Plasmid and siRNA Preparation. Preparation of the human NCLX-encoding plasmid was described previously (1). NCLXS468T- and NCLX-6His-encoding plasmids were generated by replacing a NotI fragment from the α2 S273T or WT α2-6His plasmid (2), respectively, with the corresponding fragment from the NCLX-encoding plasmid. The murine NCLX variant and the NCKX4-encoding plasmids were provided by Jonathan Lytton (Calgary, AB, Canada) (3). The RP-mt plasmid was provided by Atsushi Miyawaki (Wako, Japan) (4). Double-stranded siRNAs used to silence NCLX expression was obtained from Ambion (Applied Biosystems). The sequence of 21 nucleotides corresponding to the sense strands used for the NCLX siRNA was CAAAGCCACUCAGAUCGCUtt and that for the control siRNA was CAAAGGCAUCUCCAGCUUt. The human NCLX shRNA plasmid was obtained from Sigma (Mission TRC shRNA Target Set TRCN-5045).

Fluorescent Measurements of Intracellular Ions and Mitochondrial Membrane Potential. Changes in inner mitochondrial membrane potential (ΔΨm) were monitored by detecting microfluorimeter changes in fluorescence (ΔF) of the membrane potential-sensitive probes TMRE (5) (for HEK293 cells). HEK293 cells loaded with 0.05 μM TMRE were washed in a static bath of Ringer solution containing the probe and then exposed to 10 μM of CCCP to obtain maximal loss of ΔΨm. TMRE-loaded cells were excited with a 530-nm (±30) band-pass filter and emission monitored at greater than 590 nm. TMRE ΔF was calculated as fluorescence values at baseline and after exposure to CCCP.

Flow Cytometry Analysis of ΔΨm. To evaluate ΔΨm in SHSY-5Y cells, 5 × 105 cells were incubated with 500 nM of 5,5′,6,6′-tetramethylrhodamine-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide (JC1; Molecular Probes) in the growth medium for 10 min at 37°C in the dark and immediately analyzed by flow cytometry using the FACS Calibur instrument (BD Bioscience). Data were processed with FlowJo software and were presented as the ratio between mean fluorescence intensities measured with the FL1 (green) and FL2 (red) photomultipliers.

Generation of Anti-NCLX Antibodies. The generation of the antibody used in Figs. 1 and 2 in the main text was previously described (1). Additional antibodies used in the experiment described in Fig. S1 was raised against peptide CSRSHTEVKLEDPDGL (CSR antibody), located close to the C terminus of NCLX, as previously described (1).

Cell Fractionation, Immunoprecipitation, and Immunoblot Analysis. Subcellular fractionation of ER/sarcoplasmic reticulum or mitochondrial fractions from HEK293 (6) cells or from rat heart using a small-scale protocol (7, 8) were performed as previously described. Briefly, rat ventricular tissue (approximately 200 g) was homogenized 3 times in Polytron, PT3000 (9,000 rpm × 30 s) with ice-cold TMS buffer (0.25 M sucrose/20 mM Tris-Cl, pH 7.4, 1 mM MgCl2, 1 mM PMSF, and proteinase inhibitor mixture). The lysates were further homogenized with a glass Douncer and then centrifuged at 800 × g for 15 min to remove the contractile proteins and nuclei. The supernatant was centrifuged twice at 8,000 × g for 20 min to pellet the mitochondrial fraction. The mitochondrial fraction was separately washed whereas the collected supernatants were centrifuged at 100,000 × g for 60 min to pellet the microsomal fraction. The pellet was suspended in the TMS buffer and the sarcoplasmic reticulum and sarcolemma membranes were separated by sucrose gradient as previously described (9). The subcellular fractions were resuspended in 0.25 M sucrose/20 mM Tris-Cl, pH 7.4, and stored at −20°C.

Purification of the plasma membrane–enriched fraction was performed using a cell surface protein isolation kit (Pierce) according to the manufacturer's instructions. Protein concentration was determined by the bicinchoninate assay (Pierce), and 20 μg of protein was separated by SDS/PAGE and immunoblotted. In the case of the plasma membrane fraction, protein samples and BSA standards were precipitated in 80% acetone, suspended in ddH2O, and then assayed by the bicinchoninate method. Expressed proteins (20 μg) were separated by 10% SDS/PAGE and transferred onto a polyvinylidene difluoride membrane (Amersham). Membranes were probed using the following antibodies: anti-NCLX [1:500 (2)], anti-ANT (1:100, Santa Cruz Biotechnology), anti-Fluc2 (1:5,000; provided by J. Sommer, Berlin, Germany), anti-Na/K ATPase [1:500 (10)], and anti–N-cadherin (1:1,000; BD Biosciences; donated by Frank Kirchner, Berlin, Germany), all diluted into 5% milk in tris-buffered saline solution with Tween 20.

Isolation of mitochondria from transfected HEK293-T cells or from native mouse tissues was performed as previously described (11), with minor modifications. Briefly, cells were homogenized in isolation buffer containing (in mM): mannitol 225, sucrose 75, EDTA 0.5, HEPES 10, with the pH being adjusted to 7.4 with KOH. The crude homogenate samples were centrifuged at 1,500 × g for 5 min in 4°C, the supernatant was collected, and the samples were washed with fresh homogenization buffer and recentrifuged. Pellet was saved (crude membranes) and the supernatant collected and centrifuged for 4 min at 12,000 × g at 4°C. Supernatant containing ER fragments and cytosolic proteins was saved (cytosolic fraction). The pellet, containing the mitochondria, was then washed 3 times with homogenization buffer. All samples were analyzed for protein concentration using the Bradford method (Bio-Rad). Equal amounts of protein were then resolved by SDS/PAGE and transferred onto nitrocellulose membranes for Western blot analysis.

Immunoprecipitation procedure was performed using the ExactaCruz kit (Santa Cruz Biotechnology) according to manufacturer's protocol. Briefly, total cell lysate from HEK-293-T cells expressing NCLX bearing a c-Myc tag, NCLX bearing a 6His-tag, or both were incubated with 20 μg of an antibody targeted against the 6His epitope (Santa Cruz Biotechnology) overnight at 4°C. Subsequently, 50 μL of immunoprecipitation matrix was added to the lysate and beads were sedimented and further processed according to manufacturer's instructions.

Imunoblot analysis was carried out as described previously (1). Antibodies used in this study included NCLX-antiserum (1:1,000–2,000 dilution), anti–β-actin (1:40,000; Sigma), monoclonal anti-VDAC antibodies against the N-terminal region of 31HL human porin (12) (1:10,000; Calbiochem), anti-ANT (1:100; Santa Cruz Biotechnology), anti-c-Myc (1:500; Santa Cruz Biotechnology), and anti-FLAG (1:1,000; VWR).

Immuonoelectron Microscopy. Brains sections were processed for pre-embedding immunoelectron microscopy according to a protocol
The NCLX antibody was used at a dilution of 1:200. The postembedding immunoelectron microscopy protocol was a modification of the method of Tokuyasu (14). Briefly, control or NCLX-overexpressing SHSY-5Y cells were fixed with 2% paraformaldehyde, 0.25% glutaraldehyde in 0.1 M phosphate buffer. Cryosections were cut on a Leica UltraCut UCT Ultramicrotome equipped with a low-temperature sectioning system. Analysis was performed on a JEOL JEM-1230 electron microscope equipped with a Tietz TemCam F214 CCD camera (TVIPS, Gauting, Germany).
Fig. S1. The dimeric form of NCLX and its cellular and subcellular distribution. (a) Immunoblot analysis of NCLX expression levels using the CSR-antibody (see SI Methods) in total cellular and mitochondrial fractions prepared from HEK-293 cells. Note that NCLX distribution detected with this antibody is similar to that seen with the antibody used in Fig. 1 in the main text. (b) Based on the well documented tendency of NCX proteins to form oligomers (15), we suggest that the 100-kDa form is a dimer of NCLX. To test this hypothesis, immunoblot analysis of rat brain mitochondrial fractions was performed before (control) and 1 h after incubation in 2× concentrated sample buffer at 50°C (heat denaturate). The apparent NCLX distribution shifts from a dominant 100-kDa form to a 50-kDa polypeptide following this denaturation procedure. This supports our hypothesis that the 100-kDa form is an SDS-stable dimer of NCLX. (c) Lysates of cells transfected to express NCLX-c-Myc, NCLX-6His, or both, as indicated, were immunoprecipitated with anti-6His antibodies and immunoblots were performed with antibodies against c-Myc (see Materials and Methods in the main text). Note that the c-Myc–tagged NCLX was coprecipitated with the 6His-tagged NCLX, further substantiating our hypothesis that NCLX is found as a dimer. (d) Representative mitochondria used for the quantitative analysis of the Immunogold labeling of overexpressed (NCLX) or endogenous (control) NCLX in SHSY-5Y cells (Arrows point to Immunogold particles). Note that NCLX labeling is localized to the inner cristae of the mitochondria.
Fig. S2. Subcellular distribution of NCLX and the NCLX-S468T mutant versus the plasma membrane–targeted NCKX4. (a) Immunoblot analysis using anti-FLAG antibodies (see Materials and Methods in the main text) of cellular fractions from HEK-293-T cells transfected with a NCKX4-FLAG–encoding plasmid or control (vector). NCKX4-FLAG staining was found in plasma membrane fraction whereas the mitochondrial protein VDAC was found mostly in the mitochondrial fraction. (b) HEK-293-T cells transfected to express either murine NCLX or NCKX4 or transfected with control plasmids were loaded with the cytoplasmic Ca²⁺-sensitive dye Fura-2 and superfused with the indicated Na⁺-containing and Na⁺-free (replaced by NMDG⁺) Ringer solution containing 0.5 mM Ca²⁺. Changes in cytosolic Ca²⁺, mediated by the reverse and forward Na⁺/Ca²⁺ exchange modes, were monitored in cells expressing NCKX4 but not in cells expressing the murine form of NCLX or vector-transfected cells. This finding is consistent with previous studies showing lack of plasma membrane activity or localization of murine NCLX (3), and strongly indicates that changes in mitochondrial Ca²⁺ transport are mediated by mitochondrial NCLX and are not related to an indirect plasma membrane activity of this protein. (c–f) Immunoblot analysis of cellular fractions from HEK-293 cells transfected with control (c), human NCLX- (d), NCLX-S468T-encoding (e), or the murine NCLX isoform (f) plasmids. Note that the expression of endogenous and ectopically expressed NCLX is highly enriched in the mitochondrial fraction.
Fig. S3. Ca\textsuperscript{2+} and pH values of intact and permeabilized cells. (a) Average of initial fluorescent signal (F\textsubscript{430}) in the indicated cells expressing RP-mt. No significant differences in steady state Ca\textsuperscript{2+} levels are observed 48 h following the transfection with the overexpression or silencing constructs of NCLX. (b) Representative image of HEK-293 cells expressing RP-mt (green) and stained with Mitotracker (red, magnification ×40). Projection of the images (Right) shows colocalization of RP-mt with Mitotracker and indicate that, in agreement with previous studies (1), RP-mt is strictly localized in the mitochondria. (c) HEK293 cells loaded with CFDA were subjected to permeabilization procedure as described in Fig. 5 in the main text (see Materials and Methods in the main text) and analyzed by flow cytometry. Bar graph depicting means of CFDA fluorescence intensities (n = 5, **P < 0.01) in control and permeabilized cells is shown (Left). (Right) Histogram overlay obtained in a typical experiment. CFDA fluorescence measured before and after digitonin treatment is represented by red and green curves, respectively. Gray curve shows a background fluorescence emitted by cells not labeled by CFDA (y axis depicts cell numbers). (d) HEK-293 cells expressing RP-mt alone (control) or together with NCLX were permeabilized and loaded with Ca\textsuperscript{2+} as described in Fig. 4A in the main text. RP-mt fluorescence (F\textsubscript{430}) was measured following Ca\textsuperscript{2+} loading. Note that, following Ca\textsuperscript{2+} loading, the Ca\textsuperscript{2+} level was similar in NCLX-expressing and control cells, thus allowing for accurate comparison between NCLX-expressing and control cells. (e) RP-mt F\textsubscript{485} measured in SHSY-5Y cells overexpressing NCLX or not (control) using the same experimental procedure as in Fig. 3A in the main text. (f) Cells were treated with 2 μM of the protonophore FCCP as indicated to diminish the mitochondrial pH gradient, and RP-mt F\textsubscript{485} was measured. (g) Average values of fluorescence at 485 nm before and after application of either ATP or FCCP (taken from E and F). Note that application of both ATP and FCCP triggered a similar fluorescent change in the F\textsubscript{485} signal of the RP-mt. Because of the concern that these fluorescent changes may be related to pH change, and consistent with previous studies (16, 17), we relied on only the Ca\textsuperscript{2+} sensitive, but pH-insensitive, 430-nm wavelength in our Ca\textsuperscript{2+} measurements.
**Fig. S4.** The effect of NCLX overexpression or silencing on steady-state Ca\(^{2+}\) levels, membrane potential, and cytoplasmic Ca\(^{2+}\) response. (a) SHSY-5Y cells transfected with NCLX-encoding or control vectors were loaded with JC1 (see SI Methods) and green and red fluorescent intensities were measured by flow cytometry in the steady state (resting, Left) and after application of 5 \(\mu\)M FCCP (Right). (b) Quantitative analysis of the ratio between red and green fluorescent intensities measured in control or NCLX-expressing cells (\(n = 9\)). No change in the JC1 red and green fluorescent ratio was apparent between control and NCLX-expressing cells, suggesting similar mitochondrial membrane potential levels in both cell types. (c) Mitochondrial membrane potential was determined with TMRE. HEK-293 cells that were transfected with the indicated constructs and loaded with TMRE were superfused with Ringer solution and subsequently with the same solution containing 10 \(\mu\)M CCCP while monitoring TMRE fluorescence. Data are presented as \(\Delta F\) fluorescence of TMRE (difference of fluorescence values at baseline resting and fluorescence values after exposure to CCCP) versus control. Note that the mitochondria of HEK293 transfected with the NCLX S468T or NCLX siRNA constructs were slightly hyperpolarized compared with control. (d) Cytosolic Ca\(^{2+}\) responses following the application of ATP (40 \(\mu\)M) in Ca\(^{2+}\)-free Ringer solution using Fura-2 loaded HEK-293 cells transfected with either NCLX siRNA or control siRNA, as described in Fig. 3 in the main text. No difference in the ATP-dependent cytosolic Ca\(^{2+}\) response was found following NCLX expression or silencing, indicating that, using this experimental paradigm, mitochondrial NCLX did not change the cytosolic Ca\(^{2+}\) response. (e) The same experiment as in d in SHSY-5Y cells expressing either NCLX or the shRNA construct aimed to silence NCLX. (f) ATP-dependent cytosolic Ca\(^{2+}\) response in HEK-293 cells expressing NCLX or controls that were superfused with Ca\(^{2+}\)-containing Ringer solution, Ca\(^{2+}\) was then removed from the superfusing solution and was then readded to monitor store-operated Ca\(^{2+}\) influx. (g) Average cytosolic Ca\(^{2+}\) levels at distinct time points (arrow, f) (\(n = 7, *P < 0.05\)). Note that, in the presence of Ca\(^{2+}\)-containing Ringer solution, expression of NCLX was associated with elevated cytosolic Ca\(^{2+}\) levels, consistent with the role of the mitochondrial exchanger in elevating cytosolic Ca\(^{2+}\) and activation of SOC.
Fig. S5. Activity of human NCLX and the NCLX-S468T mutant, and the effect of endogenous NCLX knockdown on mitochondrial Na\(^+\) or Li\(^+\)-dependent Ca\(^{2+}\) efflux. (a) HEK-293 cells transfected with either NCLX-S468T–encoding or control plasmids and expressing RP-mt were superfused with ATP (40 μM) containing Ringer solution while monitoring mitochondrial Ca\(^{2+}\) fluorescence (as in Fig. 3A in the main text). Note that expression of NCLX-S468T exerted a dominant negative effect on mitochondrial Ca\(^{2+}\) efflux and inhibited endogenous activity, similar to the effect shown in Fig. 3 in the main text. (b) The average of mitochondrial Ca\(^{2+}\) efflux rates (n = 11, *P < 0.05). (c) The same experimental paradigm as in a repeated with cells loaded with Fura-2. Similar cytosolic Ca\(^{2+}\) changes were triggered by ATP in both control and NCLX-S468T-expressing cells. (d) Endogenous NCLX expression was assessed by immunoblot analysis of CHO cells transfected with siNCLX or siControl constructs; β-actin served as loading marker (40 μg). (e) Mitochondrial Ca\(^{2+}\) responses triggered by ATP (40 μM, as in Fig. 3A in the main text) were measured in cells transfected with siNCLX or siControl together with the RP-mt-encoding plasmid. (f) The average rates of mitochondrial Ca\(^{2+}\) efflux are shown (n = 7, *P < 0.05). (g) RP-mt fluorescence was monitored in digitonin-permeabilized cells transfected with siNCLX or siControl. Mitochondrial Ca\(^{2+}\) uptake was induced by superfusion with Na\(^+\)-free solution containing Ca\(^{2+}\) (60 μM). Ca\(^{2+}\) efflux was then monitored following superfusion in Ca\(^{2+}\)-free solution in the presence of Na\(^+\). (h) The same experimental paradigm as described in G but replacing Na\(^+\) with Li\(^+\), as indicated. Note that endogenous NCLX mediates both Na\(^+\) or Li\(^+\)-dependent Ca\(^{2+}\) efflux. (i) Average initial rates of mitochondrial Ca\(^{2+}\) efflux (n = 8 for all groups, *P < 0.05).