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Supplemental Information

Inactivation of Mechanically Activated Piezo1 Ion Channels Is Determined by the C-Terminal Extracellular Domain and the Inner Pore Helix

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Figure S1

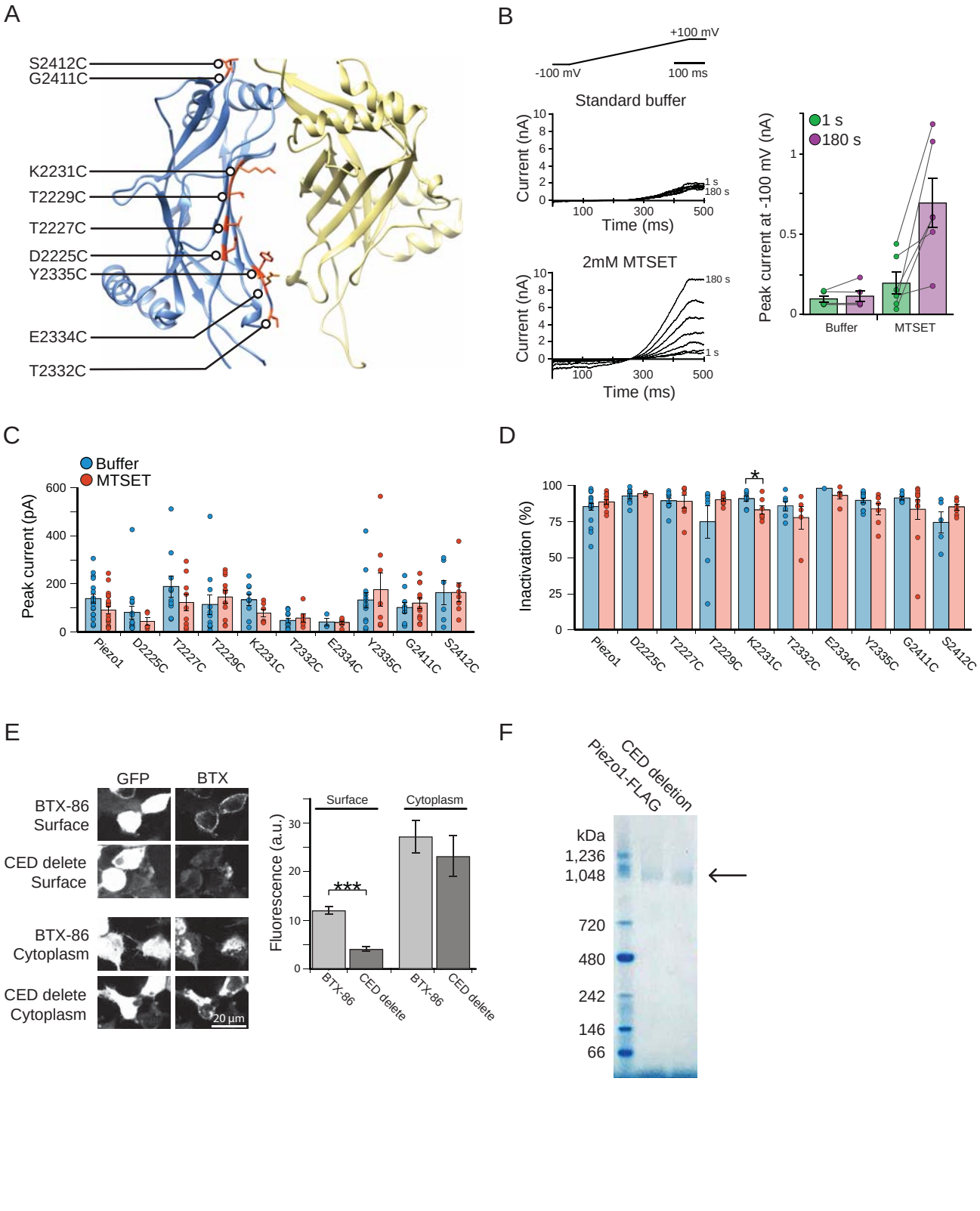
A
S2412C
G2411C
K2231C
T2229C
T2227C
D2225C
Y2335C
E2334C
T2332C
B
+100 mV
-100 mV
100 ms
Standard buffer
Current (nA)
Time (ms)
1 s
180 s
2mM MTSET
Peak current at -100 mV (nA)
Buffer
MTSET
C
Buffer
MTSET
Peak current (pA)
Piezo1
D2225C
T2227C
T2229C
K2231C
T2332C
E2334C
Y2335C
G2411C
S2412C
D
Inactivation (%)
E
GFP
BTX
BTX-86 Surface
CED delete Surface
BTX-86 Cytoplasm
CED delete Cytoplasm
20 µm
Surface
Cytoplasm
Fluorescence (a.u.)
***
BTX-86
CED delete
F
CED deletion
Piezo1-FLAG
kDa
1,236
1,048
720
480
242
146
66

Figure S1. Functional role of the Piezo1 CED. Related to Figure 2.

(A) Illustration of Piezo1 CED structure (only two subunits shown for clarity) with residues mutated individually to cysteines highlighted in red. (B) Voltage-ramp stimulation protocol and elicited currents recorded from HEK293T transiently transfected with wild-type mouse TRPA1 in standard buffer and 2 mM MTSET to demonstrate efficacy of MTSET reagent (left). Individual and mean current amplitudes of TRPA1 under indicated MTSET labeling conditions (right) (n = 6 cells). (C) Individual and mean peak current amplitudes and (D) percent inactivation from cell-attached recordings of HEK293T-P1KO cells transiently transfected with Piezo1 cysteine mutants in 'A' stimulated with a -60 mmHg pressure stimulus (n = 3-15 cells, * $p \leq 0.05$, unpaired Student's t-test). (E) Representative images of HEK293T-P1KO cells transiently transfected with bungarotoxin-taged Piezo1 (BTX-86) or CED deletion construct ΔQ2222-D2451 (CED delete). GFP indicates transfected cells, BTX indicates labeling with α-bungarotoxin conjugated with AlexaFluor 555 for surface labeling or AlexaFluor 647 for cytoplasmic labeling. Mean fluorescence intensity of α-bungarotoxin on the plasma membrane surface or in the cytoplasm for indicated conditions (n = 18 cells, *** $p \leq 0.0005$, unpaired Student's t-test). (F) FLAG-purified wild-type and CED deletion Piezo1 separated on a 3%-12% bis-tris non-denaturing gel and visualized by coomassie G-250 staining. Expected molecular weights of wild-type and CED delete Piezo1 are 876 kDa and 800 kDa, respectively (arrow). All data are mean ± SEM.

Supplemental Experimental Procedures

Cell Culture

Cells were cultured at a seeding density of 10,000 cells per well in a 24-well plate in DMEM-HG (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Clontech Laboratories), 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin (Life Technologies), and grown at 37 °C and 5% CO_2 on poly-L-lysine and laminin coated coverslips (Sigma Aldrich). Cells were transiently transfected with Piezo1 and Piezo1 mutants (1.5 μg) in the presence of 10 μM ruthenium red using Fugene6 (Promega) according to manufacturer protocol. Piezo2 and Piezo2 mutants were co-transfected with EGFP at a 1:0.5 mass ratio. Transfected cells were recorded 24-72 hours post-transfection.

Electrophysiology

For whole-cell experiments, borosilicate glass pipettes (1.5 OD, 0.85 ID; Sutter Instrument Company) had a resistance of 2-4 MΩ when filled with pipette buffer solution containing (in mM) 133 CsCl, 10 HEPES, 5 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, 4 MgATP, and 0.4 Na_2GTP, pH=7.3 with CsOH. The bath solution for whole-cell experiments contained (in mM), 130 NaCl, 3 KCl, 1 $MgCl_2$, 10 HEPES, 2.5 $CaCl_2$, and 10 glucose, pH=7.3 with NaOH. For '$NMDG^+$ intracellular' recordings, all CsCl in the pipette solution was replaced with 133 mM $NMDG^+$ and recorded with standard bath buffer. For '$NMDG^+$ extracellular' recordings, all NaCl and KCl in the bath buffer was replaced with 133 mM NMDG and recorded with standard pipette buffer. For whole-cell recordings, internal solution was allowed to dialyze for at least five minutes before recording to allow for GTP-mediated run-up of Piezo2 currents (Jia et al., 2013).
Cell-attached recordings for all experiments were performed at RT in bath solution containing (in mM) 140 KCl, 10 HEPES, 1 $MgCl_2$ and 10 Glucose, pH=7.3 with KOH. Pipette buffer for Piezo1 experiments contained (in mM) 130 NaCl, 5 KCl, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$ and 10 TEA-Cl, pH=7.3 with NaOH.

Fluorescence imaging

The CED deletion construct 'Q2222-D2451delinsTG' and the previously reported construct Piezo1-BTX-86 (Wu, 2016) were transiently transfected into HEK293T-P1KO cells for 48 hours. For membrane surface labelling, cells were live-labeled by washing twice with PBS, then incubating at 37 °C for 15 min in PBS containing α -bungarotoxin conjugated with AlexaFluor-555 (B35451; Molecular Probes) at a final concentraion of 10 μg/mL and 10 mM HEPES. Cells were then washed 3 times for 5 min each and fixed with 4% paraformaldehyde for 30 min at room temperature in the dark. For cytoplasmic protein labeling, cells were first fixed in 4% PFA and then permeabilized with 0.25% Triton X-100 (28314; Thermo Fisher) and incubated with PBS containing 10 μg/mL α-bungarotoxin conjugated with AlexaFluor-647 (B35452; Molecular Probes) for 15 min in the dark at 37 °C for labeling in cytoplasmic protein. Cells for both experiments were washed 3 times for 5 min each and mounted with Fluoromount-G (SouthernBiotech) on glass slides, and imaged on a Zeiss 780 inverted confocal microscope at x63 magnification. Mean fluorescence intensity was measured along the bounding cell membrane or within the cytoplasm with a custom-written script in Fiji image processing software as previously described (Wu, 2016).

Protein purification and NativePAGE gel

The mouse Piezo1-Flag construct was obtained from Ardem Patapoutian and purified as previously described (Syeda et al., 2016). The CED deletion construct 'P2223-S2450delinsTG-FLAG' was purified in the same manner. Purified protein samples were analyzed by non-denaturing gel electrophoresis using the NativePAGE novex (Invitrogen) system in accordance with the user manual. Specifically, samples were mixed with NativePAGE sample buffer and NativePAGE 5% G-250 sample additive and run on a 3%-12% bis-tris gel at 150 V for 90 minutes. Following electrophoresis, the native gel was visualized by coomassie G-250 staining.

Supplemental References

Jia, Z., Ikeda, R., Ling, J., and Gu, J.G. (2013). GTP-dependent run-up of Piezo2-type mechanically activated currents in rat dorsal root ganglion neurons. Molecular brain *6*, 57.

Syeda, R., Florendo, M.N., Cox, C.D., Kefauver, J.M., Santos, J.S., Martinac, B., and Patapoutian, A. (2016). Piezo1 Channels Are Inherently Mechanosensitive. Cell reports *17*, 1739-1746.

Wu, J., Goyal, R, and Jorg Grandl (2016). Localized force application reveals mechanically sensitive domains of Piezo1. Nat Commun *32*, 57-71.