Store-operated Ca\(^{2+}\) entry regulates Ca\(^{2+}\)-activated chloride channels and eccrine sweat gland function

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Eccrine sweat glands are essential for sweating and thermoregulation in humans. Loss-of-function mutations in the Ca\(^{2+}\)-release–activated Ca\(^{2+}\) (CRAC) channel genes Orai1 and Stim1 abolish store-operated Ca\(^{2+}\) entry (SOCE), and patients with these CRAC channel mutations suffer from anhidrosis and hyperthermia at high ambient temperatures. Here we have shown that CRAC channel–deficient patients and mice with ectodermal tissue–specific deletion of Orai1 (Orai1\(^{−/−}\)) or Stim1 and Stim2 (Stim1/2\(^{−/−}\)) failed to sweat despite normal sweat gland development. SOCE was absent in agonist-stimulated sweat glands from Orai1\(^{−/−}\) and Stim1/2\(^{−/−}\) mice and human sweat gland cells lacking ORAI1 or STIM1 expression. In Orai1\(^{−/−}\) and Stim1/2\(^{−/−}\) mice, abolishment of SOCE was associated with impaired chloride secretion by primary murine sweat glands. In human sweat gland cells, SOCE mediated by ORAI1 was necessary for agonist-induced chloride secretion and activation of the Ca\(^{2+}\)-activated chloride channel (CaCC) anoctamin 1 (ANO1, also known as TMEM16A). By contrast, expression of TMEM16A, the water channel aquaporin 5 (AQP5), and other regulators of sweat gland function was normal in the absence of SOCE. Our findings demonstrate that Ca\(^{2+}\) influx via store-operated CRAC channels is essential for CaCC activation, chloride secretion, and sweat production in humans and mice.

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Introduction
Sweating is a physiological process primarily intended for thermoregulation in humans. Sweat is produced by eccrine sweat glands, which develop from the epidermal basal layer during embryogenesis (1). Each eccrine sweat gland is composed of 2 portions, the secretory coil and duct, which differ in structure and function (2). The secretory coil contains dark cells, clear cells, and myoepithelial cells, whereas the duct wall consists of a stratified cuboidal epithelium (2). Sweat secretion is mediated by a coordinated network of ion channels and transporters, which orchestrate the movement of Na\(^{+}\) and Cl\(^{−}\) ions from the blood to the apical membrane of the secretory coil cells (3, 4). Increasing luminal salt concentrations establish an osmotic gradient that provides the driving force for the movement of water into the sweat gland lumen (5–7). Salt-enriched fluid produced by the secretory cells is collected in the ducts, which partially reabsorb Na\(^{+}\) and Cl\(^{−}\) before the fluid is secreted via the sweat pore to reduce salt loss (1). Sweating is triggered in response to a variety of stimuli including heat as well as cholinergic and adrenergic agonists, which bind to muscarinic acetylcholine (ACh) receptors or α- and β-adrenergic receptors, respectively, that are expressed on secretory coil cells of sweat glands (8, 9). Reduced or abolished sweating is a rare human genetic condition referred to as hypohidrotic or anhidrotic ectodermal dysplasia (EDA, OMIM #305100), which in some cases occurs together with immunodeficiency (EDA-ID, OMIM #300291). Mutations in ectodysplasin A (EDA), its receptor ectodysplasin A receptor (EDAR), and the adaptor protein EDAR-associated death domain (EDARADD) cause EDA, whereas mutations in molecules of the nuclear factor κ light chain–enhancer of activated B cells (NF-κB) signaling pathway, NEMO (encoded by IKKBG) and IkBα (encoded by NFKBIA), are responsible for EDA-ID (10, 11). Anhidrosis is a serious medical problem particularly under conditions when sweating is required for thermoregulation, and may result in hyperthermia, stroke, and death (12, 13).

Sudorific agonists such as ACh increase the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) (14–16). The resulting Ca\(^{2+}\) signals mediate the opening of Ca\(^{2+}\)-activated chloride channels (CaCCs) in secretory cells from different organs, including eccrine sweat glands (7, 17, 18). Several CaCCs, such as anoctamin 1 (ANO1, or TMEM16A) and bestrophin 2 (BEST2), have been reported to mediate fluid secretion in parotid acinar cells, submandibular salivary gland acinar cells, and colon and airway epithelia (19–23). In mice, targeted deletion of the Best2 gene abolishes spontaneous sweat production...
in the absence of agonist stimulation (3, 24). In human eccrine sweat glands, the molecular nature of the CaCC responsible for chloride secretion is still debated (3). Also debated is the source of Ca2+ signals for the activation of CaCCs in eccrine sweat glands (3). Ca2+ release from the endoplasmic reticulum (ER) through inositol 1,4,5-trisphosphate receptors (IP3Rs) and the subsequent increase in [Ca2+]i have recently been shown to be required for eccrine sweat gland function, as patients with mutations in the type 2 IP3R were anhidrotic (16). By contrast, earlier studies had shown that Ca2+ influx from the extracellular space is essential for sweating in response to cholinergic and α-adrenergic stimulation (14). However, the channels mediating Ca2+ influx in secretory sweat gland cells and the mechanism by which these channels regulate sweat secretion remain unknown (25).

Store-operated Ca2+ entry (SOCE) is a universal Ca2+ influx mechanism in a large variety of cell types (26). It is mediated by the activation of Ca2+ release–activated Ca2+ (CRAC) channels that are composed of Orai1, and potentially Orai2 and Orai3, subunits localized in the plasma membrane (PM). CRAC channels are activated following agonist stimulation of cell surface receptors such as the G protein–coupled ACh receptor. Its engagement results in anhidrosis, in both human and mice. We demonstrate how they regulate the function of the immune system and other cell functions. The importance of CRAC channels is evident in human patients with autosomal recessive loss-of-function or null mutations in ORAI1 and STIM1 genes that abolish SOCE. These patients present with a unique syndrome, termed CRAC channelopathy, which is characterized by severe immunodeficiency, autoimmunity, congenital muscular hypotonia, and EDA. Besides anhidrosis, EDA in these patients is characterized by defects in dental enamel formation (29, 30). Although studies of human patients with CRAC channelopathy and mice with conditional deletion of Orai1, Stim1, and Stim2 genes have greatly increased our understanding of the physiological role of CRAC channels and how they regulate the function of the immune system and other organs (31), their role in sweat glands remains largely undefined.

We show here that deletion of CRAC channels and SOCE abolish the function, but not the development, of eccrine sweat glands, resulting in anhidrosis, in both human and mice. We demonstrate that Ca2+ influx in response to agonist stimulation of eccrine sweat glands depends on ORAI1, STIM1, and STIM2, which are required for the activation of CaCCs and Cl– secretion. Our results identify TMEM16A as the CaCC in human sweat gland cells and SOCE as a conserved Ca2+ influx pathway that is critical for CaCC function, sweat secretion, and thermoregulation.

### Results

Anhidrosis in SOCE-deficient patients despite normal development of eccrine sweat glands. Mutations in different components of the
NF-κB signaling pathway were shown to cause EDA-ID (32, 33). Affected patients have abnormal development of ectodermal tissues including the skin, hair, teeth, and sweat glands. Most patients have fewer sweat glands, accounting for their anhidrosis (34). The EDA-ID phenotype is reminiscent of patients with CRAC channelopathy caused by loss-of-function or null mutations in \( \text{ORAI1} \) or \( \text{STIM1} \), who also present with EDA-ID and tooth defects (30). However, their immunodeficiency is caused predominantly by T cell dysfunction, and their tooth defect is characterized by reduced enamel calcification in contrast to small, conical teeth in patients with defective NF-κB signaling. Hypohidrosis or anhidrosis has been found in all patients with mutations in \( \text{ORAI1} \) or \( \text{STIM1} \) when sweat secretion was studied directly by iontophoresis, similar to other patients with mutations in \( \text{ORAI1} \) (p.G98R, p.L194P, or compound p.A103E/p.L194P) and \( \text{STIM1} \) (p.R429C, p.L374P, or p.L74P) that abolish SOCE (Table 1 and Figure 1, A–C). None of the 3 patients was able to sweat when tested by iontophoresis following cutaneous pilocarpine stimulation, similar to other patients with mutations in \( \text{ORAI1} \) (p.G98R, p.L194P, or compound p.A103E/p.L194P) and \( \text{STIM1} \) (p.R429C, p.L374P, or p.L74P) that abolish SOCE (Table 1).

Figure 1. Sweat glands are present in CRAC channel–deficient patients with EDA-ID. (A) H&E staining of eccrine sweat glands in the dermis of a healthy control donor (HD) and a patient with \( \text{ORAI1} \) p.R91W loss-of-function mutation at low magnification. (B) Alcian blue staining of the same biopsies shown in A to detect acid mucopolysaccharides in dark cells (green arrowheads). Arrows indicate different cell types in the secretory portion of sweat glands (CC, clear cells; DC, dark cells; and MC, myoepithelial cells). (C) H&E staining of eccrine sweat glands in the dermis of an HD and patients with \( \text{ORAI1} \) p.R91W (magnification of boxed area in A), \( \text{ORAI1} \) p.V181SfsX8, and \( \text{STIM1} \) p.P165Q mutations that abolish SOCE. Asterisks indicate the lumen of secretory sweat glands. Scale bars in A–C: 50 μm. (D) Quantification of sweat gland lumens from human skin biopsies shown in C. Bars represent the means of 3 HDs and 3 individual patients. Between 2 and 4 coiled nests of eccrine sweat glands were analyzed per skin biopsy. Statistical analyses were performed by 1-way ANOVA using HDs as a reference and multiple comparisons. ***P < 0.001.
skin, teeth, and sweat glands. Histological analysis of eccrine sweat glands in the footpads of Orai1fl/fl, K14-Cre (hereafter referred to as Orai1K14Cre) and Stim1fl/fl, Stim2fl/fl, K14-Cre (hereafter referred to as Stim1/2 K14Cre) mice showed glands with a morphology similar to that in WT control mice with the exception of significantly smaller sweat gland lumens (Figure 2A and Supplemental Figure 1, B–D), thus resembling the findings in human patients with CRAC channelopathy. Orai1 and Stim1 mRNA expression was substantially reduced in sweat glands isolated from Orai1K14Cre and Stim1/2K14Cre mice, respectively (Figure 2B). The residual mRNA levels observed are likely due to contamination of sweat gland tissue with other cells in the footpad preparation that are not of ectodermal origin and do not express keratin 14. This interpretation is supported by the complete lack of ORAI1 and STIM1 protein observed by immunohistochemistry in eccrine sweat glands of Orai1K14Cre and Stim1/2K14Cre mice compared with WT controls.

Deletion of Orai1 or Stim1/Stim2 genes in murine sweat glands abolishes sweating. (A–D) Analysis of sweat gland morphology in Orai1 and Stim1/2-deficient mice. (A) H&E staining of hind paw skin biopsies of WT, Orai1K14Cre, and Stim1/2K14Cre mice. Images are representative of 8 WT, 4 Orai1K14Cre, and 4 Stim1/2K14Cre mice. Scale bars: 50 μm. (B) Orai1 (left) and Stim1 and Stim2 (right) mRNA expression in sweat glands isolated from paws of WT (n = 14), Orai1K14Cre (n = 7), and Stim1/2K14Cre (n = 10) mice analyzed by quantitative real-time PCR. Hprt was used to normalize mRNA expression levels. Data are shown relative to mRNA levels in WT and as mean ± SEM. Statistical analysis by 2-tailed Student’s t test. ***P < 0.001. (C and D) Immunohistochemical staining of ORAI1 (C) and STIM1 (D) in eccrine sweat glands in footpads of WT, Orai1K14Cre, and Stim1/2K14Cre mice. Images are representative of 2 mice per genotype. Scale bars: 50 μm. (E–G) Impaired sweating in Orai1 and Stim1/2-deficient mice. ACh induced sweat responses in the hind paws of WT, Orai1K14Cre, and Stim1/2K14Cre mice. The paws of mice were coated with starch-iodine solution and injected s.c. with 100 μM ACh, and sweat dots were counted 5 minutes afterward. (E) Representative images. (F) Averaged number of sweat dots on the paws of 16 WT, 8 Orai1K14Cre, and 4 Stim1/2K14Cre mice. Each dot represents 1 hind paw from an individual mouse. (G) ACh induced sweat responses in the hind paws of WT mice that were pretreated epicutaneously with 1 μM or 100 μM CRAC channel inhibitor BTP2 or vehicle (ethanol) 4 hours and 2 hours before ACh injection. Each dot represents 1 hind paw from an individual mouse. Statistical analyses in F and G were performed by 1-way ANOVA using multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001.
The application of the CRAC channel inhibitor BTP2 to the footpads of WT mice. BTP2 treatment resulted in a significant reduction in sweat production over a 100-fold concentration range (Figure 2G). Taken together with the findings in mice with conditional deletion of \( \text{Orai1} \) and \( \text{Stim1/2} \) genes, these data suggest that CRAC channels regulate the function, but not the development, of murine eccrine sweat glands.

ORAI1, STIM1, and STIM2 mediate agonist-induced Ca\(^{2+}\) influx in sweat glands but are not required for expression of ion channels mediating Cl\(^{-}\) secretion.

To assess the role of ORAI1, STIM1, and STIM2 in Ca\(^{2+}\) signaling in response to agonist stimulation of eccrine sweat glands, we isolated individual glands from the paws of \( \text{Orai1K14Cre} \) or \( \text{Stim1/2K14Cre} \) mice and analyzed them for \([\text{Ca}^{2+}]_i\). Stimulation with ACh in Ca\(^{2+}\)-free media induced a comparable Ca\(^{2+}\) release from ER stores in the secretory coils of sweat glands of WT and \( \text{Orai1} \)- and \( \text{Stim1/2} \)-deficient mice (Figure 3, A and B, and Supplemental Figure 3). Readdition of extracellular Ca\(^{2+}\) caused robust Ca\(^{2+}\) influx in sweat glands of WT but not \( \text{Orai1K14Cre} \) or \( \text{Stim1/2K14Cre} \) mice (Figure 3, A–C), indicating that ORAI1 and STIM1/2 mediate SOCE in murine eccrine sweat glands upon cholinergic stimulation. While Ca\(^{2+}\) entry following ACh stimulation could potentially be mediated by channels other than CRAC, this which show strong ORAI1 and STIM1 expression (Figure 2, C and D). Together, mice with conditional deletion of CRAC channel genes in ectodermal tissues have sweat glands of largely normal morphology with the exception of reduced sweat gland lumens.

We next investigated the effects of CRAC channel deletion on eccrine sweat gland function by inducing sweat secretion in WT, \( \text{Orai1K14Cre} \), and \( \text{Stim1/2K14Cre} \) mice via cholinergic stimulation (46). Since innervated sweat glands are restricted to the footpads in mice (47), we tested sweat secretion in the hind paws of mice, which were painted with iodine-starch solution and injected s.c. with ACh. Analyzing the number of dark spots on the paw surface, which correspond to active sweat pores, 5 minutes after ACh treatment, we found about 30 spots per paw in WT mice but hardly any spots in \( \text{Orai1K14Cre} \) and \( \text{Stim1/2K14Cre} \) mice (Figure 2E and F), indicating that CRAC channels are required for eccrine sweat gland function in mice. It is noteworthy that while \( \text{Orai2} \) mRNA expression was detected in sweat gland tissue from WT mice (Supplemental Figure 1A), we did not observe a defect in sweat secretion in \( \text{Orai2}\text{-}\text{/-} \) mice (Supplemental Figure 2), suggesting that ORAI1 is the main CRAC channel homolog mediating SOCE in eccrine sweat gland cells. We next tested the effects of acute CRAC channel inhibition on sweat secretion by epicutaneous application of the CRAC channel inhibitor BTP2 to the footpads of WT mice. BTP2 treatment resulted in a significant reduction in sweat production over a 100-fold concentration range (Figure 2G). Taken together with the findings in mice with conditional deletion of \( \text{Orai1} \) and \( \text{Stim1/2} \) genes, these data suggest that CRAC channels regulate the function, but not the development, of murine eccrine sweat glands.

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is unlikely given the fact that deletion of either Orai1 or Stim1/2 resulted in a similar Ca\(^{2+}\) influx defect.

To understand the mechanisms underlying the sweating defect in CRAC channel–deficient mice and patients, we first tested whether SOCE controls the expression of molecules that mediate Cl\(^{-}\) and water secretion by eccrine sweat glands. In mice, the CaCC BEST2 is required for sweat production, as its deletion in Best2\(^{-/-}\) mice causes severe hypohidrosis (3, 24). Based on studies in the NCL-SG3 sweat gland cell line, TMEM16A has been implicated as a molecular component of CaCCs in human eccrine sweat glands (7). Besides BEST2 and TMEM16A, the intracellular Cl\(^{-}\) concentration ([Cl\(^{-}\)]) in sweat gland cells is regulated by the electroneutral Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter NKCC1 (encoded by the SLC12A2 gene), which mediates Cl\(^{-}\) influx across the basolateral membrane of secretory cells (24, 48, 49). Cl\(^{-}\) secreted via CaCCs into the sweat gland lumen results in the consecutive secretion of water into the acini lumen mediated by the opening of the water channel aquaporin 5 (AQP5) (5). Expression levels of Best2 and Tmem16a mRNA and BEST2 and TMEM16A protein as analyzed by immunofluorescence were normal in eccrine sweat glands isolated from CRAC channel–deficient mice (4).
lated from *Orai1* and *Stim1* mice compared with WT controls (Figure 4, A–C). As expected, TMEM16A was mainly localized in the apical membrane of secretory sweat glands (Figure 4B and Supplemental Figure 4), whereas BEST2 was distributed throughout the cytoplasm (Figure 4C), consistent with findings in a previous study (24). Furthermore, we found normal levels of *Nkcc1* and *Aqp5* mRNA in sweat glands from *Orai1* and *Stim1* mice (Figure 4A). Expression of other *Cl*– permeable channels and transporters such as TMEM16B, CFTR, and NKCC2 that might contribute to *Cl*– transport and sweating was very low and barely detectable by quantitative real-time PCR (data not shown). Normal transcript levels of *Cl*– channels and transporters are consistent with normal expression of the transcription factors FOXA1, which was shown to regulate the expression of BEST2 and NKCC1 (24), and NFATc3, which is regulated by *Ca*2+ signals and activates FOXA1 (SOCE mediates *Cl* secretion in mouse and human sweat gland cells. We next tested the functional role of SOCE in *Cl* secretion by primary murine sweat glands. Isolated sweat glands from WT and *Orai1* and *Stim1* mice were loaded with the intracellular *Cl* indicator MQAE and stimulated with ACh in buffer containing physiological *Ca*2+ (2 mM) and *Cl* (100 mM) concentrations followed by removal of extracellular *Cl* to force *Cl* secretion (Figure 4D). Before stimulation, WT and *Orai1*–deficient sweat glands had comparable [Cl–], (WT: 62 ± 12 mM; *Orai1*; 61 ± 8 mM). After ACh stimulation, [Cl–] significantly decreased in WT glands consistent with *Cl* secretion (Figure 4, D and E). By contrast, sweat glands from *Orai1* mice showed a moderate increase in [Cl–], which suggests a predominance of *Cl* influx mechanisms in the absence of *Cl* secretion. When extracellular *Cl* was replaced with isethionate to force *Cl* secretion from cells, WT sweat glands showed a strong reduction of [Cl–], consistent with *Cl* efflux as expected (Figure 4, D and F). *Orai1* sweat glands also showed reduced [Cl–], but the decrease was much less pronounced compared with that in WT cells. There was a striking difference in the overall net reduction of [Cl–] at the end of experiments compared with baseline [Cl–] in WT sweat gland cells (~50 mM), whereas only a small decrease in *Orai1* cells was observed (~10 mM) (Figure 4G). The residual reduction in [Cl–] in *Orai1* sweat glands after removal of extracellular *Cl* is likely due to *Ca*2+-independent *Cl* efflux mechanisms that are preserved in ORAI1–deficient cells. These results demonstrate that SOCE is required to mediate *Cl* secretion in murine eccrine sweat glands.

To test whether human sweat glands also require SOCE for *Cl* secretion, we used the human eccrine sweat gland cell line NCL-SG3 (51), which has been widely used to measure epithelial ion transport (52). NCL-SG3 cells abundantly expressed ORAI1 and STIM1 mRNA (data not shown) and protein (Supplemental Figure 5, A–C). Suppression of ORAI1 and STIM1 in NCL-SG3 cells by shRNA strongly reduced their mRNA (Figure 5A) and protein (Supplemental Figure 5, A–C) expression. We found strongly reduced SOCE in NCL-SG3 cells stably transduced with shORAI1 and shSTIM1 after ionomycin stimulation, whereas *Ca*2+ release from ER stores was unaffected (Figure 5, B and C), indicating that ORAI1 and STIM1 regulate SOCE in human sweat gland cells.

We next analyzed [Cl–] in NCL-SG3 cells lacking ORAI1 or STIM1 expression following induction of SOCE. One particular characteristic of NCL-SG3 cells is their inability to respond to cholinergic stimulation with ACh (17, 51, 53, 54). They do, however, have anion fluxes in response to the *Ca*2+ ionophores calcimycin and ionomycin (17, 54). Ionomycin stimulation of NCL-SG3 cells in 100 mM [Cl–]o (where [Cl–]o indicates the extracellular concentration of Cl–) resulted in a moderate reduction of [Cl–], which was strongly enhanced when *Cl*– secretion was forced by removal of *Cl* from the extracellular buffer (Figure 4, D and E). NCL-SG3 cells transduced with shORAI1 or shSTIM1 lacked a comparable reduction of [Cl–], suggesting that *Cl*– secretion by NCL-SG3 cells depends on SOCE (Figure 4, D and E). Defective *Cl*– secretion in SOCE-deficient NCL-SG3 cells was not due to altered expression of CaCCs, since the reduction of SOCE by shORAI1 or shSTIM1 did not decrease *TMEM16A* and *BEST2* mRNA levels (Figure 5F). These results are consistent with our findings in murine sweat glands and demonstrate that SOCE is a conserved mechanism for *Cl*– secretion in eccrine sweat glands.

**SOCE is required for activation of CaCCs in human sweat glands.** To characterize the mechanism by which SOCE regulates *Cl* secretion, we measured *Cl* currents in NCL-SG3 cells. Using the whole-cell patch-clamp configuration, we observed that *Cl* currents are activated in the presence of 1 μM *Ca*2+ in the patch pipette, and thus the cytosol, whereas no *Cl* currents were observed in the absence of *Ca*2+ (Figure 6, A and B). The *Cl* currents displayed hallmarks of epithelial CaCC currents, including fast activation followed by a smaller slow time-dependent component, deactivating tail currents upon repolarization (Figure 6B), and strong outward rectification (Figure 6C). CaCC currents with similar properties were detected in SOCE-deficient NCL-SG3 cells after transduction with shORAI1 when the patch pipette contained 1 μM *Ca*2+ (Figure 6, B and C). These results show that ORAI1-deficient NCL-SG3 cells can activate CaCC currents similarly to SOCE-sufficient NCL-SG3 cells when cytotoxic [*Ca*2+]i is elevated directly through the patch pipette, thus confirming the presence of functional CaCCs in NCL-SG3 cells. Stimulation of WT NCL-SG3 cells with ionomycin in the presence of extracellular *Ca*2+, which depletes ER *Ca*2+ stores and induces SOCE (Figure 5B), resulted in robust and sustained *Cl* currents (Figure 6, D and E). The currents had properties similar to those induced by inclusion of 1 μM *Ca*2+ in the patch pipette (Figure 6, B and C) and were consistent with reported CaCC currents (23, 55). By contrast, ORAI1-deficient NCL-SG3 cells showed only transient and attenuated *Cl* currents upon ionomycin stimulation (Figure 6, D and E). Transient *Cl* currents parallel the transient increase in [*Ca*2+]i resulting from ER *Ca*2+ release in the absence of SOCE in ORAI1-deficient cells (Figure 5B), indicating that SOCE is required for sustained activation of CaCCs, whereas *Ca*2+ release from ER stores can only transiently and partially activate CaCCs.

To investigate whether SOCE is required for CaCC function in agonist-stimulated sweat glands, we treated WT and ORAI1-deficient NCL-SG3 cells with trypsin, an agonist of proteinase-activated receptor 2 (PAR2), which has previously been shown to evoke CaCC currents in NCL-SG3 cells (56). Stimulation of WT NCL-SG3 cells with trypsin in *Ca*2+-containing media resulted in stronger and more sustained elevation of [*Ca*2+]i, in comparison with those treated with vehicle (Figure 6D).
SOCE mediates Cl⁻ secretion in human sweat gland cells by activating TMEM16A. The identity of the CaCCs in eccrine sweat gland cells is controversial (3, 7, 24). In mice, deletion of Best2 gene expression impairs sweating (24), whereas TMEM16A was suggested to mediate CaCC-dependent Cl⁻ secretion in human sweat gland cells (7). We found TMEM16A expression in primary human eccrine sweat glands from a healthy donor (Supplemental Figure 6A). Its expression was not altered in CRAC channel–deficient patients with ORAI1 p.V181SfsX8 null or STIM1 p.P165Q loss-of-
Figure 6. SOCE activates Ca²⁺-dependent Cl⁻ channels (CaCCs) in human sweat gland cells. NCL-SG3 cells were transduced with shORAI1 (red) or left untransduced (Ctrl, black) and Cl⁻ currents measured by whole-cell patch-clamp. (A) Currents were elicited by 20-mV steps from –80 mV to 120 mV (from a holding potential of –50 mV) followed by a 0.5-second hyperpolarizing step to –80 mV. (B) Representative current traces recorded in individual Ctrl and shORAI1-transduced NCL-SG3 cells with 0 μM Ca²⁺ (top) or 1 μM Ca²⁺ (bottom) present in the patch pipette. Dotted lines indicate the zero-current level. (C) Current density as a function of voltage at the end of each test pulse from experiments shown in B (mean ± SEM, n = 5 per cell line). The reversal potential of the current is close to the equilibrium potential of Cl⁻ (E_Cl ~ –24 mV). (D) Representative Cl⁻ current traces extracted at 0, 60 and 300 seconds from the experiment shown in B. (E) Representative [Ca²⁺] traces from NCL-SG3 cells stimulated with 10 μM trypsin (left) and quantitation of [Ca²⁺] at 60 seconds (peak) and 300 seconds (sustained phase). The AUC was integrated between 50 and 300 seconds (mean ± SEM of 3 independent experiments, n = 15 cells analyzed per experiment). Statistical significance was determined using a 2-tailed Student’s t test. *P < 0.05, **P < 0.001. (G) Current densities were recorded in NCL-SG3 cells stimulated with 10 μM trypsin using an identical pulse protocol to E (mean ± SEM, n = 5). (H) Representative Cl⁻ current traces extracted at 0, 60 and 300 seconds from the experiment shown in G. TEA-Cl, tetraethylammonium chloride.
function mutations (not shown). TMEM16A was also detectable by Western blot in NCL-SG3 cells (Supplemental Figure 6B). To functionally assess which CaCC mediates Cl– secretion in human eccrine sweat gland cells, we stably deleted TMEM16A or control shRNA (shCtrl). (A) TMEM16A and BEST2 mRNA expression measured by quantitative real-time PCR and normalized to levels in shCtrl-transduced cells. GAPDH was used as a housekeeping control. Mean ± SEM of 3 independent experiments done in triplicate. (B) [Ca2+]i measurements in Fura-2-loaded NCL-SG3 cells stimulated with 1 μM ionomycin (Iono) in Ca2+-free Ringer solution followed by readaddition of 1 mM Ca2+ to induce SOCE. Representative [Ca2+]i traces (left) and AUC (right) after readdition of 1 mM Ca2+ (Ca2+ influx phase). For additional details see Supplemental Methods. Bar graphs represent mean ± SEM of 3 independent experiments done in triplicate. (C) [Cl–]i traces averaged from 50 cells in 1 experiment, and representative of 6–7 independent experiments. (D) Analysis of Cl– efflux rates (420–600 seconds, left), reduction in [Cl–]i (middle; calculated as the integrated area420–720s relative to baseline), and net Cl– efflux (right) after removal of extracellular Cl– at 420 seconds from data shown in C. For additional details see Figure 5E and Supplemental Methods. Data in D are mean ± SEM of 7 independent experiments for shCtrl, and 6 for both shBEST2 and shTMEM16A. Statistical analyses in A and D were performed using 1-way ANOVA followed by Bonferroni post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7. TMEM16A mediates SOCE-induced Cl– secretion in human sweat gland cells. NCL-SG3 cells were stably transduced with shBEST2, shTMEM16A, or control shRNA (shCtrl). (A) TMEM16A and BEST2 mRNA expression measured by quantitative real-time PCR and normalized to levels in shCtrl-transduced cells. GAPDH was used as a housekeeping control. Mean ± SEM of 3 independent experiments done in triplicate. (B) [Ca2+]i measurements in Fura-2-loaded NCL-SG3 cells stimulated with 1 μM ionomycin (Iono) in Ca2+-free Ringer solution followed by readaddition of 1 mM Ca2+ to induce SOCE. Representative [Ca2+]i traces (left) and AUC (right) after readdition of 1 mM Ca2+ (Ca2+ influx phase). For additional details see Supplemental Methods. Bar graphs represent mean ± SEM of 3 independent experiments done in triplicate. (C) [Cl–]i traces averaged from 50 cells in 1 experiment, and representative of 6–7 independent experiments. (D) Analysis of Cl– efflux rates (420–600 seconds, left), reduction in [Cl–]i (middle; calculated as the integrated area420–720s relative to baseline), and net Cl– efflux (right) after removal of extracellular Cl– at 420 seconds from data shown in C. For additional details see Figure 5E and Supplemental Methods. Data in D are mean ± SEM of 7 independent experiments for shCtrl, and 6 for both shBEST2 and shTMEM16A. Statistical analyses in A and D were performed using 1-way ANOVA followed by Bonferroni post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001.

Discussion

We identified CRAC channels and SOCE as a critical signaling pathway for eccrine sweat gland function in humans and mice.
CRAC channels were required to activate Ca\(^{2+}\)-activated Cl\(^{-}\) channels, in particular TMEM16A, and to induce Cl\(^{-}\) secretion. This pathway is disrupted in human patients with CRAC channelopathy due to null or loss-of-function mutations in the ORAI1 and STIM1 genes. Their disease is characterized by EDA as well as severe immunodeficiency, skeletal myopathy, and hypocalcified dental enamel (29, 30). The patients’ anhidrosis presents clinically with hyperthermia especially in hot summer months and was reported in most, but not all, ORAI1- or STIM1-deficient patients. However, all patients in whom sweating was tested directly by pilocarpine iontophoresis uniformly lacked sweat production (35, 38, 41, 43). In those CRAC channel–deficient patients in whom
anhidrosis was not reported, it may have gone unnoticed because of a focus on their severe immunodeficiency disease and early death (35–37, 39, 41, 42). Alternatively, several patients with hypomorphic ORAI1 mutations and reduced but not abolished SOCE may have hypohidrosis that is more difficult to diagnose (40, 42). Skin biopsies of patients with ORAI1 and STIM1 mutations show eccrine sweat glands with morphologies and cellular compositions similar to those of healthy donors but significantly reduced lumen of the sweat acini, which is consistent with similar findings in Orai1<sup>Itpr2</sup>−/− and Stim1/2<sup>Itpr2</sup>−/− mice. CRAC channels and SOCE therefore do not appear to be required for the development of eccrine sweat glands, but instead control their secretory function. This is in contrast to patients with EDA-ID resulting from mutations in IKBKGNFKBIA (OMIM #300291) (32–34, 57, 58), who have abnormal development of ectoderm-derived tissues including the skin, hair, teeth, and eccrine sweat glands. Thus, mutations of genes in the NF-κB signaling pathway (IKBKG, NFKBIA) and in the CRAC channel complex (ORAI1, STIM1) both cause EDA-ID, but the underlying causes of anhidrosis are different. Whereas the NF-κB pathway controls the development of sweat glands, CRAC channels regulate sweat gland function via the activation of CaCCs.

Ca<sup>2+</sup> signals control the transcriptional regulation of gene expression in many cell types (59). It was therefore possible that CRAC channel deficiency interferes with eccrine sweat gland function by altering the expression of ion channels or transporters. However, we did not find evidence that SOCE is required for the expression of molecules involved in sweat production such as TMEM16A, BEST2, NKCC1, and AQP5 (3–5, 7, 24, 49). The 2 main CaCCs that have been implicated in sweat gland function, TMEM16A and BEST2, were expressed normally at transcript and protein levels in murine and human sweat gland cells that lack SOCE. Further supporting the role of SOCE in regulating the function but not the expression of Cl<sup>−</sup> channels involved in sweat secretion was our finding that acute inhibition of SOCE in the paws of mice with the CRAC channel inhibitor BTP2 significantly reduced ACh-stimulated sweat secretion. A direct Ca<sup>2+</sup>-dependent regulation of CaCCs is also consistent with the fact that addition of 1 μM Ca<sup>2+</sup> inside the patch pipette was sufficient for the activation of Cl<sup>−</sup> currents even in the absence of ORAI1 expression.

CaCCs have long been recognized to be important for sweat gland function (7, 17), and Cl<sup>−</sup> secretion in the human eccrine sweat gland cell line NCL-SG3 was shown to depend on increases in [Ca<sup>2+</sup>]<sub>i</sub> (7, 15, 17). However, the source of Ca<sup>2+</sup> to activate CaCCs remains debated. Recently, patients with missense mutations in the ITPR2 gene encoding IP<sub>R</sub>2 were reported to suffer from a generalized, isolated hypohidrosis without other symptoms characteristic of EDA such as skin, hair, or tooth defects (16). IP<sub>R</sub>2 is 1 of 3 IP<sub>R</sub> homologs that are localized in the ER membrane and function as nonselective Ca<sup>2+</sup> release channels whose opening results in a transient [Ca<sup>2+</sup>]<sub>i</sub> increase. Sweat gland cells from Itpr2<sup>−/−</sup> mice had reduced ACh-induced Ca<sup>2+</sup> signals, and Itpr2<sup>−/−</sup> mice showed impaired sweat secretion in response to cholinergic stimulation (16). It was therefore concluded that Ca<sup>2+</sup> released from the ER via IP<sub>R</sub>2 controls sweat gland function. This is intriguing because IP<sub>R</sub>S were shown to colocalize with CaCCs required for Cl<sup>−</sup> secretion in several cell types (60–62). However, Ca<sup>2+</sup> release from the ER via IP<sub>R</sub>2 not only increases [Ca<sup>2+</sup>]<sub>i</sub> and may thus contribute to CaCC activation but also decreases [Ca<sup>2+</sup>]<sub>ER</sub>, which is the trigger for the activation of STIM1 and STIM2 and the opening of store-operated CRAC channels. Thus, mutations or deletion of IP<sub>R</sub>2 that inhibit Ca<sup>2+</sup> release from the ER will also impair the activation of SOCE. Our data support the notion that the hypohidrosis observed in patients with IP<sub>R</sub>2 mutation is due to defective CRAC channel activation and furthermore that SOCE is essential for sweat gland function whereas Ca<sup>2+</sup> released from the ER is not sufficient. This conclusion is based on the fact that the release of Ca<sup>2+</sup> from intracellular stores is intact in (i) primary sweat gland cells isolated from Orai1<sup>Itpr2</sup>−/− and Stim1/2<sup>Itpr2</sup>−/− mice, (ii) NCL-SG3 cells transduced with shRNAs against STIM1 or ORAI1, and (iii) cells from patients with loss-of-function mutations in ORAI1 or STIM1 genes (35, 38, 44, 63). This conclusion is further supported by the fact that the transient Ca<sup>2+</sup> signals in ORAI1-deficient NCL-SG3 cells after ionomycin or tryptic stimulation, which are due to Ca<sup>2+</sup> release from ER stores, are not sufficient for sustained activation of Cl<sup>−</sup> currents and Cl<sup>−</sup> secretion. The hypohidrotic phenotype in IP<sub>R</sub>2 mutant patients is fully consistent with a critical role of SOCE in eccrine sweat gland function because of the role of IP<sub>R</sub>2 as an ER leak channel upstream of CRAC channel activation. Interestingly, a recent study proposed a model of how CRAC channels and IP<sub>R</sub>S may work in tandem to regulate the Ca<sup>2+</sup>-dependent activation of CaCCs (64). Based on studies in Xenopus oocytes, Ca<sup>2+</sup> entering the cytosol through CRAC channels is taken up by the ER via sarco/endoplasmic Ca<sup>2+</sup> ATPase (SERCA) pumps and released at different sites by IP<sub>R</sub>S to activate CaCCs. In this model, IP<sub>R</sub>S but not CRAC channels colocalize with CaCCs. Whether a similar mechanism plays a role in eccrine sweat gland cells remains to be investigated.

Not only the source of Ca<sup>2+</sup> for CaCC activation but also the molecular nature of CaCCs themselves in eccrine sweat glands is debated. The most likely candidates are TMEM16A and BEST2. TMEM16A has been proposed to be the prototypical CaCC responsible for Ca<sup>2+</sup>-dependent Cl<sup>−</sup> secretion in several epithelia (20, 65, 66). TMEM16A was found to be strongly expressed in the apical membrane of mouse salivary gland acinar cells (18, 20, 23), and conditional deletion of Tmem16a abolished cholinergic-induced fluid secretion (18). Moreover, Tmem16a<sup>−/−</sup> mice had impaired Ca<sup>2+</sup>-activated Cl<sup>−</sup> secretion in airway and colon epithelia, and pancreatic acinar cells (21). Recent findings in NCL-SG3 cells and isolated human eccrine sweat gland cells from biopsy samples showed mRNA expression of several splice variants of Tmem16a (7). Nevertheless, evidence for Tmem16A protein expression in primary human and mouse eccrine sweat glands has been missing. We found that Tmem16A protein is strongly expressed in the apical membrane of murine eccrine sweat glands, consistent with its potential role as a CaCC. In human eccrine sweat glands, Tmem16A localization was not restricted to the apical membrane but was found to be more homogenously distributed in cells, which may have to do with the different placement of clear and dark cells in mouse and human eccrine sweat glands (1, 45, 49). The Cl<sup>−</sup> currents we recorded in NCL-SG3 cells closely resembled those reported for Tmem16A, in particular the Tmem16A(ac) splice variant, which displays larger instantaneous current and smaller time-dependent activation at positive membrane potential and higher Ca<sup>2+</sup> sensitivity compared with other splice variants (55).
and which has been reported to be expressed in NCL-SG3 cells (7). Most importantly, deletion of TMEM16A in NCL-SG3 sweat gland cells abolished Cl− secretion and Cl− currents activated by either Ca2+ in the patch pipette or the PAR2 agonist trypsin. These data demonstrate that TMEM16A is essential for CaCC function and Cl− secretion in human eccrine sweat gland cells and that its activation depends on SOCE.

BEST2 belongs to a small family of anion channels (BEST1–4), which have been linked to bicarbonate transport in intestinal epithelial cells (67), regulation of voltage-dependent Ca2+ channels (68), and Cl− transport in the ER (60). A role for BEST2 in sweat secretion is supported by the lack of spontaneous sweating in Best2−/− mice (24), although it is noteworthy that Best2−/− mice were not tested for agonist-induced sweating, and it is possible that they have residual sweat production in response to cholinergic stimulation, which might point to an additional role of other CaCCs such as TMEM16A in Cl− secretion by sweat gland cells in mice. We found BEST2 protein to be expressed in murine eccrine sweat glands, although it was localized predominantly in the cytoplasm, similar to published observations (24). It has been reported that BEST2 is not expressed by all mouse sweat gland secretory cells but only in dark cells (24) that support sweat secretion by clear cells (3).

In human NCL-SG3 cells, shRNA-mediated deletion of BEST2 had no effect on Cl− secretion or Cl− currents activated by Ca2+ in the patch pipette or trypsin stimulation, suggesting that BEST2 does not contribute to SOCE-induced Cl− secretion in these cells. Compared with TMEM16A, mRNA expression of BEST2 in NCL-SG3 cells was much lower, potentially explaining its negligible role.

In addition to controlling CaCC function and Cl− secretion, SOCE may regulate additional processes involved in sweating. For instance, Ca2+ signals in epithelial cells were shown to mediate the translocation of the water channel AQPS to the apical membrane of salivary (69) and sweat gland cells (70). AQPS has been proposed to be the main channel mediating water transport in eccrine sweat glands (5, 49, 70). However, despite consensus about the localization of AQPS in the apical membrane of the mouse sweat gland cells (5, 71), Aqp5−/− mice produced ambiguous results, as they had either normal (71) or impaired (3) sweat production. Another potential role for SOCE in eccrine sweat glands is the regulation of myoepithelial cell contraction. Myoepithelial cells are smooth muscle cells found in exocrine glands such as sweat, salivary, lacrimal, and mammary glands. In sweat glands, myoepithelial cells surround the secretory coil, and their contraction has been suggested to facilitate sweat secretion in response to ACh (72). Lack of SOCE in Orai1−/− mice abolishes lactation due to impaired milk ejection from mammary gland secretory alveoli, which in turn was caused by decreased myoepithelial cell contraction (73). The role of SOCE in sweat gland myoepithelial cell function remains to be elucidated. While defects in AQPS trafficking to the PM or myoepithelial cell contraction may potentially contribute to anhidrosis in CRAC channel-deficient patients and mice, we show in this study that sweat production already fails at an earlier step because of impaired SOCE-dependent CaCC function and Cl− secretion.

In summary, we identify CRAC channels and SOCE as an essential pathway required for sweating and thermoregulation. Whereas ORAI1 and STIM1 do not appear to be required for sweat gland development, their function is critical for the activation of CaCCs and Cl− secretion. We identify TMEM16A as the SOCE-activated CaCC responsible for Cl− secretion in human eccrine sweat gland cells. Our data demonstrate that Ca2+ influx through store-operated CRAC channels is the source of Ca2+ required for CaCC activation, Cl− secretion, and sweat gland function and thus reveal the molecular mechanisms underlying the anhidrosis in patients with loss-of-function mutations in Orai1 and Stim1. CRAC channelopathy represents a new form of EDA-ID that is due not to an absence of sweat glands but to their impaired function. Besides sweat gland cells, the function of other secretory cells may be regulated by SOCE-dependent activation of CaCCs and Cl− secretion. Our findings add to the existing evidence supporting an important physiological role of SOCE in regulating the function of exocrine cells including those in the salivary (74), lacrimal (75), and mammary glands (73). From a translational perspective, topical CRAC channel inhibition may be a therapeutic approach for clinical conditions associated with excessive sweating like hyperhidrosis.

**Methods**

**Human samples.** Skin biopsies of patients with CRAC channelopathy who are homozygous for Orai1 p.R91W (35, 44), Orai1 p.V181fsX8, and Stim1 p.F165Q (41) mutations and healthy donors were used for histology and immunohistochemistry.

**NCL-SG3 cells and shRNA transduction.** The human eccrine sweat gland cell line NCL-SG3 was a gift of Roland Lang (Paracelsus Medical University Salzburg, Salzburg, Austria). NCL-SG3 cells were established by infection of primary human eccrine sweat glands with simian virus 40 (SV40) (51). For knockdown of Orai1, Stim1, BEST2, and TMEM16A expression, NCL-SG3 cells were stably transduced with lentiviral particles expressing ORAI1-, STIM1-, BEST2-, or TMEM16A-specific shRNAs (Supplemental Table I). The knockdown efficiency was evaluated by quantitative real-time PCR, flow cytometry (Orai1), and Western blotting (STIM1 and TMEM16A). Two to three shRNAs per gene were tested, and those with the best knockdown efficiency were used for experiments. Additional details regarding cell culture, transduction of NCL-SG3 cells, flow cytometry, and Western blotting can be found in Supplemental Methods.

**Mice.** Orai1−/− (76) and Stim1−/− Stim2−/− mice (77) have been described previously. They were crossed to K14-Cre mice (78) (The Jackson Laboratory, strain 004782) to generate Orai1−/− K14-Cre (Orai1<sup>K14cre</sup>) and Stim1<!sub>−/−</sub> Stim2<sub>−/−</sub> K14-Cre (Stim1<sup>K14cre</sup>) mice. Orai1−/− mice were generated using VGB6 ES cells (C57BL/6<sup>N</sup>Tac) obtained from the Knockout Mouse Project (KOMP, www.komp.org) repository at UC Davis (project ID VG14962, Orai2tm1(KOMP)Vclg).

All mice were maintained on a C57BL/6 genetic background and used between 6 and 16 weeks of age.

**Sweat testing.** Sweat secretion in mice was measured using the iodine-starch sweat test as previously described (46). Additional details can be found in Supplemental Methods.

**Histology, immunohistochemistry, immunofluorescence, and image analysis.** Details regarding sample processing, histology, immunohistochemistry, immunofluorescence, and image analysis are described in Supplemental Methods.

**Sweat gland isolation from mice.** Tips of mouse digits were dissected with micro-scissors and fine-tip forceps under a stereo microscope, and pieces of skin tissue were digested with 0.25 mg/ml Liber-
ase TM (Roche) in DMEM (Corning) for 45 minutes at 37°C. Digested tissue was passed through a 40-μm cell strainer (Fisher Scientific), and gland tissue retained in the filter was collected for quantitative real-time PCR analysis and intracellular Ca²⁺ and Cl⁻ measurements.

**Measurement of intracellular Ca²⁺ levels in isolated sweat glands and cell lines.** Measurements of intracellular Ca²⁺ levels were performed as described previously (63). Additional details can be found in Supplemental Methods.

**Measurement of intracellular Cl⁻ levels in isolated sweat glands and cell lines.** [Cl⁻] was measured using the MQA E [N-(ethoxycarbonyl-methyl)-6-methoxyquinolinium bromide] dye as described (79). Additional details regarding [Cl⁻] measurements can be found in Supplemental Methods.

**Real-time PCR.** Total RNA from freshly isolated mouse sweat glands and NCL-SG3 cell lines was extracted with TRIzol (Invitrogen), reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad), and analyzed by quantitative real-time PCR using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) and gene-specific primers (Supplemental Table 2). Values of mRNA expression were normalized to the human GAPDH or the mouse hypoxanthine guanine phosphoribosyl transferase (Hprt) housekeeping gene, respectively, using the 2⁻ΔΔCT method.

**Patch clamp electrophysiology.** For measurements of Cl⁻ currents, NCL-SG3 cells were grown on glass coverslips for 1–3 days before experimentation. Coverslips were transferred to a chamber containing extracellular bath solution (155 mM tetraethylammonium chloride to block K⁺ channels, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.2). Cl⁻ currents in individual cells were measured in the whole-cell patch clamp configuration using pClamp 9 and an Axopatch 200B amplifier (Molecular Devices). Recordings were sampled at 2 kHz and filtered at 1 kHz. Pipette resistances were 3–5 MΩ, and seal resistances were greater than 1 gigohm. Pipette solutions (pH 7.2) contained 60 mM tetraethylammonium chloride, 90 mM tetraethylammonium glutamate, 10 mM HEPES, and either 1 mM EGTA without added Ca²⁺ to yield negligible free Ca²⁺ or 5 mM EGTA with 4.3 mM CaCl₂ added to yield 1 μM free Ca²⁺. Alternatively, 1 mM HEDTA (N-(2-hydroxyethyl)lithiendiamine-N,N',N' triacetic acid) and 20 μM CaCl₂ were used in the internal pipette solution to better mimic physiological buffering and basal [Ca²⁺], conditions (~100 nM Ca²⁺). Free [Ca²⁺] was estimated using Maxchelator freeware (http://maxchelator.stanford.edu/). Agonists were directly perfused onto individual cells using a multibarrel perfusion pipette.

**Statistics.** Data are expressed as mean ± SEM. Normally distributed variables according to the Kolmogorov-Smirnov or the Shapiro-Wilk test were analyzed by 2-tailed Student’s t test for comparisons between 2 groups and 1-way ANOVA and Bonferroni post hoc test for comparisons between more than 2 groups. Statistical analyses were performed using GraphPad Prism 6 software. A value of P less than 0.05 was considered statistically significant.

**Study approval.** All animal procedures were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of New York University Langone Medical Center. For experiments using human tissue, informed consent for the studies was obtained from the patients’ families in accordance with the Declaration of Helsinki and Institutional Review Board approval of the New York University School of Medicine.

**Author contributions**

ARC, DIY, and SF designed the research; ARC, MV, LEW, ME, LH, JY, HPS, and CW performed experiments; MS, SC, SET, TI, IM, MC, and SF collected samples and obtained clinical data; ARC, MV, LEW, HPS, DIY, and SF analyzed data; DC and RSL contributed reagents; and ARC and SF wrote the paper.

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