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 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 IKKB), and IkBa (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 Ca2+ concentration ([Ca2+]i) (14–16). The resulting Ca2+ signals mediate the opening of Ca2+-activated chloride channels (CaCCs) in secretory cells from different organs, including eccrine sweat glands (7, 17, 18). Several CaCCs, such as anoctamin 1 (ANO1, also known as TMEM16A), and other regulators of sweat gland function was normal in the absence of SOCE. Our findings demonstrate that Ca2+ influx via store-operated CRAC channels is essential for CaCC activation, chloride secretion, and sweat production in humans and mice.
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 that activate CaCCs in eccrine sweat glands (3). Ca2+ influx from the extracellular space is essential for sweating in response to cholinergic and -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 IP3R activation following agonist stimulation of cell surface receptors such as the G protein–coupled ACh receptor. Its engagement results in IP3R activation and opening of ORAI1-CRAC channels that mediate the sustained Ca2+ influx from the extracellular space, which is required for many 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 abolishes 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

### Table 1. Summary of patients with loss-of-function or null mutations in human ORAI1 or STIM1 genes that result in impaired SOCE and anhidrosis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Patient</th>
<th>Mutation</th>
<th>Protein</th>
<th>SOCE</th>
<th>Anhidrosis</th>
<th>Clinical</th>
<th>Sweat test</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORAI1</td>
<td>1</td>
<td>p.R91W</td>
<td>+</td>
<td>–</td>
<td>Y</td>
<td>n.t.</td>
<td>Dead (11 mo)</td>
<td>McCarl et al. 2009 (35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>p.H165PfsX1</td>
<td>–</td>
<td>residual</td>
<td>n.r.</td>
<td>n.t.</td>
<td>Alive after HSCT at 9 mo</td>
<td>Chou et al. 2015 (40)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>p.R270X</td>
<td>+</td>
<td>residual</td>
<td>n.r.</td>
<td>n.t.</td>
<td>Dead (7 mo)</td>
<td>Badran et al. 2016 (42)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>p.V1815fsX8</td>
<td>–</td>
<td>–</td>
<td>Y</td>
<td>Y</td>
<td>Alive after HSCT (8 yr)</td>
<td>Unpublished</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>p.C98R</td>
<td>–</td>
<td>–</td>
<td>Y</td>
<td>Y</td>
<td>Alive after HSCT (20 mo)</td>
<td>Unpublished</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>p.C98R</td>
<td>n.t.</td>
<td>n.t.</td>
<td>Y</td>
<td>Y</td>
<td>Dead (2.5 yr)</td>
<td>Unpublished</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>p.L194P</td>
<td>–</td>
<td>–</td>
<td>Y</td>
<td>Y</td>
<td>Dead (7.5 mo)</td>
<td>Unpublished</td>
<td></td>
</tr>
<tr>
<td>STIM1</td>
<td>1</td>
<td>p.E129RfsX9</td>
<td>–</td>
<td>–</td>
<td>n.r.</td>
<td>n.t.</td>
<td>Dead (9 yr)</td>
<td>Picard et al. 2009 (36)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>c.1538-1 G&gt;A</td>
<td>–</td>
<td>–</td>
<td>n.r.</td>
<td>n.t.</td>
<td>Dead (2 yr)</td>
<td>Byun et al. 2010 (37)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>p.R429C</td>
<td>+</td>
<td>–</td>
<td>Y</td>
<td>Y</td>
<td>Alive after HSCT (6 yr)</td>
<td>Fuchs et al. 2012 (38)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>p.R429C</td>
<td>+</td>
<td>n.t.</td>
<td>Y</td>
<td>Y</td>
<td>Dead (21 mo)</td>
<td>Fuchs et al. 2012 (38)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>p.R426C</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.r.</td>
<td>n.t.</td>
<td>Lost to follow-up at 5 yr</td>
<td>Wang et al. 2014 (39)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>p.P165Q</td>
<td>+</td>
<td>–</td>
<td>Y</td>
<td>n.t.</td>
<td>Alive (8 yr)</td>
<td>Schaballie et al. 2015 (41)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>p.P165Q</td>
<td>+</td>
<td>–</td>
<td>Y</td>
<td>Y</td>
<td>Alive (21 yr)</td>
<td>Schaballie et al. 2015 (41)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>p.L74P</td>
<td>n.t.</td>
<td>–</td>
<td>Y</td>
<td>Y</td>
<td>Alive (11 yr)</td>
<td>Parry et al. 2016 (43)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>p.L74P</td>
<td>n.t.</td>
<td>–</td>
<td>Y</td>
<td>Y</td>
<td>Alive (21 yr)</td>
<td>Parry et al. 2016 (43)</td>
<td></td>
</tr>
</tbody>
</table>

*Hypohidrosis. HSCT, hematopoietic stem cell transplantation; n.r., not reported; n.t., not tested; p, protein; SOCE, store-operated Ca2+ entry; Y, yes.
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 ORAI1 or 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 ORAI1 and STIM1 when sweat secretion was tested directly by iontophoresis, and was present clinically in most patients as an overt inability to sweat at high ambient temperatures and/or hyperthermia (Table 1) (35–43). To investigate whether anhidrosis in CRAC channel–deficient patients was due to abnormal development of sweat glands as in other patients with EDA-ID, we analyzed skin biopsies from patients with mutations in ORAI1 and STIM1 that abolish SOCE. Eccrine sweat glands were present in the dermis of a patient homozygous for an ORAI1 p.R91W loss-of-function mutation (44), an ORAI1 p.V181SfsX8 null mutation that abolishes ORAI1 expression, and a STIM1 p.P165Q loss-of-function mutation (41) (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 ORAI1 (p.G98R, p.L194P, or compound p.A103E/p.L194P) and STIM1 (p.R429C, p.L374P, or p.L74P) 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.
compared with a healthy donor identified by Alcian blue staining, suggesting impaired function of dark cells of sweat glands (45). Collectively, these data indicate that anhidrosis in CRAC channel–deficient patients is not due to impaired eccrine sweat gland development but is instead a defect in their secretory function.

Deletion of Orai1 or Stim1/Stim2 genes in murine sweat glands abolishes sweating despite normal sweat gland development. In contrast to humans, eccrine sweat glands in mice are found only in the footpads. We first investigated whether CRAC channel genes are present in sweat glands of WT mice. Orai1 and Stim1 mRNA was highly expressed, whereas transcript levels of Orai2, Orai3, and Stim2 were less abundant (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI89056DS1). To study the role of CRAC channels in murine eccrine sweat glands, we generated mice with conditional deletion of CRAC channel genes in tissues of ectodermal origin, including skin, teeth, and sweat glands. Histological analysis of eccrine sweat glands in the footpads of Orai1fl/fl and Stim1fl/fl Stim2fl/fl K14-Cre (hereafter referred to as Orai1K14Cre and Stim1/2K14Cre 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 Stim1/2 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. Compared with a healthy donor identified by Alcian blue staining, suggesting impaired function of dark cells of sweat glands (45). Collectively, these data indicate that anhidrosis in CRAC channel–deficient patients is not due to impaired eccrine sweat gland development but is instead a defect in their secretory function.

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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 \textit{Orai1} and \textit{Stim1/2} genes, these data suggest that CRAC channels regulate the function, but not the development, of murine eccrine sweat glands.

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

To assess the role of \textit{ORAI1}, \textit{STIM1}, and \textit{STIM2} in Ca\textsuperscript{2+} signaling in response to agonist stimulation of eccrine sweat glands, we isolated individual glands from the paws of \textit{Orai1\textsuperscript{K14Cre}} or \textit{Stim1/2\textsuperscript{K14Cre}} mice and analyzed them for [Ca\textsuperscript{2+}]i. Stimulation with ACh in Ca\textsuperscript{2+}-free media induced a comparable Ca\textsuperscript{2+} release from ER stores in the secretory coils of sweat glands of WT and \textit{Orai1}- and \textit{Stim1/2}-deficient mice (Figure 3, A and B, and Supplemental Figure 3). Readdition of extracellular Ca\textsuperscript{2+} caused robust Ca\textsuperscript{2+} influx in sweat glands of WT but not \textit{Orai1\textsuperscript{K14Cre}} or \textit{Stim1/2\textsuperscript{K14Cre}} mice (Figure 3, A–C), indicating that \textit{ORAI1} and \textit{STIM1/2} mediate SOCE in murine eccrine sweat glands upon cholinergic stimulation. While Ca\textsuperscript{2+} entry following ACh stimulation could potentially be mediated by channels other than CRAC, this

which show strong \textit{ORAI1} and \textit{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, \textit{Orai1\textsuperscript{K14Cre}}, and \textit{Stim1/2\textsuperscript{K14Cre}} 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 \textit{Orai1\textsuperscript{K14Cre}} and \textit{Stim1/2\textsuperscript{K14Cre}} mice (Figure 2, E and F), indicating that CRAC channels are required for eccrine sweat gland function in mice. It is noteworthy that while \textit{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 \textit{Orai2\textsuperscript{K14Cre}} mice (Supplemental Figure 2), suggesting that \textit{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

Figure 3. Deletion of \textit{Orai1} or \textit{Stim1/Stim2} in murine sweat glands abolishes SOCE. (A–C) Sweat glands isolated from the footpads of WT (top), \textit{Orai1\textsuperscript{K14Cre}} (middle), and \textit{Stim1/2\textsuperscript{K14Cre}} (bottom) mice were loaded with CellTracker Orange (CMRA) and Fura-2 for 30 minutes. Intracellular Ca\textsuperscript{2+} levels were determined by time-lapse microscopy of F340/F380 values that were calibrated to [Ca\textsuperscript{2+}]. (A) Representative F340/F380 ratios before stimulation in Ca\textsuperscript{2+}-free buffer ("basal"), after stimulation with 1 μM ACh in Ca\textsuperscript{2+}-free buffer ("store depletion"), and after perfusion with 2 mM Ca\textsuperscript{2+} ("Ca\textsuperscript{2+} influx"). (B) Representative traces of [Ca\textsuperscript{2+}]i from an experiment similar to the one shown in A. (C) Averaged peak [Ca\textsuperscript{2+}], (left) and integrated Ca\textsuperscript{2+} response (area under the curve, AUC, right) after readdition of 2 mM Ca\textsuperscript{2+} (420–600 seconds). Bars represent the mean of 15 WT, 5 \textit{Orai1\textsuperscript{K14Cre}}, and 10 \textit{Stim1/2\textsuperscript{K14Cre}} mice. Each dot represents data from 1 gland of an individual mouse. Statistical analyses in C were performed by 1-way ANOVA followed by Bonferroni post hoc test. **P < 0.01, ***P < 0.001.
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 eccrine sweat glands isolated from WT and Orai1\(^{K14Cre}\) mice. Sweat glands were loaded with MQAE and stimulated with 1 \(\mu\)M ACh in buffer containing 100 mM Cl\(^{-}\) followed by perfusion with Cl\(^{-}\)-free buffer ("0 mM Cl\(^{-}\") to force Cl\(^{-}\) secretion. MQAE signals were calibrated to absolute [Cl\(^{-}\)]. (D) [Cl\(^{-}\)] traces represented as mean ± SEM of glands isolated from 11 WT and 7 Orai1\(^{K14Cre}\) mice. (E) Quantification of the Cl\(^{-}\) influx rate ("slope\(_{420-520s}\)") and change of [Cl\(^{-}\)], measured as integrated area ("area\(_{420-720}\)", relative to baseline) after stimulation with 1 \(\mu\)M ACh. (F) Quantification of the Cl\(^{-}\) efflux rate ("slope\(_{420-520s}\)") and change of [Cl\(^{-}\)], measured as integrated area ("area\(_{420-720}\)", relative to baseline) after removal of extracellular Cl\(^{-}\). (G) Quantification of net Cl\(^{-}\) efflux calculated as the difference between [Cl\(^{-}\)] at the beginning of the experiment and after stimulation with 1 \(\mu\)M ACh, or after removal of extracellular Cl\(^{-}\) at the end of the experiment, respectively. For additional details see Supplemental Methods. Data in E–G are the mean ± SEM of 11 WT and 7 Orai1\(^{K14Cre}\) mice. Statistical analysis by 2-tailed Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001.
lated from \textit{Orai1\textsuperscript{K14Cre}} and \textit{Stim1\textsuperscript{2 \textsuperscript{K14Cre}} mice compared with WT controls (Figure 4, A–C). As expected, TEMEM16A 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 \textit{Nkcc1} and \textit{Aqp5} mRNA in sweat glands from \textit{Orai1\textsuperscript{K14Cre}} and \textit{Stim1\textsuperscript{2 \textsuperscript{K14Cre}} mice (Figure 4A). Expression of other Cl–-permeable channels and transporters such as TEMEM16B, 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 NKCCI (24), and NFATc3, which is regulated by Ca\textsuperscript{2+} signals and activates FOXA1 (50) (Figure 4A). Collectively, these data show that impaired sweat secretion in CRAC channel-deficient mice is not due to abnormal expression of the CaCCs BEST2 and TEMEM16A or other channels that regulate Cl– and water secretion.

\textit{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 \textit{Orai1\textsuperscript{K14Cre}} mice were loaded with the intracellular Cl– indicator MQAE and stimulated with ACh in buffer containing physiological Ca\textsuperscript{2+} (2 mM) and Cl– (100 mM) concentrations followed by removal of extracellular Cl– to force Cl– secretion (Figure 4D). Before stimulation, WT and \textit{Orai1}\textsuperscript{-deficient sweat glands had comparable [Cl–], (WT: 62 ± 12 mM; \textit{Orai1\textsuperscript{K14Cre}}: 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 \textit{Orai1\textsuperscript{K14Cre}} 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). \textit{Orai1\textsuperscript{K14Cre}} 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 \textit{Orai1\textsuperscript{K14Cre}} cells was observed (<10 mM) (Figure 4G). The residual reduction in [Cl–] in \textit{Orai1\textsuperscript{K14Cre}} sweat glands after removal of extracellular Cl– is likely due to Ca\textsuperscript{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 (7, 51, 52). NCL-SG3 cells abundantly expressed \textit{Orai1} and \textit{STIM1} mRNA (data not shown) and protein (Supplemental Figure 5, A–C). Suppression of \textit{Orai1} and \textit{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 sh\textit{Orai1} and sh\textit{STIM1} after ionomycin stimulation, whereas Ca\textsuperscript{2+} release from ER stores was unaffected (Figure 5, B and C), indicating that \textit{Orai1} and \textit{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\textsuperscript{2+} ionophores calcimycin and ionomycin (17, 54). Ionomycin stimulation of NCL-SG3 cells in 100 mM [Cl–]\textsubscript{o} (where [Cl–]\textsubscript{o} indicates the extracellular concentration of Cl–) resulted in a moderate reduction of [Cl–] in, which was strongly enhanced when Cl– secretion was forced by removal of Cl– from the extracellular buffer (Figure 5, D and E). NCL-SG3 cells transduced with sh\textit{ORAI1} or sh\textit{STIM1} lacked a comparable reduction of [Cl–], suggesting that Cl– secretion by NCL-SG3 cells depends on SOCE (Figure 5, 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 sh\textit{ORAI1} or sh\textit{STIM1} did not decrease TEMEM16A and \textit{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.

\textit{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 \textmu M Ca\textsuperscript{2+} in the patch pipette, and thus the cytosol, whereas no Cl– currents were observed in the absence of Ca\textsuperscript{2+} (Figure 6, A and B). The Cl– currents displayed hallmarks of epithelial CaCC currents, including fast activation followed by a slower 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 sh\textit{ORAI1} when the patch pipette contained 1 \textmu M Ca\textsuperscript{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 cytosolic [Ca\textsuperscript{2+}] 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\textsuperscript{2+}, which depletes ER Ca\textsuperscript{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 \textmu M Ca\textsuperscript{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\textsuperscript{2+}] resulting from ER Ca\textsuperscript{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\textsuperscript{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 protease-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\textsuperscript{2+}-containing media resulted in stronger and more sustained elevation of [Ca\textsuperscript{2+}], in comparison
SOCE mediates Cl\(^-\) secretion in human eccrine 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 \(\text{Best2}\) 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 \(\text{ORAI1}\) p.V181SfsX8 null or \(\text{STIM1}\) p.P165Q loss-of-function with \(\text{ORAI1}\)-deficient cells (Figure 6F), consistent with residual Ca\(^{2+}\) store depletion but impaired SOCE in the absence of \(\text{ORAI1}\) (Figure 5B). CaCC currents in trypsin-stimulated WT NCL-SG3 cells were robust and sustained, whereas those in \(\text{ORAI1}\)-deficient cells were smaller and more transient (Figure 6, G and H), similar to results obtained after ionomycin stimulation. Taken together, CRAC channel function and SOCE triggered either by passive depletion of Ca\(^{2+}\) stores or by agonist stimulation are essential to activate CaCCs and Cl\(^-\) secretion.
Figure 6. SOCE activates Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels (CaCCs) in human sweat gland cells. NCL-SG3 cells were transduced with shORAI1 (red) or left untransduced (Ctrl, black) and Cl\textsuperscript{-} 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 Ca\textsuperscript{2+} (top) or 1 μM Ca\textsuperscript{2+} (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\textsuperscript{-} (E_{Cl} ~ –24 mV). (D) Current densities in NCL-SG3 cells stimulated with 1 μM ionomycin (Iono). Currents were elicited by consecutive 2-second voltage steps to 80 mV, followed by a 0.5-second hyperpolarizing step to –80 mV (mean ± SEM, n = 5 cells per cell line). (E) Representative Cl\textsuperscript{-} current traces extracted at 0, 60 and 300 seconds from the experiment in D. (F) Representative [Ca\textsuperscript{2+}]\textsubscript{i} traces from NCL-SG3 cells stimulated with 10 μM trypsin (left) and quantitation of [Ca\textsuperscript{2+}]\textsubscript{i} 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\textsuperscript{-} current traces extracted at 0, 60 and 300 seconds from the experiment shown in G. TEA-Cl, tetraethylammonium chloride.
We next measured Ca\(^{2+}\)-activated Cl\(^{-}\) currents in TMEM16A- and BEST2-deficient NCL-SG3 cells. In whole-cell current recordings, the presence of 1 μM Ca\(^{2+}\) in the patch pipette, and thus the cytosol, we observed robust Cl\(^{-}\) currents in shBEST2-transduced cells, which had properties identical to those in untransduced NCL-SG3 cells (Figure 8, A and B, and Figure 6). By contrast, no Cl\(^{-}\) currents or barely detectable Cl\(^{-}\) currents were found in shTMEM16A-transduced cells under the same conditions (Figure 8, A and B), indicating that TMEM16A is the main CaCC in human eccrine sweat gland cells.

It is possible that activation of BEST2, unlike that of TMEM16A, requires additional signals besides Ca\(^{2+}\) that are generated by agonist stimulation. To verify that TMEM16A is the physiological CaCC in human eccrine sweat gland cells, we stimulated NCL-SG3 cells with the PAR2 agonist trypsin. Neither deletion of TMEM16A nor that of BEST2 affected Ca\(^{2+}\) influx in NCL-SG3 cells after trypsin stimulation (Figure 8C). Trypsin induced strong Cl\(^{-}\) currents in shBEST2-transduced cells (Figure 8, D and E) that had properties similar to those in WT NCL-SG3 cells (Figure 6). By contrast, deletion of TMEM16A almost completely abolished the trypsin-induced Cl\(^{-}\) currents in NCL-SG3 cells during both the store-depletion and the SOCE phase of the Ca\(^{2+}\) response (Figure 8, D and E). A similar defect in trypsin-induced CaCC currents was detected in shTMEM16A-transduced but not in WT and shBEST2-transduced NCL-SG3 cells when we used a weak Ca\(^{2+}\)-buffer in the patch pipette (Supplemental Figure 7). The Cl\(^{-}\) current properties were similar to those observed in previous experiments (Figure 8D and Supplemental Figure 7), but the transient current kinetics mimicked more closely those of trypsin-induced SOCE (Figure 8C and Supplemental Figure 7A). It is noteworthy that we observed residual Cl\(^{-}\) currents before trypsin stimulation in WT and shBEST2-transduced NCL-SG3 cells (Supplemental Figure 7, A and B). These results are consistent with the expression of the TMEM16A(ace) splicing variant in NCL-SG3 cells (7), which has high affinity for Ca\(^{2+}\) and is active at negative membrane potentials (55). Taken together, our results show that SOCE mediated by ORAI1 and STIM1 is essential for Cl\(^{-}\) secretion via the Ca\(^{2+}\)-activated Cl\(^{-}\) channel TMEM16A, and thus controls eccrine sweat gland function and thermoregulation.

**Discussion**

We identified CRAC channels and SOCE as a critical signaling pathway for eccrine sweat gland function in humans and mice.
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 CRAC channels were required to activate Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels, in particular TMEM16A, and to induce Cl\textsuperscript{-} 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 CRAC channels were required to activate Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels, in particular TMEM16A, and to induce Cl\textsuperscript{-} 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 CRAC channels were required to activate Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels, in particular TMEM16A, and to induce Cl\textsuperscript{-} 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 CRAC channels were required to activate Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels, in particular TMEM16A, and to induce Cl\textsuperscript{-} 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 CRAC channels were required to activate Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels, in particular TMEM16A, and to induce Cl\textsuperscript{-} 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 Orai1K14Cre and Stim1/2K14Cre 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 IKBKG and NFKBIA (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.

Ca2+ 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- 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 Ca2+-dependent regulation of CaCCs is also consistent with the fact that addition of 1 μM Ca2+ inside the patch pipette was sufficient for the activation of Cl- currents even in the absence of Orai1 expression.

CaCCs have long been recognized to be important for sweat gland function (7, 17), and Cl- secretion in the human eccrine sweat gland cell line NCL-SG3 was shown to depend on increases in [Ca2+]i (7, 15, 17). However, the source of Ca2+ 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 Ca2+-dependent Cl- 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 cholingeric-induced fluid secretion (18). Moreover, Tmem16a−/− mice had impaired Ca2+-activated Cl- 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- currents we recorded in NCL-SG3 cells closely resembled those reported for Tmem16a, in particular the Tmem16a(ace) splice variant, which displays larger instantaneous current and smaller time-dependent activation at positive membrane potential and higher Ca2+ 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 Cl⃦ secretion and Cl⃦ currents activated by either Ca2+ in the patch pipette or the PAR2 agonist tripsin. These data demonstrate that TMEM16A is essential for CaCC function and Cl⃦ 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 Cl⃦ 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 Cl⃦ 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 Cl⃦ secretion or Cl⃦ currents activated by Ca2+ in the patch pipette or tripsin stimulation, suggesting that BEST2 does not contribute to SOCE-induced Cl⃦ 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 Cl⃦ 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), Aqup5−/− mice produced ambiguous results, as they had either normal (71) or impaired (5) 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 Cl⃦ 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 Cl⃦ secretion. We identify TMEM16A as the SOCE-activated CaCC responsible for Cl⃦ 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, Cl⃦ 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 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 Cl⃦ 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 secretory 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 1). 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.** Orai1fl/fl (76) and Stim1fl/fl (77) have been described previously. They were crossed to K14-Cre mice (78) (The Jackson Laboratory, strain 004782) to generate Orai1fl/fl K14-Cre (Orai1loxcre) and Stim1fl/fl K14-Cre (Stim1loxcre) mice. Orai1−/− mice were generated using VG8 E5 cells (C57BL/6NTac) obtained from the Knockout Mouse Project (KOMP, www.komp.org) repository at UC Davis (project ID VG14962, Orai2tm1(KOMP)Vlcg).

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 stereoscope 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 transferred to a chamber containing extracellular bath solution (155 mM tetraethylammonium chloride, 90 mM tetraethylammonium glutamate, 10 mM HEPES, pH 7.2). Intracellular Cl– levels were measured using the MQAE [N-(ethoxycarbonyl-methyl)-6-methoxyquinolinium iodide] dye as described (79). Additional details are available in Supplemental Methods.

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–]i was measured using the MQAE [N-(ethoxycarbonyl-methyl)-6-methoxyquinolinium iodide] dye as described (79). Additional details regarding [Cl–]i 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 genespecific primers (Supplemental Table 2). Values of mRNA expression were normalized to the human GAPDH or the mouse hypoxanthine guanine phosphoribosyl transferase (Hpirt) housekeeping gene, respectively, using the 2ΔΔCT 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 megohms, 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)ethylenediamine-N,N,N′,N′′-tetraacetic acid) and 20 μM CaCl2 were used in the internal pipette solution to better mimic physiological buffering and basal [Ca2+]i conditions (~100 nM Ca2+). Free [Ca2+]i 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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Address correspondence to: Stefanie Feske, Department of Pathology, New York University School of Medicine, 550 First Avenue, Smilow 316, New York, New York 10016, USA. Phone: 212. 263.9066; E-mail: feskes01@nyumc.org.


