinned humans, which has a threonine (Fig. 8, A and B). Consistent with the notion that native NCKX5 is localized to an intracellular membrane, no NCKX activity could be measured

FIGURE 4. NCKX5 co-fractionates with melanosomal and trans-Golgi markers. Dark NHM cultures were extracted and fractionated by sucrose density centrifugation as described under “Experimental Procedures.” Fractions 1–7 were collected from different points of the sucrose gradient, corresponding to steps of 0.8, 1, 1.2, 1.4, 1.6, 1.8, and 2 m sucrose, respectively. Fractions were probed by Western blotting with anti-TGN46, anti-NCKX5 (C), and anti-TYRP1 antisera. Arrows indicate reactive bands.

FIGURE 3. NCKX5 is partially localized to the trans-Golgi network in NHM. A, NHM grown on coverslips were fixed and probed with anti-NCKX5-(I) (green) and anti-TGN46 (red) antisera. Staining with each antibody was visualized by high magnification immunofluorescence confocal microscopy, and co-localization is seen as yellow in the merged images. Two merged images of different optical sections from the same cell are shown (500 nm apart). B, NHM grown on coverslips were fixed and probed with anti-NCKX5-(I) (green) and anti-GM130 (red) antisera and imaged by high magnification immunofluorescence microscopy. The red and green and merged images of part of a cell are shown. C, NHM were grown on coverslips and transfected with Lipofectamine alone (vehicle control), siRNA 492, or scrambled siRNA control. After 5 days cells were fixed and probed by low magnification immunofluorescence confocal microscopy with anti-NCKX5-(I) (green) and anti-TGN46 (red) antisera. Staining with each antibody was visualized by high magnification immunofluorescence microscopy. The red and green and merged images of part of a cell are shown. D, NHM grown on coverslips were fixed and probed with anti-NCKX5-(I) (green) and anti-Tyr, Mel-5, anti-GP100, or HMB45 (red) antisera and imaged by high magnification immunofluorescence microscopy. Merged images are shown.
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A

untransfected

vehicle control

scrambled siRNA

siRNA 762

FIGURE 5. SLC24A5 knockdown disrupts melanogenesis in cultured B16 melanocytes. A, B16 melanocytes growing in quadruplicate in 48-well plates were transfected with Lipofectamine alone (vehicle control), scrambled siRNA control, siRNA 762 cultured for 72 h and photographed alongside untransfected cells. B, the ratio of total melanin (µg) in the media to total protein (µg) in the cells recovered 72 h after B16 cells were transfected with the scrambled siRNA control or siRNA 762. Individual data points are plotted for four replicates of three experiments. A two-way analysis of variance taking account of variability between experiments and siRNA duplexes indicated that there is a difference in normalized melanin content between cells transfected with siRNA 762 and scrambled siRNA control (p < 0.0001).

B

FIGURE 6. SLC24A5 knockdown disrupts melanogenesis in cultured NHM. A, comparison of the ratio of total melanin (µg) to total protein (µg) extracted from light NHM treated with the scrambled siRNA control (SC), siRNA 185, and siRNA 492. Individual data points are plotted for three replicates of three experiments. A two-way analysis of variance taking account of variability between experiments and siRNA duplexes indicated that there is a difference in normalized melanin content between each of the siRNA duplexes and the scrambled siRNA control (p < 0.0001) after a Dunnett’s adjustment for multiple comparisons to the common control. B, light NHM were cultured for 1 week in melanoblast medium, plated into 6-well plates, and transfected with siRNA 185, siRNA 492, or scrambled siRNA control (SC) or untransfected (MGM). These cells were then grown in MGM media for 5 days or left in melanoblast media (MB) and trypsinized, and 450,000 cells from each treatment were pelleted in Eppendorf tubes and photographed.

Recent publications have focused on the importance of SLC24A5 in the variation of human skin color across global populations (9–11). However, little is understood about the biological mechanisms that mediate the association between SLC24A5 polymorphisms and vertebrate pigmentation.

SLC24A5 Plays a Key Role in Melanogenesis—In vivo epidermal melanocytes synthesize melanin within specialized
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FIGURE 7. Effect of SLC24A5 knockdown on expression of melanosomal protein expression. Whole cell protein extracts from melanoblast SLC24A5 knockdown experiments were probed with anti-Tyr, anti-TYRP1, anti-MART-1, anti-PMEL17, anti-LAMP-1, and anti-β-actin antisera. Lanes were loaded as follows: MB, melanoblast; MGM, re-pigmented melanoblast/melanocyte; VC, knockdown vehicle control; SC, knockdown with scrambled siRNA control; 185, knockdown with siRNA 185; 492, knockdown with siRNA 492. For pmel17, the arrow indicates the 85 kDa of the protein found in stage 1 melanosomes. For MART-1 and HMB45 blots, 5 μg of protein were loaded per well.

NCKX5 Possesses NCKX Activity That Is Altered by the nsSNP in SLC24A5—The unique intracellular localization of NCKX5 necessitates the development of new assays as the currently available methodologies are not suitable for measuring intracellular sodium-calcium exchange activity. The cell surface-localized recombinant NCKX5 generated by heterologous expression of SLC24A5 cDNA in insect High Five cells enabled us to demonstrate for the first time that NCKX5 possesses authentic NCKX activity (Fig. 8C). However, the level of activity conferred by SLC24A5 was much lower than that generated by SLC24A2 in the same system, possibly reflecting differences in subcellular distribution or the considerably lower expression level observed for NCKX5 compared with NCKX2. Because the activity of NCKX5 in this heterologous expression system was low, we used both NCKX2 and NCKX5 assays to test the effect of the amino acid change encoded by the SLC24A5 nsSNP on NCKX activity. In both proteins, switching alanine to threonine at the critical residue (NCKX5 residue 111 and NCKX2 residue 177) caused a substantial reduction in exchanger activity. This suggests that this residue is important for the exchanger activity of both NCKX2 and NCKX5, and a high level of cross-species and cross-family sequence conservation around this residue implies is likely to be important for all NCKX proteins. These data also support the hypothesis that the nsSNP associated with skin color variation is causative, directly mediating phenotypic variation in skin color by altering NCKX5 exchanger activity.

SLC24A5, a pH Regulator?—Intracellular sodium-calcium exchange raises potential questions as to how the necessary ion gradients are maintained across an intracellular membrane and in which direction the exchanger operates under physiological conditions. Lamason et al. (7) propose a model based upon a melanosomal localization for NCKX5. They suggest that NCKX5 moves Ca\(^{2+}\) from the cytoplasm to the extracellular space (26–28).

Using our affinity-purified polyclonal antisera suggest that, unlike other previously characterized members of the sodium-calcium exchanger and NCKX families, native NCKX5 is located in a subcellular compartment that partially overlaps with a region of the TGN (Figs. 2 and 3). We have found no evidence to suggest that NCKX5 is located at the plasma membrane of cultured melanocytes, consistent with previous findings (7), which reported an intracellular localization for tagged recombinant zebrafish NCKX5. In a proteomic analysis of fractionated melanocytes, NCKX5 was detected in melanosomal-enriched fractions (18), although we have not detected any co-localization between NCKX5 and melanosomal markers by immunofluorescence microscopy with our antisera (Fig. 3D). Fractionation of our NHM extracts using a sucrose density gradient similar to that used in the proteomic study (18) revealed that NCKX5, dark pigment, and TGN46 were all enriched in the fractions enriched for melanosomal marker TYRP1. These data, supporting an intracellular, partial TGN localization, are surprising since all other NCKX proteins have been shown to traffic to the plasma membrane where they operate to remove Ca\(^{2+}\) from the cytoplasm to the extracellular space (26–28).

NCKX5 Is Localized to Subcellular Membranes—Multiple sequence alignments predict that the protein encoded by SLC24A5 is a member of the NCKX protein family (~42% similarity across the whole protein and 70% similarity in the conserved α1 and α2 regions). However, despite a high degree of similarity at the amino acid level (Fig. 8B), the credentials of NCKX5 as a sodium-calcium-potassium exchanger have not previously been established experimentally. Data generated...
ing of the melanosomal scaffold protein pmel17. If this were the case, a reduction in NCKX5 exchange activity, which we predict would result from the threonine 111 substitution encoded by the nsSNP, would cause a net acidification of the melanosomes as the activity of the Na\(^+/\)H\(^+\) exchanger is impaired. This is consistent with the observation that melanosomal pH is higher in cultured melanocytes derived from Negroid donors compared with those derived from Caucasian donors (29). The more alkaline environment of Negroid melanosomes is believed to favor the activity of tyrosinase, a rate-limiting catalyst of melanin synthesis that operates optimally at neutral pH. This might account for the reduced tyrosinase activity measured in Caucasian compared with Negroid-derived melanocytes (30). Although our studies cannot rule out a cryptic melanosomal localization for NCKX5, our work has definitively demonstrated that NCKX5 is partially localized to the TGN, which leads us to propose two alternative models for the role of NCKX5 in pigmentation.

A Role for SLC24A5 in the TGN?—The Golgi apparatus is an established calcium store (31), and its role in sorting and processing secretory and membrane proteins is highly sensitive to changes in calcium concentration within the lumen. Recent work has shown that the calcium concentration of the Golgi compartment is highly regulated in keratinocytes (32, 33), and the calcium gradients across the Golgi play a fundamental part in intracellular calcium signaling and homeostasis (for review, see Ref. 34). In addition, the Na\(^+/\)H\(^+\) exchanger NHE7 is located in the TGN and post-Golgi vesicles (35, 36) and is differentially expressed in light and dark NHM (37). The model proposed by Lamason et al. (7) in which SLC24A5 modulates...
melanosomal pH could also be applied to the TGN. Data on the role of yeast Nhx1 in protein trafficking (38) and the effects of alkalization of tyrosinase on its maturation in melanoma cells and mouse melanocytes (39, 40) led Smith et al. (37) to suggest that NHE-7 might regulate tyrosinase maturation by raising the pH of the TGN to allow correct folding and maturation of tyrosinase. This complex process is tightly regulated, and there is evidence that it is essential that it is navigated correctly for active tyrosinase to be localized appropriately (41, 42). In our first alternative model we propose that the nsSNP in SLC24A5 reduces the Na\(^+/\)Ca\(^{2+}\) exchange activity of NCKX5 located in the TGN, which alters Na\(^+/\)H\(^{+}\) exchange activity through NHE-7 coupled to a V-ATPase, acidifying the TGN, potentially impairing the maturation of tyrosinase. In addition, the accompanying changes to calcium gradients in the Golgi lumen could impact a number of processes, including the activation of calcium-dependent furin-like protease activity in a manner analogous to the melanosomal model proposed by Lamason et al. (7). The Golgi Ca\(^{2+}\) may also act as a store for Ca\(^{2+}\)-mediated signaling such as the proposed melanogenic response to agouti signaling protein (43) or the UV-induced response to endothelin-1 (44). Ca\(^{2+}\) is also a co-factor for phenylalanine hydroxylase (45); the latter has been suggested to protect the redox balance and prevent DNA damage (46).

**SLC24A5 in Melanosome Biogenesis?**—Our alternative model takes into account the dramatic effect of SLC24A5 knockdown on pigmentation in re-differentiating melanocytes, and the effect that this has on the expression of pmel17 and Lamp1. The 85-kDa form of pmel17 that is down-regulated by SLC24A5 knockdown is a marker of early melanosomes (25), whereas Lamp1 is enriched in lysosomes (47). In the model proposed by Raposo et al. (47, 48), the coated endosome (also referred to as a stage 1 melanosome) serves as a critical sorting point between endocytic and pre-melanosomal pathways, from which macromolecules are specifically directed into melanosomal or lysosomal pathways.

We, therefore, propose a second model in which NCKX5 modulates melanogenesis through a role in the trafficking/sorting decisions, which occur within the coated endosome. In this model NCKX5 is required for the correct delivery of melanosomal components such as pmel17 into the melanosomal pathway. Thus, when SLC24A5 is disrupted through siRNA knockdown, melanosomal maturation is blocked, leading to a down-regulation of pmel17 and late melanosomal markers TYR and TYRP1. Consequently, protein and membrane resources from within the coated endosome are diverted into the endosome-lysosome pathway, leading to an up-regulation of Lamp1. Endosomal membrane fusion events, which are essential processes in melanosome and lysosome-related organelle biogenesis, are Ca\(^{2+}\)-mediated (49, 50) and are likely modulated by changing local Ca\(^{2+}\) gradients. We propose that NCKX5 could play a role in maintaining local calcium concentrations in or near to the coated endosomes, which are closely associated with the TGN (47), to facilitate appropriate membrane fusion “kiss and run” events and ensure the delivery of important proteins such as pmel17 into the melanosomal system. The importance of correctly regulating such intracellular trafficking mechanisms to melanogenesis is highlighted by the de-pigmenting effects of certain tricyclic compounds that elicit the mis-trafficking of tyrosinase leading to its co-localization with Lamp1 (51). A more precise examination of NCKX5 localization by immunoelectron microscopy and of the effect of SLC24A5 knockdown on different components of the endosomal, lysosomal, and melanosomal pathways would help to further our understanding of the mechanisms by which NCKX5 regulates melanogenesis and human skin color variation.

We have shown that NCKX5 is localized to the TGN of epidermal melanocytes and plays a direct and important role in human melanogenesis. Furthermore, the nsSNP in SLC24A5 associated with natural human skin color variation encodes an amino acid change that alters both NCKX2 and NCKX5 activity. This strongly suggests that NCKX5 exchange function modulates melanogenesis in epidermal melanocytes and thereby in human skin.

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