Anoctamin 8 Tether the Endoplasmic Reticulum and Plasma Membranes to Assemble Ca^{2+} Signaling Complexes at ER/PM compartment

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Supplementary Methods

Constructs, antibodies and chemicals: STIM1, Orai1 and SARAF clones have been previously described (Maleth, Choi et al., 2014). Ist2 was a kind gift from Dr. James Rothman (Yale university), mCherry-PMCA4 was a kind gift from Dr. Agnes Enyedi (Semmelweis University, Budapest, Hungary) and mCherry-IP_3R3 was a kind gift from Dr. David Yule (Rochester University). The ANO8 clone was obtained from Open Biosystems, cat# 3711771 and ANO8-YFP was obtained from Origene, cat# MG21901. The primers listed in appendix table 2 were obtained from Integrated DNA Technologies, Inc (IDT). All point mutations were generated using the QuikChange Lightning site-directed mutagenesis kit from Agilent Technologies. The FKBP12 and FRB constructs are described in(Korzeniowski, Popovic et al., 2009) and were a kind gift from Dr. Tamas Balla (NIH). PLCδ1 PH-EGFP (Cat #21179) and ER-GECO 1 (Cat # 61244) were purchased from Addgene. In all experiments total cDNA in all transfections was kept the same by supplementing the control condition with appropriate empty vector.

Antibodies used in the present work are: polyclonal anti-GFP (Life Technologies, Cat # A11122) 1:1000 dilution, monoclonal anti-FLAG (Sigma Cat #F3165) 1:1000 dilution; monoclonal anti-MYC (Cell Signaling Inc., Cat # 2276) 1:1000 dilution; anti-HA (Cell Signaling Inc., Cat # 2367S) 1:1000 dilution; anti STIM1 (BD biosciences Cat # 610954) 1:1000 dilution; anti-Orai1 (Thermofisher scientific, Cat # PA1-74181) 1:1000 dilution; anti IP3R (BD transduction Laboratories, Cat #610312) 1:1000 dilution; anti-PMCA4a (Santa Cruz, Cat # sc-20028); anti- SERCA (Novus Laboratories, Cat # NB300-581) 1:1000 dilution; Anti-ANO8 (Protein tech Cat # 19485-i-AP) 1:1000 dilution. Carbachol (Sigma, Y0000113), Adenosine 5′-triphosphate magnesium salt (A9187) and Atropine (A0132) were obtained from Sigma-Aldrich. Cyclopiazonic acid (CPA) was from Alomone lab (Cat # C0750).

siRNA probes and qPCR: HEK293 cells were obtained from ATCC and are routinely verified to be clear of mycoplasma. The cells were plated at 70–80% confluence and transfected with duplexes after 12 h (100 nM/well) in a 6-well plate. The cells were harvested after 48 h; RNA was extracted using the TRIZOL reagent and the mRNA levels were determined by quantitative PCR. In brief, isolated mRNA was reverse-transcribed into cDNA by the iscript cDNA synthesis kit from Bio-Rad Laboratories. The primers for qPCR for the different ANOs and GAPDH listed in appendix table 1 were purchased from Applied Biosystems. The fold change in the transcript levels of the ANOs were calculated by normalizing the Ct values from control and siRNA-transfected cells (threshold values) to GAPDH. The plasmids for STIM1 and the mutants were transfected after 48 h of siRNA transfection and the cells were imaged for Ca^{2+} or used for current measurement 24 h after transfection.

Co-immunoprecipitation and biotinylation: Full blots for all experiments are given in source data. For co-immunoprecipitation, the cells were harvested in 500 µl of binding buffer (10 mM $NaVO_3$, 10 mM $Na_4P_2O_7$, 50 mM NaF, pH 7.4, and 1% Triton X-100 in PBS), sonicated, and spun down at 13,000 rpm for 5 min. Cell extracts were incubated with the indicated antibodies overnight at 4°C and then incubated with Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Cat #17-0618-01) for 2 h at 4°C. Beads were collected and washed three times with lysis buffer, and proteins were released by heating in 40 µl sample buffer at 56°C for 20 min. Ten µl of each sample was subjected to SDS-PAGE and subsequently transferred to methanol-soaked Nitrocellulose membranes for Western blot analysis.

For biotinylation, cells were incubated with 0.5 mg ml−1 EZ-Sulfo-NHS-LC-biotin (Thermo Scientific, Waltham, MA, USA, Cat #21335) for 30 min on ice, then incubated with 100 mM glycine for 10 min to quench the free biotin and washed with PBS. Lysates were prepared in lysis buffer (contained (mM) 20 Tris, 150 NaCl, 2 EDTA, with 1% Triton X-100, and a protease inhibitor mixture). After sonication, the lysates were centrifuged at 13,000 rpm for 20 min at 4°C, and protein concentration in the supernatants was determined. Biotinylated proteins were isolated with High Capacity NeutrAvidin Agarose beads (Thermo Scientific, Cat #29204) by incubation for 4 h on ice. The beads were washed with lysis buffer and proteins were recovered by heating with sample buffer at 56°C for 20 min. After separation by SDS-PASE the blots were analyzed for Orai1, STIM1 and ANO8.

PI(4,5)P$_2$ depletion: Depletion of plasma membrane PI(4,5)P$_2$ was accomplished with the FRB/FKBP system as described in (Toth, Toth et al., 2012, Varnai, Thyagarajan et al., 2006). Lyn- FRB localized to the plasma membrane and 5'-phosphatase fused to FKBP12 tagged with mRFP is in the cytosol. The two constructs were co-expressed in the cells. Upon exposure of the cells to 0.2 µM rapamycin for 2 mins, the FRB and the FKBP12 heterodimerize to recruit the phosphatase to the plasma membrane, which hydrolyzes the plasma membrane PI(4,5)P$_2$.

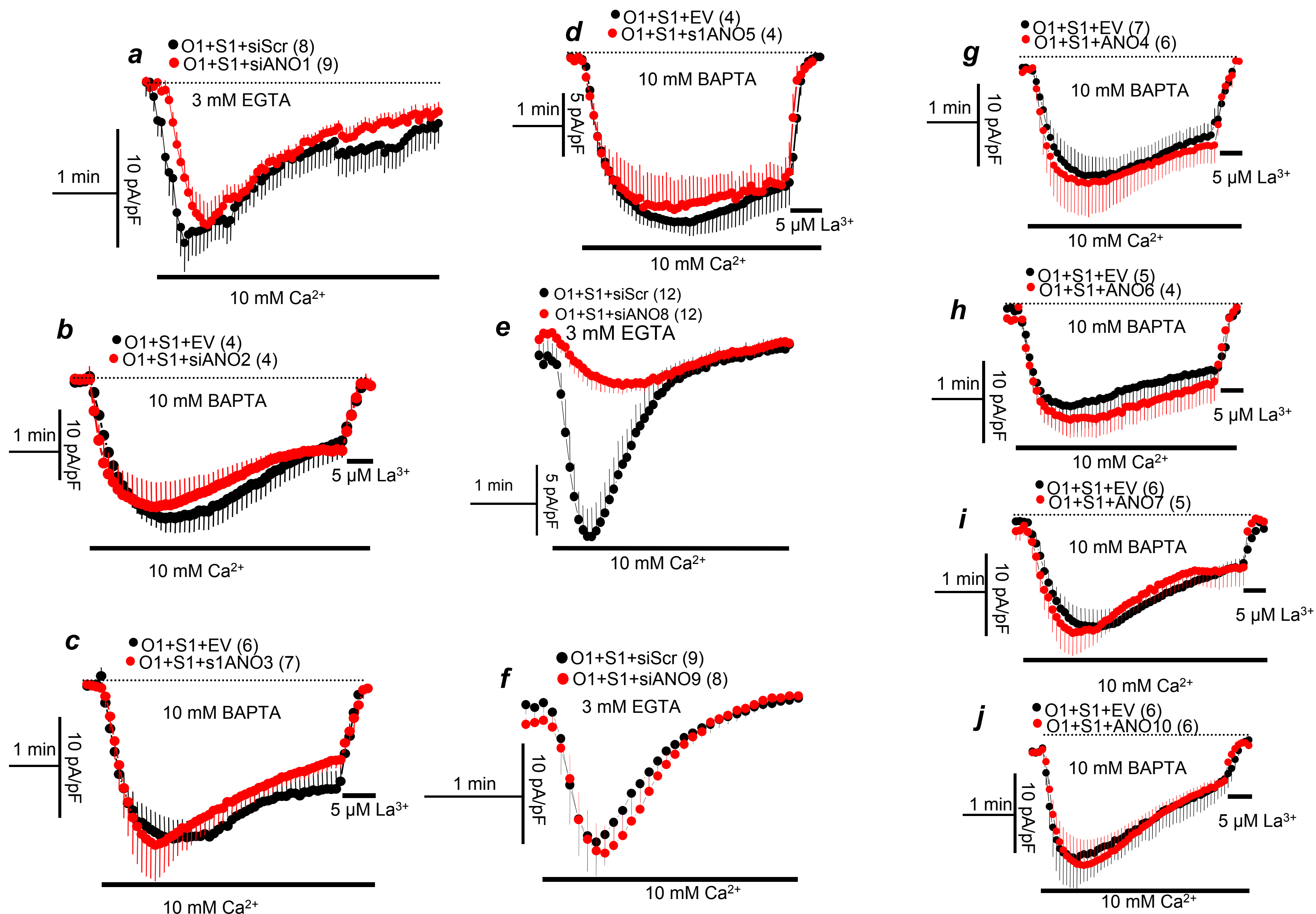
Confocal imaging: HeLa or HEK cells were plated on glass bottom dishes and transfected with the indicated construct for 24 h. Cells were washed and incubated with media containing 1 mM Ca^{2+} (control) or media containing 0.2 mM EGTA (Ca^{2+} free) and 25 µM CPA for 7-10 min (store depleted) before imaging. The images were captured at room temperature with a confocal system (FV1000; Olympus) equipped with a UplanSApo 60× oil immersion objective (NA 1.35; Olympus) at 3× zoom. CFP was recorded with 440 nm laser line, YFP was recorded with a 488 nm laser line and mCherry with 568 nm laser line. When more than one color was used, CFP was recorded with 440 nm laser line and YFP with 515 nm laser line and images were recorded sequentially to prevent bleed-through between channels.

Measurement of free cytoplasmic Ca^{2+}: HEK293 cells were plated on 18 mm coverslips. After 24 h of transfection the cells were loaded with Fura-2 by incubation with 5 µM Fura-2/AM (and 0.02% Pluronic acid (Teff labs) for 30-40 min at 37°C in culture media. Coverslips were assembled into a perfusion chamber and the cells continually perfused with warm (37 ^{o}C) media. Fura-2 fluorescence was measured with a TILL photonics Ca^{2+} imaging system at excitation wavelengths of 340 and 380 nm and light emitted at above 500 nm was collected. Collected images were analyzed using MetaFluor and the results are given as the 340/380 ratio. The standard bath solution contained (mM) 140 NaCl, 10 HEPES, 10 Glucose, 5 KCl, 1 $MgCl_2$, and either 1 mM $CaCl_2$ or 0.2 mM EGTA (Ca^{2+}-free). Results are presented as the mean ± SEM from at least three experiments and 30–60 cells/experiment.

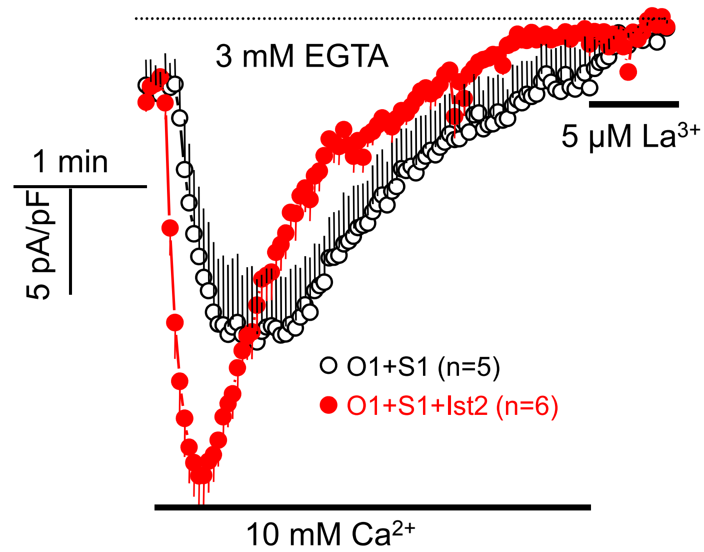
References

Brunner JD, Lim NK, Schenck S, Duerst A, Dutzler R (2014) X-ray structure of a calcium-activated TMEM16 lipid scramblase. *Nature* 516: 207-12
Korzeniowski MK, Popovic MA, Szentpetery Z, Varnai P, Stojilkovic SS, Balla T (2009) Dependence of STIM1/Orai1-mediated calcium entry on plasma membrane phosphoinositides. *J Biol Chem* 284: 21027-35

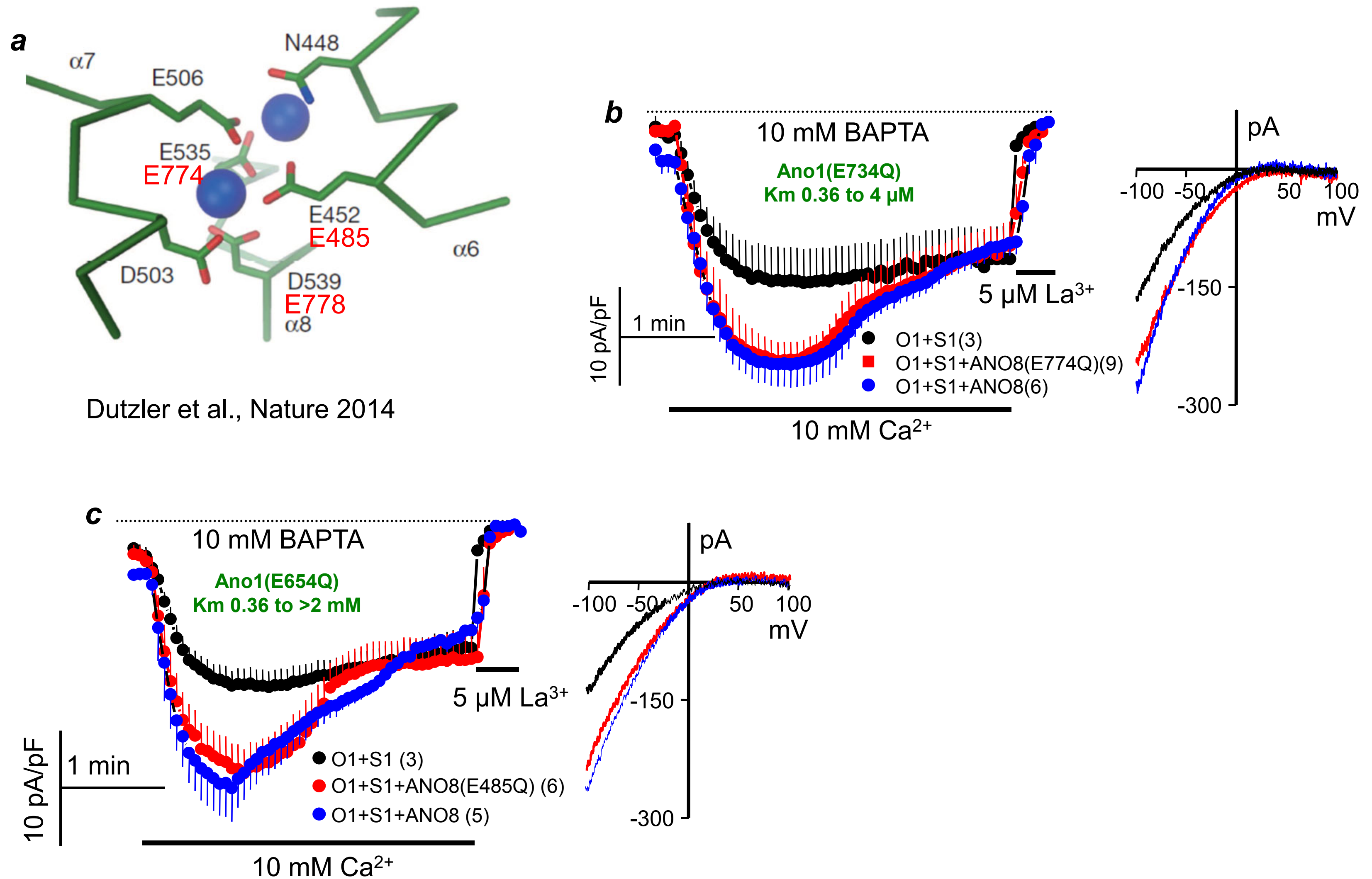
Maleth J, Choi S, Muallem S, Ahuja M (2014) Translocation between PI(4,5)P2-poor and PI(4,5)P2-rich microdomains during store depletion determines STIM1 conformation and Orai1 gating. *Nat Commun* 5: 5843



Appendix Figure S1: Effect of knockdown (a-f) or over-expression (g-j) of ANO proteins on STIM1-Orai1 current density and inactivation Panels (**a-f**): CRAC current was measured in HEK cells transfected with STIM1-CFP and Orai1-mCherry and treated with scrambled (all black traces) or siRNA to knockout ANO1 (**a**), ANO2 (**b**), ANO3 (**c**), ANO5 (**d**), ANO8 (**e**) or ANO9 (**f**). Panels (**g-j**): CRAC current was measured in HEK cells transfected with STIM1-CFP and Orai1-mCherry and empty vector (EV, all black traces) or ANO4 (**g**), ANO6 (**h**), ANO7 (**i**) or ANO10 (**j**). The numbers in parenthesis indicate the number of measurements.



Appendix Figure S2: Ist2 increases Orai1 current density and Slow Ca2+-Dependent Inactivation (SCDI): CRAC current was measured in HEK cells transfected with STIM1-CFP, Orai1-mCherry and with (red) or without (black) Ist2. Pipette solution contained 3 mM EGTA.



Appendix Figure S3: The effects of ANO8 are independent of its Ca^{2+} binding site: Panel (**a**): shows the Ca^{2+} binding sites of the TMEM16 family member from the fungus *Nectria haematococca* (Brunner, Lim et al., 2014) and the corresponding residues of ANO8 (red). Panels (**b, c**): The ANO1(E734Q) and ANO1(E654Q) mutations shifted the ANO1 Km for Ca^{2+} from 0.36 to 4 µM (**b**) and to more than 2 mM (**c**), respectively (green). The equivalent ANO8(E774Q) (**b**) and ANO8(E485Q) (**c**) mutations had no effect on increased STIM1-Orai1 current density and inactivation measured with pipette solution containing 10 mM BAPTA.

17 37
AEAASGAGGTSLEGE**RGKR**PPPEGEPAAPASGVLD**KLFGKRLLQAGR**YLVS

245 265
HKAWMKTVP……**IAMYFAWLGFYTSAMVYPAVF** (TM1)………

861 877 897 900
AIPDIPGWVAEEMAKLEYQRREAFKRHERQAQQRFQQQQRRRREEEERQR

948
HAEQQARRERDTGGREEARAEAPGPDPVAERGAAKAKGSE**RPRRP**GALLP

PGPVLRLKQIIPLQTRPPAPTGCAPPPRSPADTRLPAFLSLRFLKAPERGPSPP

1011
RPGKLFAFSAREPSANGAPGGGARAHRSAGDEPAAAEPEPRPEDAGHRP

Appendix Figure S4: Predicted potential lipid binding site on ANO8: ANO8 structural domains (Cytoplasmic N and C terminus and transmembrane domain sector) were predicted by ROBETTA online full-chain protein structure prediction server and the lipid binding sites by the the BHsearch program (http://helixweb.nih.gov/bhsearch). The sites predicted to bind phospholipids with the highest score are shown in red and the other potential sites are shown in green. The first transmembrane domain (starting with I245) and last residue of the last transmembrane domain (D861) are shown in blue.

Appendix table S1: List of siRNAs used in the present study.

Construct	Primers	% knockdown
siANO1	rCrGrGrGrUrCrUrCrArUrUrArArUrGrUrGrGrUrArCrArUrCrUrUrC rArGrArUrGrUrArCrCrArCrArUrUrArArUrGrArGrArCrCCG	85
siANO2	rUrArGrCrArGrCrUrUrCrCrUrGrUrCrArUrUrCrArUrArUrCrGrUrC rCrGrArUrArUrGrArArUrGrArCrArGrGrArArGrCrUrGrCTA	93
siANO3	rUrCrUrArCrCrArUrArCrArUrUrUrArCrUrUrCrArGrCrUrCrUrUrC rArGrArGrCrUrGrArArGrUrArArArUrGrUrArUrGrGrUrAGA	60
siANO4	rArArCrCrUrGrCrUrUrArUrUrUrGrUrUrUrArUrCrGrArUrCrCrUrG rGrGrArUrCrGrArUrArArArCrArArArUrArArGrCrArGrGTT	72
siANO5	rUrArGrArGrUrUrUrArGrCrArGrUrCrUrUrUrCrArArUrCrCrCrArA rGrGrGrArUrUrGrArArArGrArCrUrGrCrUrArArArCrUrCTA	65
siANO6	rGrArCrArGrArUrArArGrGrUrUrArGrArUrUrCrGrUrArUrGrCrUrU rGrCrArUrArCrGrArArUrCrUrArArCrCrUrUrArUrCrUrGTC	67
siANO8	rGrUrGrGrArGrArGrCrGrArGrCrUrArCrGrCrUrUrCrUrUCA rUrGrArArGrArArGrCrGrUrArGrCrUrCrGrCrUrCrUrCrCrArCrArU	91
siANO9	rGrCrUrGrArArGrArArGrGrCrArArCrUrCrUrArCrUGT rArCrArGrUrArGrArGrUrUrGrCrCrUrUrCrUrUrUrCrArGrGrCrArU	62
siANO10	rCrUrArGrUrArArCrArUrUrCrUrArArUrCrUrUrGrGrArGrGrCrArC rGrCrCrUrCrCrArArGrArUrUrArGrArArUrGrUrUrArCrUAG	70
siSARAF	rCrCrArGrGrArGrArCrGrArUrArArArGrUrArGrArArArGrTrT rCrCrArGrGrArGrArCrGrArUrArArArGrUrArGrArArArGrTrT	91
Scrambled	rCrGrUrArArUrCrGrCrGrUrArArUrArCrGrCrGrUrArT- rArUrArCrGrCrGrUrArUrUrArCrGrCrGrArUrUrArArCrGrArC	0

Appendix table S2: List of primers used in the present study.

R948,950,951Q	F: 5’ GCCAAGGCCAAGGGCAGCGAGCAGCCCCAACAGCCCGGAGCGCTGCTG CCACCC 3’ R: 5’ GGGTGGCAGCAGCGCTCCGGGCTGTTGGGGCTGCTCGCTGCCCTTGGCCTTGGC 3’
ANO8 E485Q	F: 5’ CAGCTGCTGCAGAACGTGCGCGCGGTGCTGCAGCCGCACCTGTAC 3’ R: 5’ GTACAGGTGCGGCTGCAGCACCGCGCGCACGTTCTGCAGCAGCTG 3’
ANO8 E774Q	F: 5’ GCCCTCGTCAACAACCTGATTCAGATCCGAAGTGATGCCTTCAAG 3’ R: 5’ CTTGAAGGCATCACTTCGGATCTGAATCAGGTTGTTGACGAGGGC 3’