Supplementary Information Zdebik *et al.*

Supplementary Figures

Figure S1

Currents and proton transport in oocytes expressing WT ClC-4 (A-C) and its 'proton glutamate' mutant E281A (D-F). Voltage was clamped in steps of 20 mV from -100 to +80 mV. Original voltage clamp traces are shown in (A) and (D) for WT ClC-4 and the E281A mutant, respectively. Averaged steady-state current-voltage (I/V) curves are given in (B) (ClC-4) (n=10) and (E) ($ClC\text{-}4_{E281A}$) (n=7). (C) (WT) and (F) (E281A) show extracellular pH changes observed before and during a depolarizing pulse trains to + 80 mV. Consistent with the data obtained with ClC-5 (Fig. 1 G, H, main text), E281A gave neither currents nor H^+ transport. H^+-transport was studied in 8 and 5 oocytes expressing WT and E281A mutant ClC-4, respectively.

Figure S2

Western blot analysis of total expression levels in *Xenopus* oocytes of ClC-5 and key mutants of the 'proton glutamate' E268, as indicated at the bottom of the lanes. All constructs were tagged with an extracellular HA-epitope as described by Schwake *et al.* (2001) (*J Biol Chem* **276**, 12049-12054). Expression times in hours are stated below, and apparent molecular weights are given in the free lane separating results from different expression times. ClC-5 proteins were detected with an antibody directed against the HA-epitope and detection of rab 4 (after stripping the membrane) served as loading control. ClC-5 appears as a doublet, probably due to differences in glycosylation. The gating glutamate mutations did not change expression levels within the experimental error.

Figure S3

Cl^-, but not H^+ transport is restored to the ClC-4 'proton glutamate' E281A mutant by additionally inserting the uncoupling mutation in the 'gating glutamate' (E224A). (**A**) I/V curves of the double mutant with different anions (n=12). SCN^- changed dramatically both the shape and the reversal potential of the

currents. Similar, but smaller currents were also observed in non-injected oocytes and mock-transfected cells (data not shown). We therefore assume that part of the current in the presence of SCN^- is endogenous. *Inset*, voltage clamp trace obtained with Cl^-. (**B**) Changing pH_o affects neither conductance nor reversal potentials, in accord with the lack of H^+ transport observed in Fluorocyte measurements (**C**)(n=14). Pulses from -60 mV (100ms) to +90 mV (400 ms) were applied repetitively as indicated by bars. Data are almost identical to the results obtained for ClC-5 (shown in Fig. 4 in the main text).

Figure S4

Average I/V curves in the presence of extracellular Cl^-, NO_3^-, or SCN^- for uninjected oocytes (n=10) (A), oocytes expressing the 'proton glutamate' mutant ClC-5_{E268A} (n=14) (B) and the mutant ClC-5_{E268D} (n=16) (C). Oocytes were from 2 batches in which at least 5 µA of WT ClC-5 currents were observed at +100 mV. Uninjected oocytes showed currents of comparable magnitude as oocytes injected with ClC-5_{E268A}. Although NO_3^- transport is largely uncoupled from H^+-fluxes in WT ClC-5, it is not detectably transported by the E268A mutant that is thought to disrupt H^+ transport to the Cl^-/H^+-exchange site. Significant, but smaller currents are observed with all anions in the conservative E268D mutant.

Figure S5

ClC-0 and ClC-1 are not converted into Cl^-/H^+ transporters by inserting a 'proton glutamate'. When the valine which they carry in the position of the "proton glutamates" in the transporter CLCs is mutated to glutamate. (**A**), typical I/V trace for ClC-0_{V227E}, (**B**), Fluorocyte recording of this mutant (n=5). No change in intracellular fluorescence was seen upon activation of the pulse protocol.

Table 1

Reversal potential changes measured upon changes of extracellular pH. Results were obtained from voltage-clamp experiments conducted on the constructs indicated in the first column. The last column indicates the expected change for a Cl^-/H^+ exchanger with a 2:1 stoichiometry (calculated as $\Delta E_H/3$, where ΔE_H is the change in the Nernst potential for protons). The measured values are clearly inconsistent with H^+ transport activity.

Channel	pH change	ΔE_{rev} (mV) (mean ± SD (n))	Expected ΔE_{rev} for a $2Cl^-:1H^+$ exchanger
ClC-0	7.4 to 5	0.3 ± 0.2 (15)	46 mV
ClC-0_{V227E}	7.3 to 6	0.1 ± 0.1 (2)	25 mV
ClC-0_{V227E}	7.5 to 5	1.8 ± 0.6 (15)	48 mV
hClC-1_{V292E}	7.3 to 6	0.5 ± 0.3 (2)	25 mV
hClC-1_{V292E}	7.3 to 5	0.7 ± 0.5 (6)	44 mV

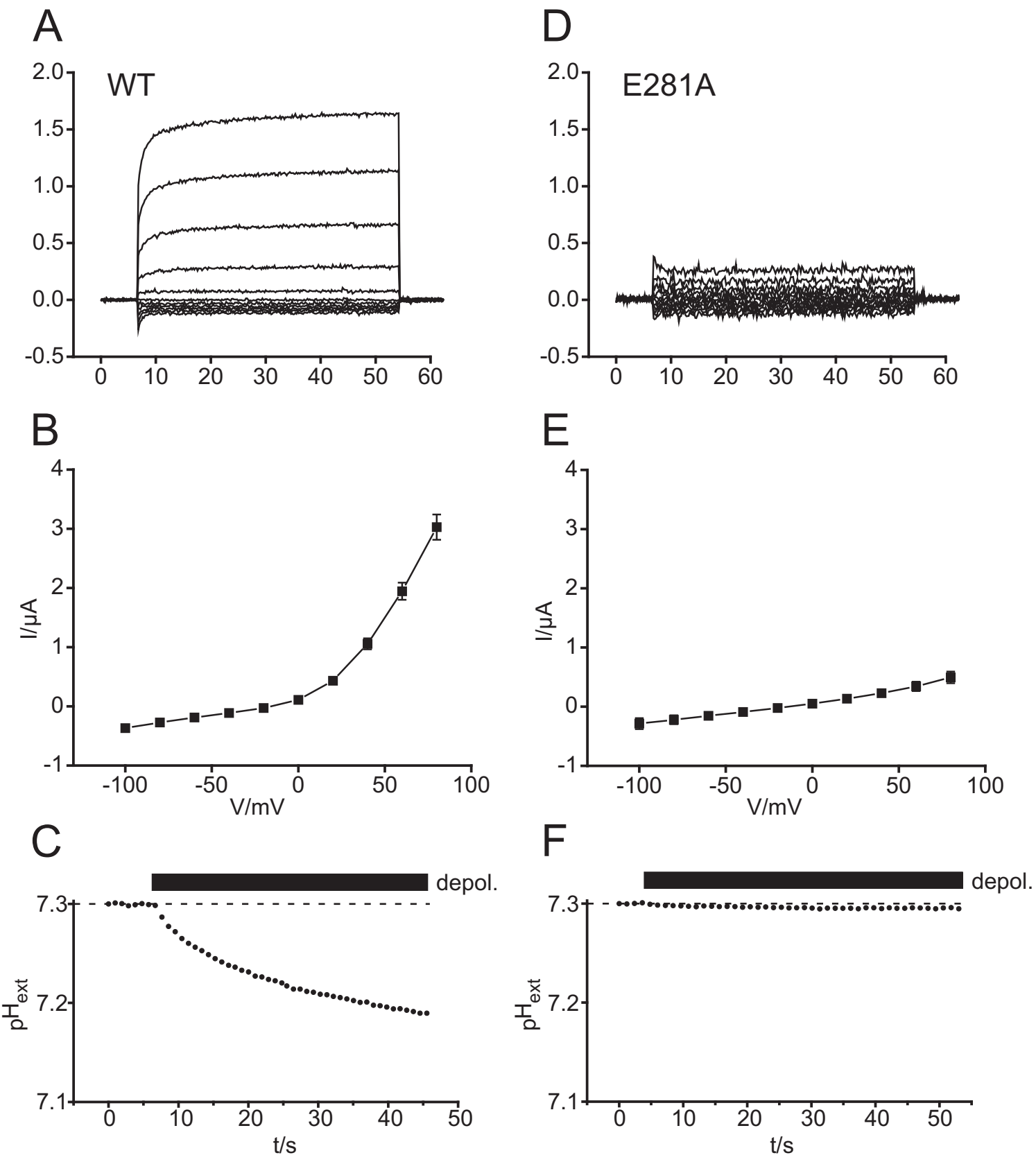
A
WT
2.0
1.5
1.0
0.5
0.0
-0.5
0
10
20
30
40
50
60
D
E281A
B
I/µA
V/mV
-100
-50
0
50
100
E
C
depol.
pH_ext
7.3
7.2
7.1
t/s
F

Fig. S1

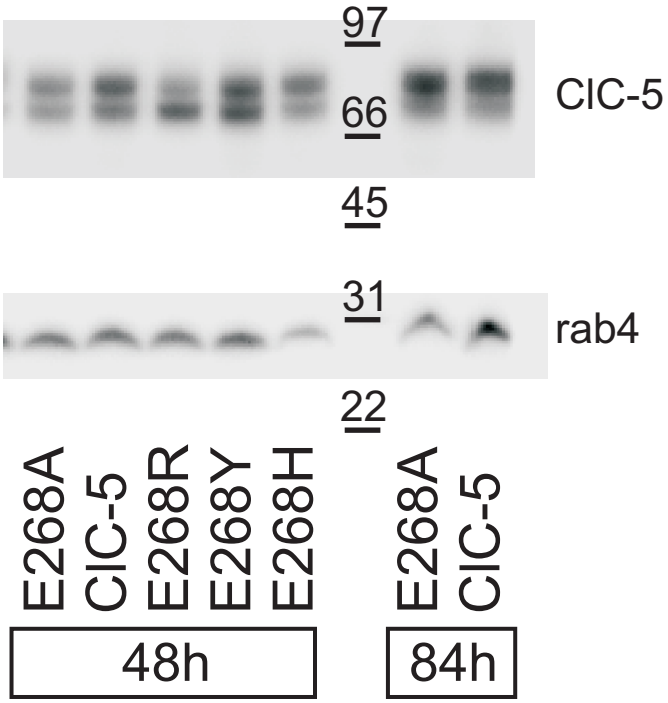
97
66
45
31
22
ClC-5
rab4
E268A
ClC-5
E268R
E268Y
E268H
E268A
ClC-5
48h
84h

Fig. S2

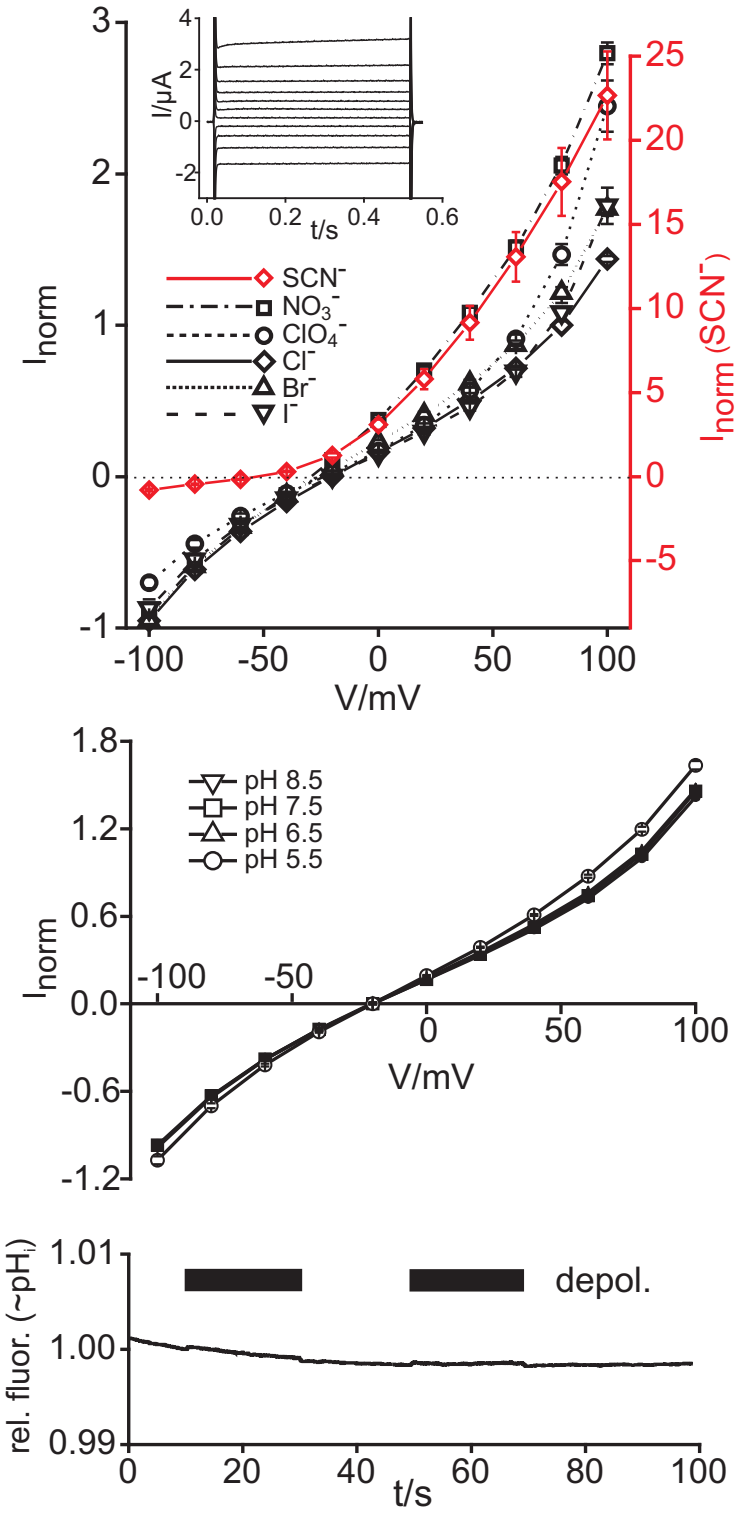
I/µA
t/s
SCN⁻
NO3⁻
ClO4⁻
Cl⁻
Br⁻
I⁻
Inorm
Inorm (SCN⁻)
V/mV
pH 8.5
pH 7.5
pH 6.5
pH 5.5
rel. fluor. (~pHi)
depol.

Fig. S3

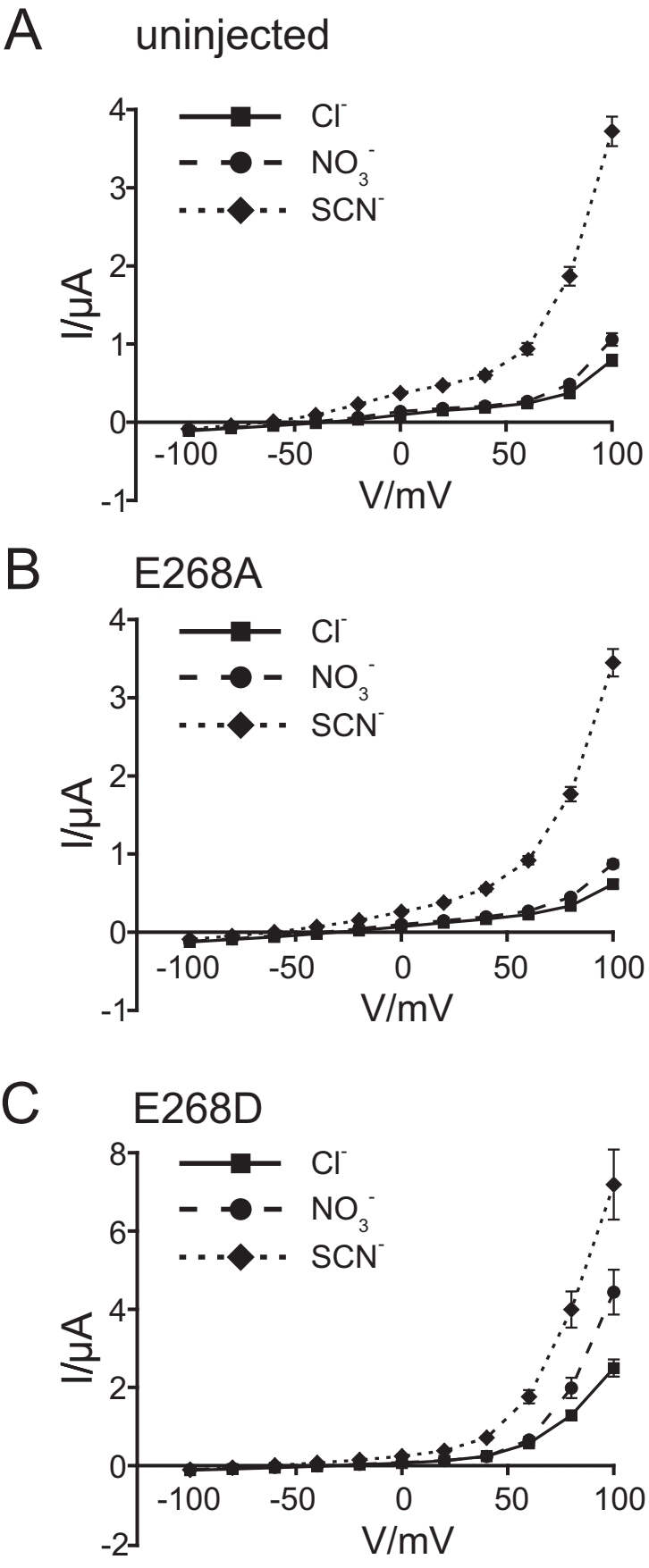

Fig. S4

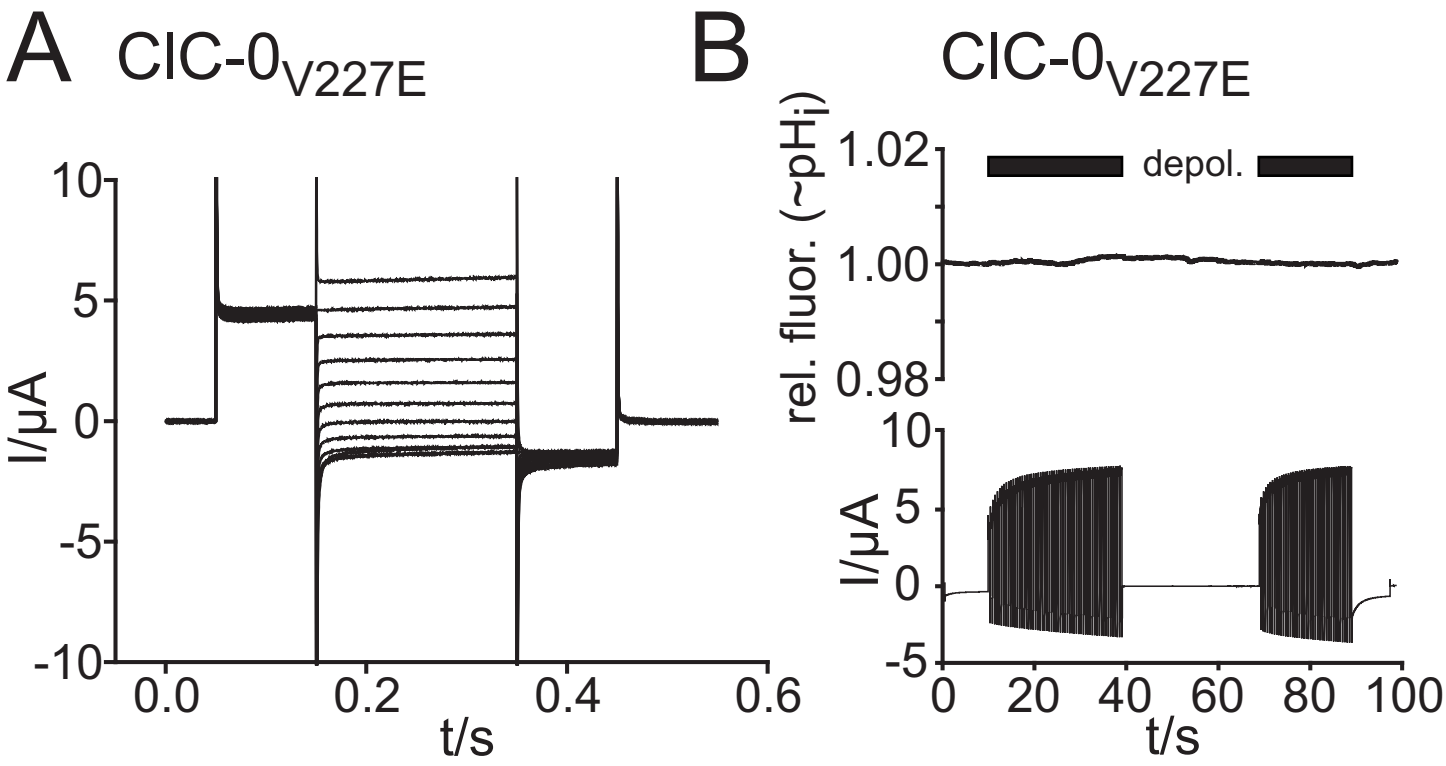
A
ClC-0V227E
10
5
0
-5
-10
I/µA
0.0
0.2
0.4
0.6
t/s
B
ClC-0V227E
rel. fluor. (~pHi)
1.02
1.00
0.98
depol.
10
5
0
-5
I/µA
0
20
40
60
80
100
t/s

Fig. S5