Anoctamin-4 is a bona fide Ca^{2+}-dependent non-selective cation channel

Nadine Reichhart[1,*], Simon Schöberl[2,*], Susanne Keckeis[1], Ahmad S. Alfaar[1], Christophe Roubeix[1], Magdalena Cordes[1], Sergio Crespo-Garcia[1,3], Akvile Haeckel[4], Norbert Kociok[1], Renate Föckler[5], Gabriele Fels[1], Anja Mataruga[6], Robert Rauh[7], Vladimir M. Milenkovic[8], Kerstin Zühlke[9], Enno Klussmann[9], Eyk Schellenberger[4], Olaf Strauß[1]

[1]Experimental Ophthalmology, Department of Ophthalmology, Charité – Universitätsmedizin Berlin, a corporate member of Freie Universität, Humboldt-University, the Berlin Institute of Health, Berlin, Germany

[2]Department of Gastroenterology, Endocrinology, Rheumatology and Infectious Diseases, University Hospital Regensburg, Regensburg, Germany

[3]Department of Ophthalmology, Maisonneuve-Rosemont Hospital Research Centre, University of Montreal, Quebec, Canada.

[4]Institute for Radiology and Children's Radiology, Charité – Universitätsmedizin Berlin, a corporate member of Freie Universität, Humboldt-University, the Berlin Institute of Health, Berlin, Germany.

[5]Experimental Ophthalmology, Eye Hospital, University Medical Center Regensburg, Regensburg, Germany.

[6]Institute of Complex Systems, Zelluläre Biophysik (ICS-4), Forschungszentrum Jülich, Jülich, Germany

[7]Institut für Zelluläre und Molekulare Physiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany.

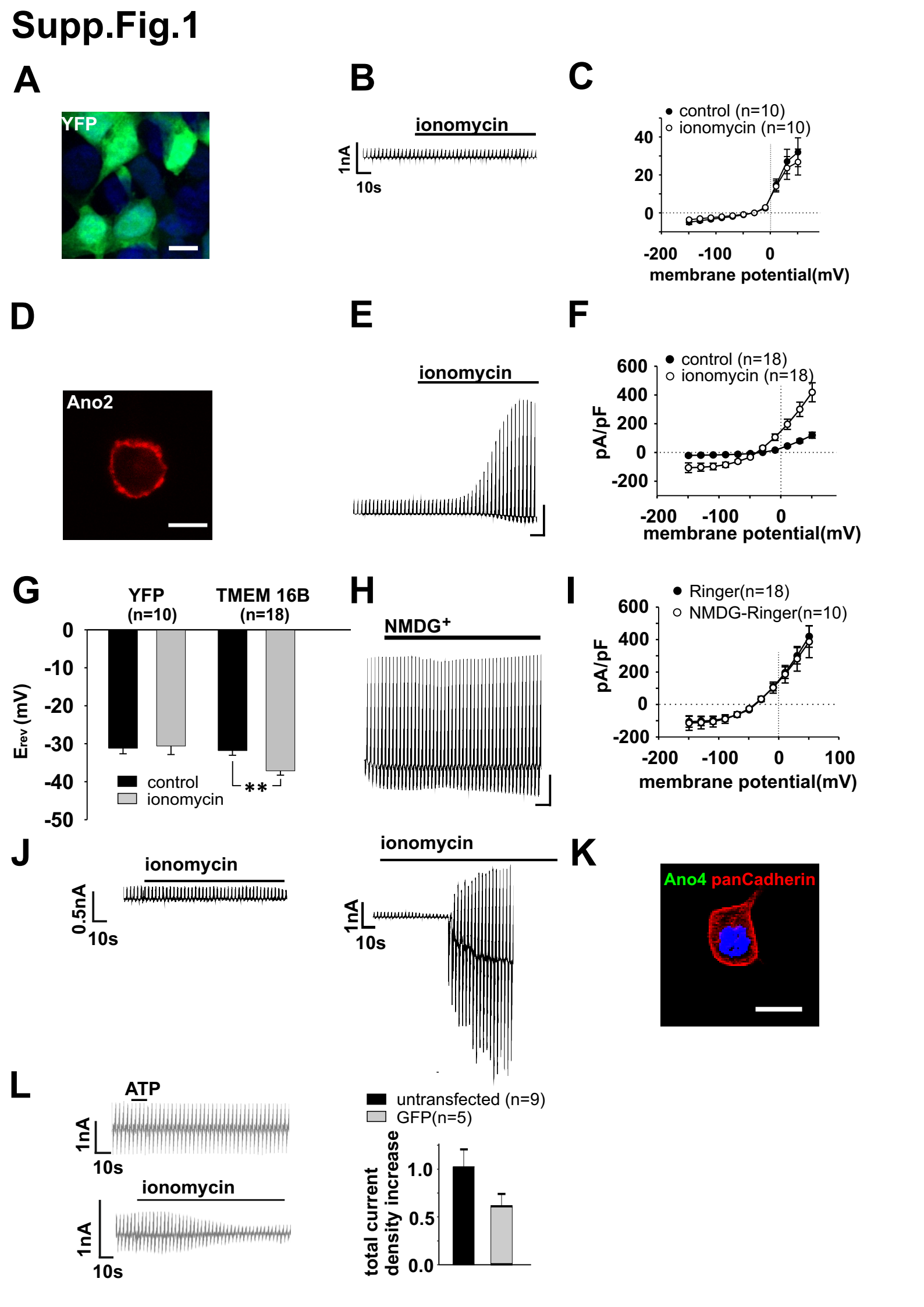
[8]Department of Psychiatry and Psychotherapy, Molecular Neuroscience, University of Regensburg, Germany
[9] Max Delbrück Center for Molecular Medicine Berlin (MDC), Berlin, Germany

*These authors contributed equally to this study.

Corresponding Author:
Olaf Strauß, PhD
Experimental Ophthalmology
Department of Ophthalmology
Charité – Universitätsmedizin Berlin, a corporate member of Freie Universität, Humboldt-University, the Berlin Institute of Health

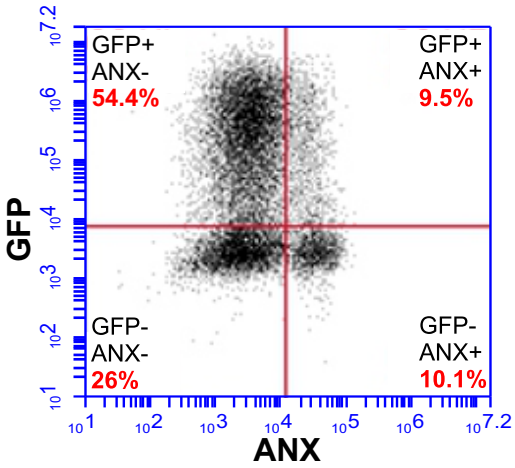
Campus Virchow Clinic
Augustenburger Platz 1
13353 Berlin
Tel.: +4930450654359
Fax: +4930450554907
olaf.strauss@charite.de

Supp.Fig.1

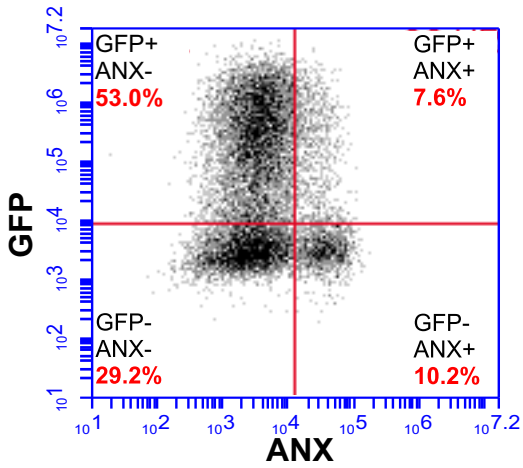

A
YFP
B
ionomycin
1nA
10s
C
control (n=10)
ionomycin (n=10)
40
20
0
-200
-100
0
membrane potential(mV)
D
Ano2
E
ionomycin
F
control (n=18)
ionomycin (n=18)
600
400
200
0
-200
pA/pF
-200
-100
0
membrane potential(mV)
G
YFP
(n=10)
TMEM 16B
(n=18)
0
-10
-20
-30
-40
-50
Erev (mV)
control
ionomycin
**
H
NMDG+
I
Ringer(n=18)
NMDG-Ringer(n=10)
600
400
200
0
-200
pA/pF
-200
-100
0
100
membrane potential(mV)
J
ionomycin
0.5nA
10s
ionomycin
1nA
10s
K
Ano4 panCadherin
L
ATP
1nA
10s
ionomycin
1nA
10s
untransfected (n=9)
GFP(n=5)
total current
density increase
1.0
0.5
0.0

Supp.Fig.2

A


GFP+
ANX-
54.4%
GFP+
ANX+
9.5%
GFP-
ANX-
26%
GFP-
ANX+
10.1%
GFP
ANX

B

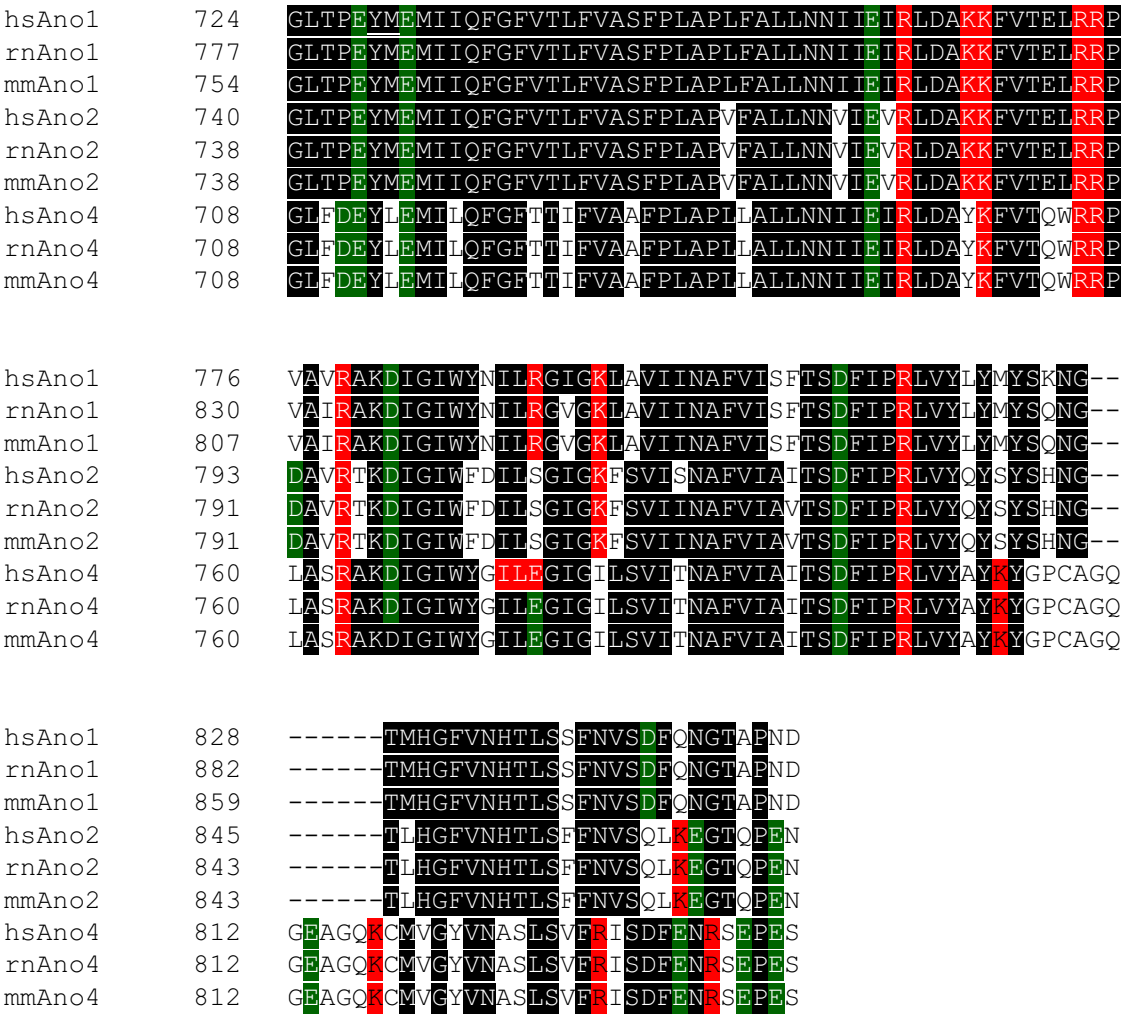

GFP+
ANX-
53.0%
GFP+
ANX+
7.6%
GFP-
ANX-
29.2%
GFP-
ANX+
10.2%
GFP
ANX

Supp.Fig.3

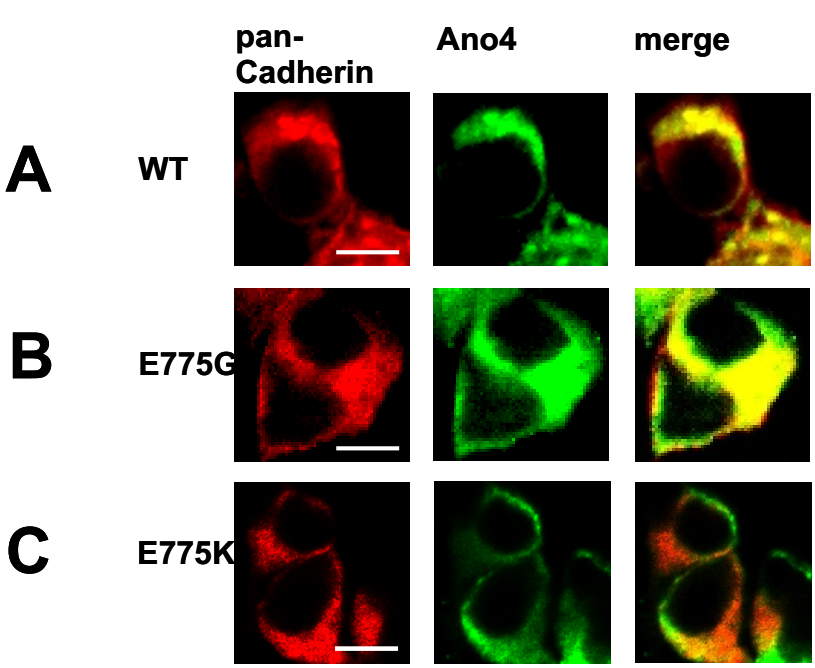
A

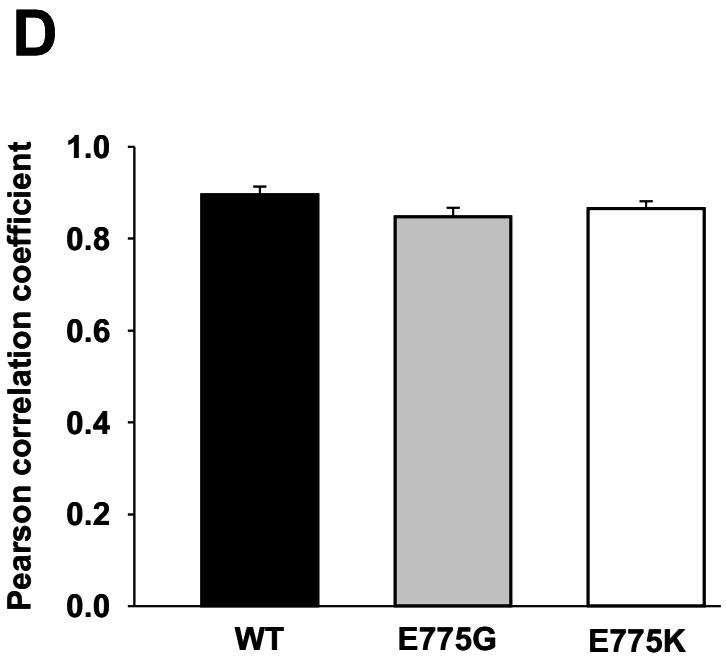
```
hsAno4      768   GIWYGILEGIGILSVITNAFVIAITSDFI   797
rnAno4      768   GIWYGILEGIGILSVITNAFVIAITSDFI   797
mmAno4      768   GIWYGILEGIGILSVITNAFVIAITSDFI   797
btAno4      733   GIWYGILEGIGILSVITNAFVIAITSDFI   762
ecAno4      781   GIWYGILEGIGILSVITNAFVIAITSDFI   810
ggAno4      775   GIWYGILEGIGILSVITNAFVIAVTSDFI   804
laAno4      794   GIWYGILEGIGILSVITNAFVIAITSDFI   823
mamuAno4    768   GIWYGILEGIGILSVITNAFVIAITSDFI   797
```

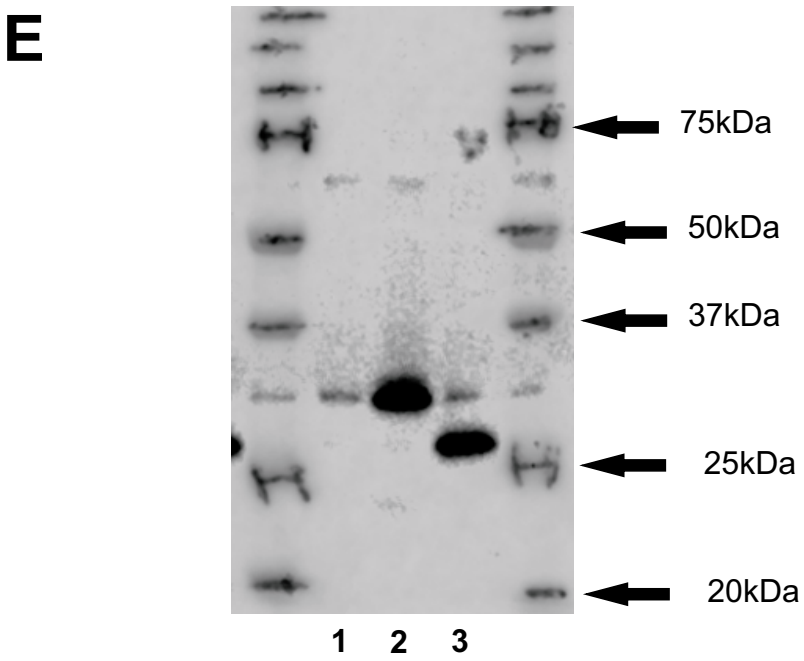
B



Supp.Fig.4

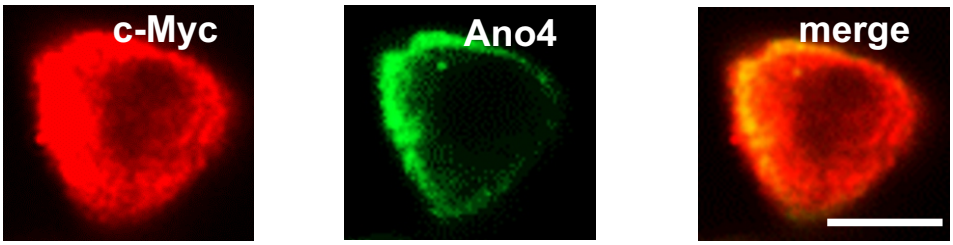

pan-Cadherin
Ano4
merge
A
WT
B
E775G
C
E775K


D
Pearson correlation coefficient
1.0
0.8
0.6
0.4
0.2
0.0
WT
E775G
E775K

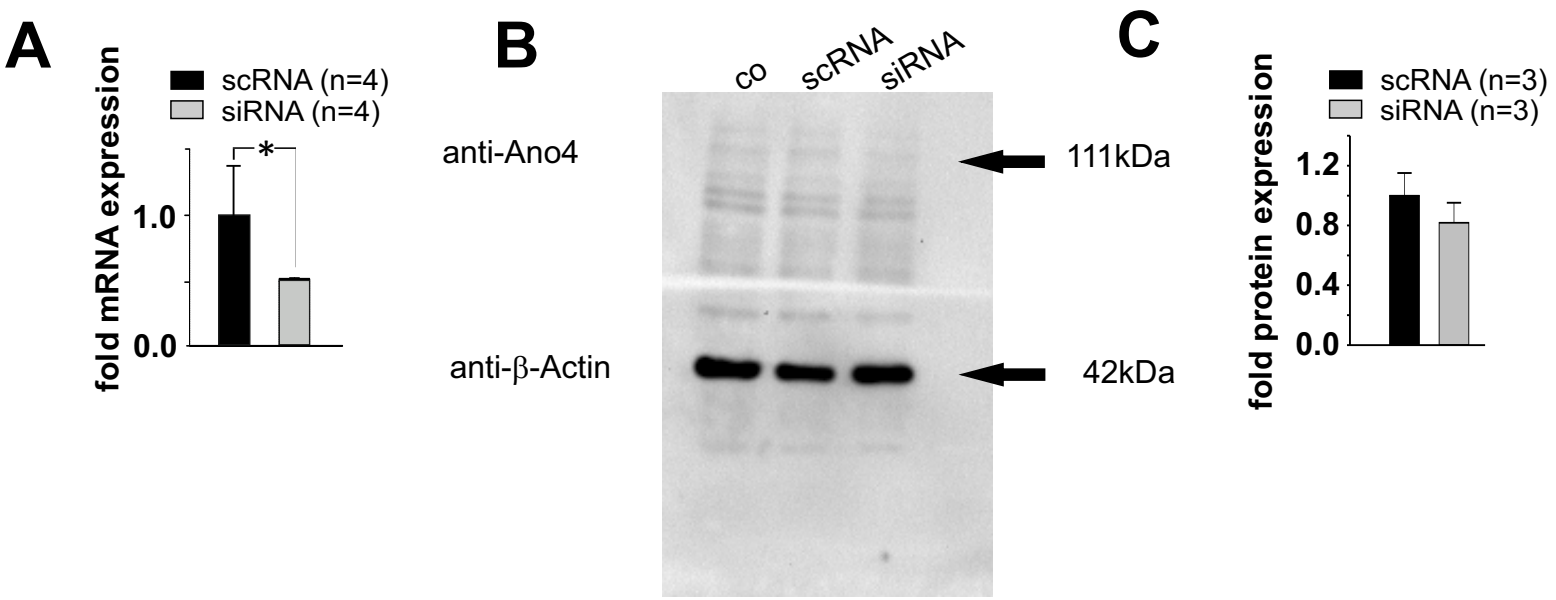

E
75kDa
50kDa
37kDa
25kDa
20kDa
1
2
3

Supp.Fig.5

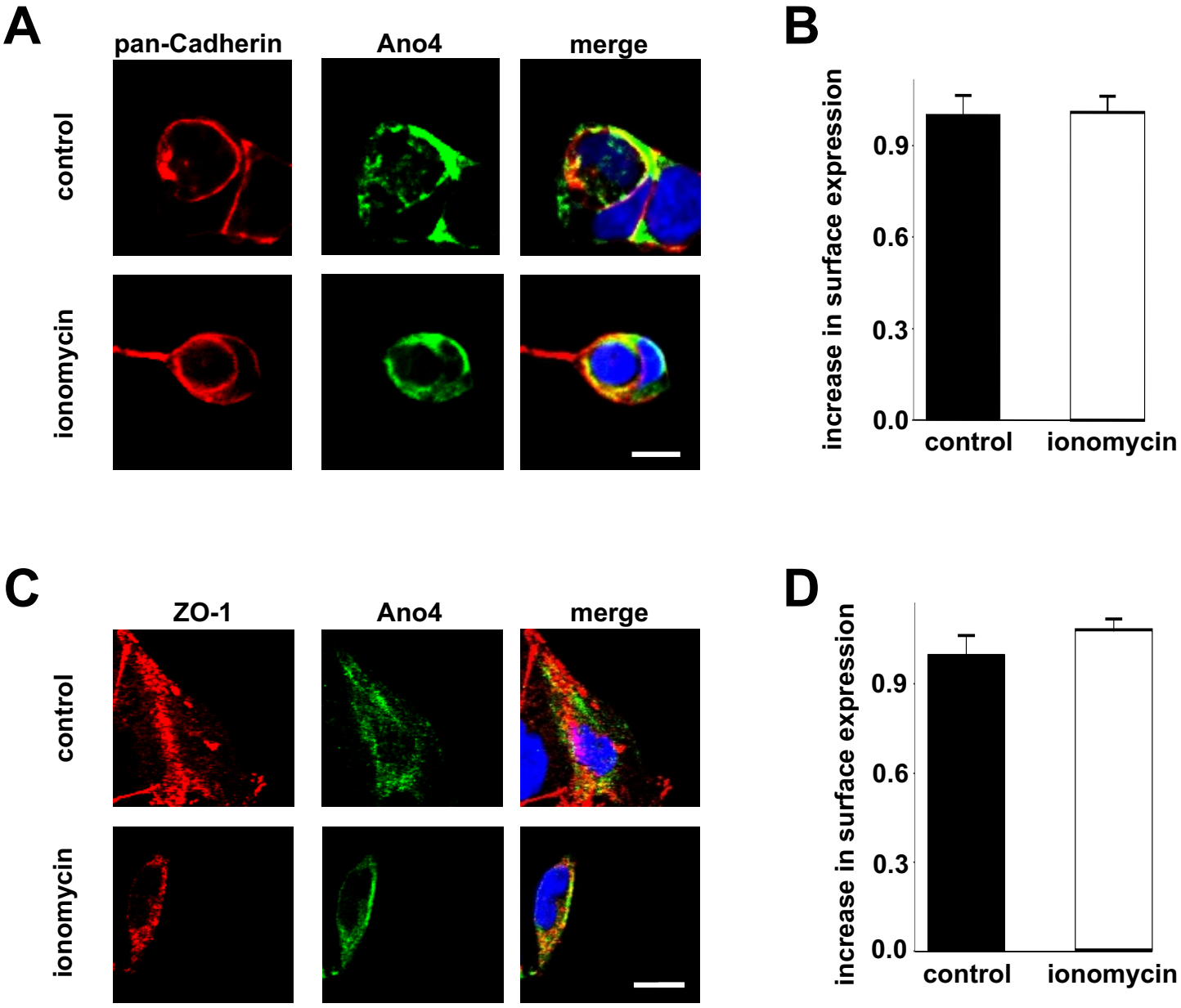
A



Supp.Fig.6


A
fold mRNA expression
scRNA (n=4)
siRNA (n=4)
*
1.0
0.0
B
co
scRNA
siRNA
anti-Ano4
111kDa
anti-β-Actin
42kDa
C
fold protein expression
scRNA (n=3)
siRNA (n=3)
1.2
0.8
0.4
0.0

Supp.Fig.7


A
pan-Cadherin
Ano4
merge
control
ionomycin
B
increase in surface expression
0.9
0.6
0.3
0.0
control
ionomycin
C
ZO-1
Ano4
merge
control
ionomycin
D
increase in surface expression
0.9
0.6
0.3
0.0
control
ionomycin

Supplement Table1:

Name	Species	Accession number	Length in amino acids
hsAno4	Homo sapiens	NP_001273544.1	955
mmAno4	Mus musculus	NP_001264117.1	955
rnAno4	Rattus norvegicus	XP_008763453.1	955
btAno4	Bos taurus	NP_001095520.1	920
eqAno4	Equus caballus	XP_005606787.1	968
ggAno4	Gallus gallus	XP_425452.4	962
laAno4	Loxodonta africana	XP_003405674.1	981
mamuAno4	Macaca mulatta	XP_001090523.1	955
hsAno2	Homo sapiens	NP_001265525.1	1003
mmAno2	Mus musculus	NP_705817.2	1002
rnAno2	Rattus norvegicus	XP_008761521.1	1002
hsAno1	Homo sapiens	NP_060513.5	986
mmAno1	Mus musculus	NP_848757.4	1017
rnAno1	Rattus norvegicus	NP_001101034.1	1040

Supplement Table1: detailed amino acid sequence information of Ano1, 2 and 4

name, species, accession number and number of amino acids. hs = homo sapiens; mm= mus musculus; rn = rattus norvegicus; bt = bos taurus; eq = equus caballus; gg = gallus gallus; la = loxodonta africana; mamu = macaca mulatta

Supplement Figure 1: Electrophysiological analysis of heterologously expressed GFP and Ano2

S1A: Confocal image of HEK cells expressing YFP. Staining with antibody against GFP (green). Scale bar represents 10µm. **S1B:** Raw currents through HEK293 cells heterologously expressing YFP before and after application of ionomycin (1µM), indicated by the bar. **S1C:** Current density-voltage plot of HEK293 cells heterologously expressing YFP before (filled circles) and after application of ionomycin (open circles). Values are given as mean ±SEM. **S1D:** Confocal image of HEK293 cells expressing Ano2-c-Myc. staining with antibody against c-Myc (red). Scale bar represents 10µm. **S1E:** Raw currents through HEK293 cells heterologously expressing Ano2 before and after application of ionomycin (1µM), indicated by the bar. **S1F:** Current density-voltage plot of HEK293 cells heterologously expressing Ano2 before (filled circles) and after application of ionomycin (open circles). **S1G:** Reversal potentials of the data shown in C (left) and F (right) before (black bars) and after (grey bars) application of ionomycin). **S1H:** Raw currents through HEK293 cells heterologously expressing Ano2 before and after application of Ringer-NMDG, indicated by the bar. **S1I:** Current density-voltage plot of HEK293 cells heterologously expressing Ano2 before (filled circles) and after application of ionomycin (open circles). **S1J**: Currents evoked in transfected HEK cells by ionomycin: left panel: cell transfected with Ano4-1-1150del-GFP that lacks the N-terminus of Ano4; right panel cell transfected with Ano4-GFP that contains a GFP at the C-terminus. **S1K:** Confocal image of untransfected HEK293 cells. Staining with antibodies against Ano4 (green) and pan Cadherin(red). Scale bar represents 20µm. **S1L:** *left side:* Currents evoked in untransfected HEK cells (upper panel) by ATP and Hek cells transfected with GFP (lower panel) by ionomycin. *Right side:* Bar chart of total current density increase after the application of ATP in untransfected HEK293 cells (black bar) or ionomycin in HEK293 cells transfected with GFP (grey bar) normalized to current density before ATP stimulation (=1). All values are given as mean ±SEM. **: $p<0.01$.

Supplement Figure 2: Lack of Scramblase activity of heterologously expressed Ano4 after stimulation with ATP

S2A-C: Assessment of scramblase activity by FACS sorting of annexin A5-labeled HEK293 cells transfected with GFP alone (**A**), with Ano4 plus GFP under control conditions (**B**) or 20 min after the application of ATP (500 µM). (**C**): X-axis: Fluorescence intensity of Anx A5-6S-IDCC (log); Y-axis: Fluorescence intensity of GFP (log). The right upper square represents the transfected, Annexin A5-positive cell fraction.

Supplement Figure 3: Alignment of conserved domains of Ano1, 2 and 4

S3A: Multiple sequence alignment of Ano4 in the region displayed in Figure 3B. For detailed sequence information see Table1. Highly conserved E (at position 775 in mmAno4) marked in green. **S3B:** Multiple sequence alignment of Anoctamin1, 2 and 4 in the area spanning from amino acid 708-843 (according to Figure 3A). Conserved amino acids are marked in black. Negatively charged amino acids highlighted in green and positively charged amino acids in red.

Supplement Figure 4: Subcellular localization of WT Ano4, E775G and E775K

S4A-C: Confocal image of HEK cells expressing Ano4 (**A**), E775G (**B**) and E775K (**C**). Staining with antibody against *pan*-Cadherin, a cell surface marker (red, left panel), Ano4 (green, middle panel).right panel: Merge of the two channels. Scale bar represents 10µm. **S4D:** Pearson Correlation Coefficient (PCC) of Ano4 and *pan*-Cadherin in HEK cells transfected with WT Ano4 (black bar), E775G (grey bar) and E775K (white bar). n=11-14 **S4E**: Western Blot of biotinylated samples: lane 1: untransfected HEK293; lane2: HEK293 transfected with GFP; lane 3:HEK293 transfected with GFP; arrows indicate different protein sizes

Supplement Figure 5: Evaluation of the Ano4 antibody

S5A: Confocal image of a HEK293 cell transfected with Ano4-c-Myc. Staining against c-Myc (red) and Ano4 (green); merge of the channels (yellow). Nuclei were stained with DAPI. Scale bar represents 10µm.

Supplement Figure 6: Modulation of gene and protein expression of Ano4 by siANO4

S6A: Bar chart comparing the relative expression of Ano4 mRNA in siRNA-transfected cells (grey bar) to scRNA (black bar) transfection. Ano4 expression in scRNA-transfected cells is normalized to 1. **S6B:** Western Blot analysis of lysates of ARPE-19 cells, either non-transfected (co) or transfected with scRNA or siANO4 using an antibody against Ano4. β-Actin served as loading control. (estimated protein size: Ano4 111kDa, β-Actin 42kDa). **S6C:** Densitometric analysis of S5B: Relative expression of Ano4 protein in siAno4-transfected (grey bar) to scRNA-transfected cells (black bar) resulting from Western Blot experiments. Ano4-expression of scRNA transfected cells was normalized to 1. n=3*: $p<0.05$.

Supplemental Figure 7: Influence of ionomycin on membrane expression of Ano4

S7A: Confocal images of HEK 293 cells heterologously expressing Ano4 after stimulation with control substance (PBS, upper panel) or ionomycin (1µM, lower panel for 5 min. Staining with antibody against *pan*-Cadherin, a cell surface marker (red, left panel), Ano4 (green, middle panel). Right panel: Merge of the channels. Nuclei were stained with DAPI. Scale bar represents 10µm. **S7B:** Bar chart depicting the increase in membrane expression of Ano4 upon ionomycin stimulation. Values are normalized to control. n_{PBS}= 29; $n_{ionomycin}$ =26. **S7C:** Confocal images of ARPE 19 cells endogenously expressing Ano4 after stimulation with control substance (PBS, upper panel) or ionomycin (1µM, lower panel for 5 min. Staining with antibody against ZO-1, a cell surface marker (red, left panel), Ano4 (green, middle panel). Right panel: Merge of the channels. Nuclei were stained with DAPI. Scale bar represents 10µm. **S7D:** Bar chart depicting the increase in membrane expression of Ano4 upon ionomycin stimulation. Values are normalized to control. n_{PBS}= 12; $n_{ionomycin}$ =20.