SUPPLEMENTARY MATERIALS

Enhancing prestin oligomerization for superior sonogenetics

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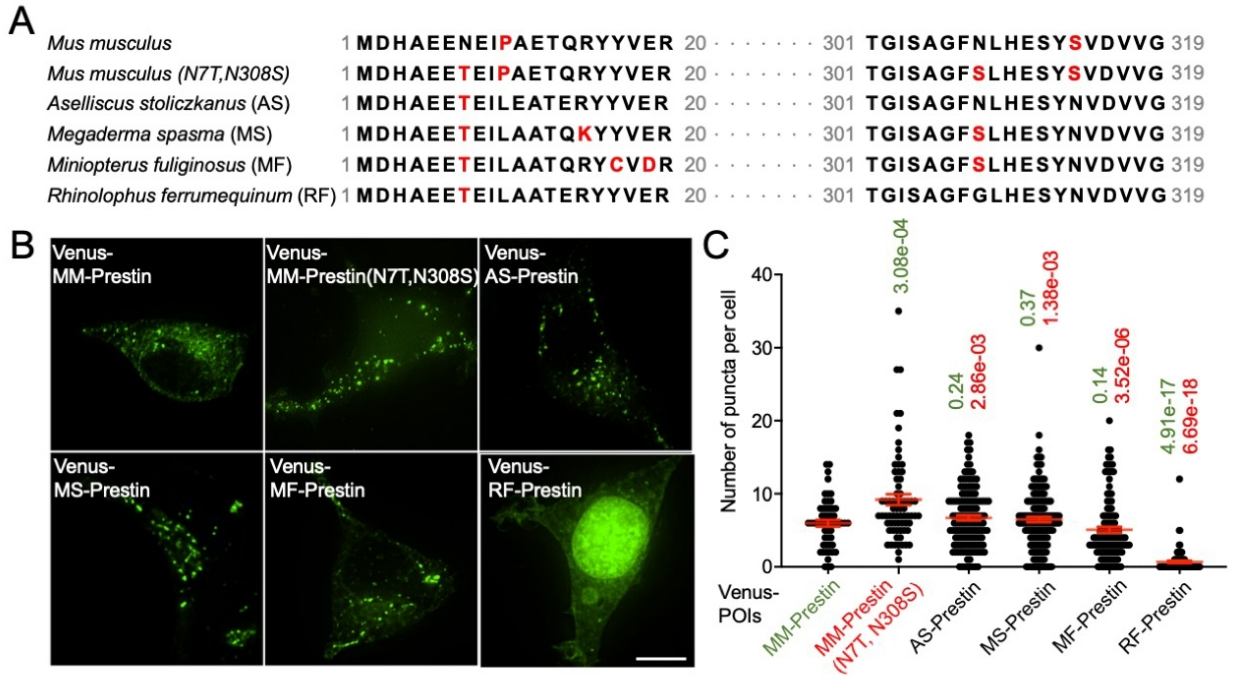

Figure S1. Puncta formation of prestins from different species. (A) Sequence alignment of prestins from four echolocating bats (*Aselliscus stoliczkanus*, *Megaderma spasma, Miniopterus fuliginosus, Rhinolophus ferrumequinum*) and mouse (Mus musculus), with or without the N7T and N308S mutations. Residues highlighted in red indicate amino acids that diverge from the conserved sequence shared by most species. (B) Representative fluorescence images of HEK293T cells transfected with the indicated constructs: Venus-MM-Prestin, Venus-MM-Prestin(N7T, N308S), Venus-AS-Prestin, Venus-MS-Prestin, Venus-MF-Prestin, and Venus-RF-Prestin. Maximum intensity z-projections were generated from 17 stacks, each 0.3 μm apart. Scale bar, 10 μm. (C) Quantification of puncta number for the indicated constructs shown in B. Data (in red) represent mean ± S.E.M. from at least three independent experiments; n = 55, 70, 141, 147, 118, and 90 cells (from left to right). Student's *t*-test was used to compare values between the indicated construct and MM-Prestin (green) or MM-Prestin(N7T, N308S) (red), respectively.

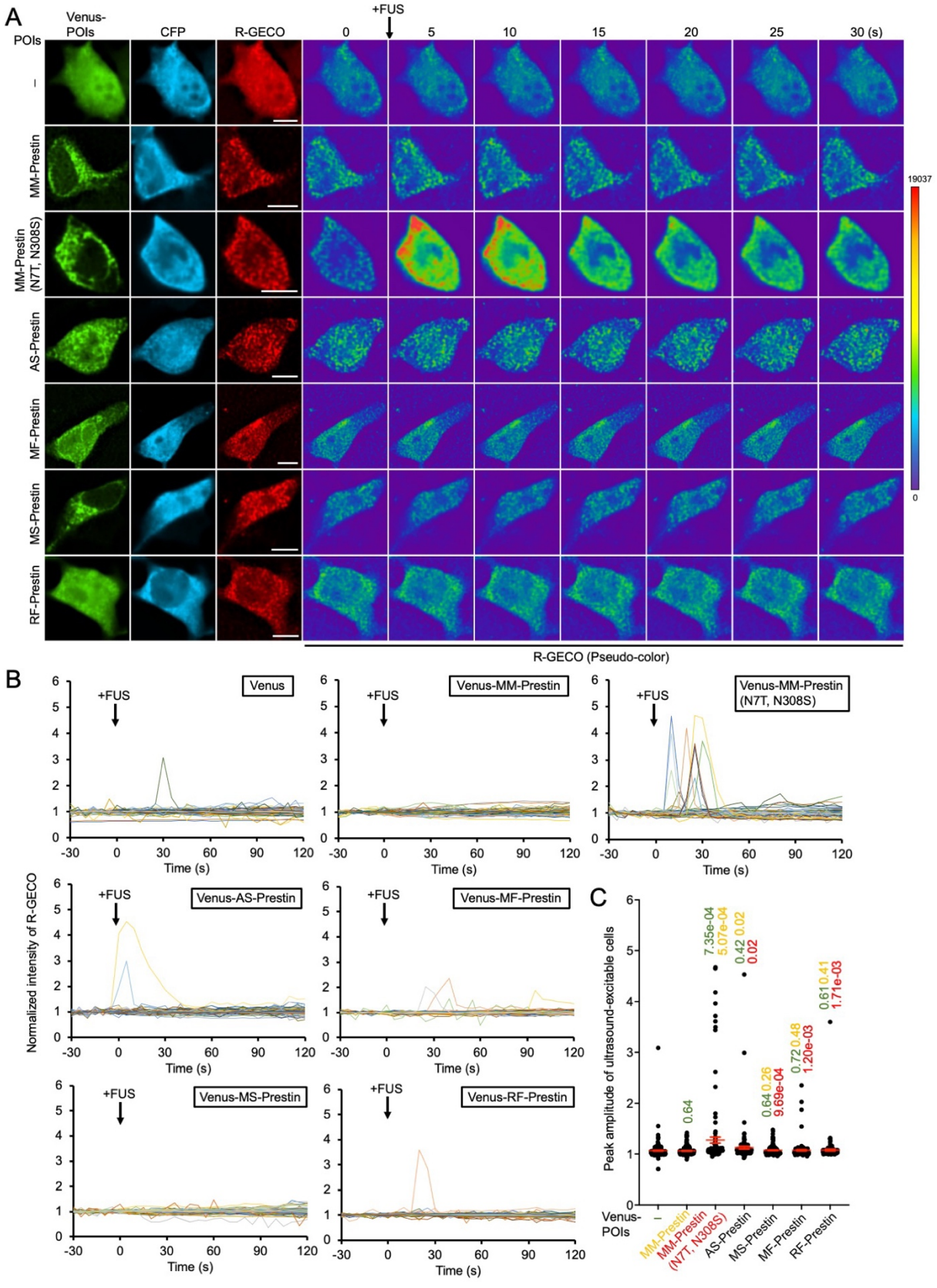
A
Venus-POIs
CFP
R-GECO
+FUS
0
5
10
15
20
25
30 (s)
POIs
-
MM-Prestin
MM-Prestin (N7T, N308S)
AS-Prestin
MF-Prestin
MS-Prestin
RF-Prestin
19037
0
R-GECO (Pseudo-color)
B
Venus
Venus-MM-Prestin
Venus-MM-Prestin (N7T, N308S)
Venus-AS-Prestin
Venus-MF-Prestin
Venus-MS-Prestin
Venus-RF-Prestin
+FUS
Time (s)
Normalized intensity of R-GECO
C
Peak amplitude of ultrasound-excitable cells
Venus-POIs
7.35e-04
5.07e-04
0.02
0.42
0.02
0.64
0.64
0.26
9.69e-04
0.72
0.48
1.20e-03
0.61
0.41
1.71e-03

Figure S2. Prestins from four echolocating bats do not confer US sensitivity to cells. (A) HEK293T cells were co-transfected with CFP-R-GECO and the indicated constructs. Twenty-four hours post-transfection, a pulse of FUS (0.5 MHz, 0.5 MPa, 10 Hz PRF, 2000 cycles, 3-sec duration) was applied, and R-GECO fluorescence intensity was monitored in real time (shown in pseudo-color). Scale bar: 10 μm. (B) Time course of normalized R-GECO fluorescence intensity in cells expressing the indicated constructs following 0.5 MHz FUS stimulation. Data were collected from at least three independent experiments; n = 255, 210, 140, 153, 136, 138, and 152 cells for Venus, Venus-MM-Prestin, Venus-MM-Prestin(N7T, N308S), Venus-AS-Prestin, Venus-MF-Prestin, Venus-MS-Prestin, and Venus-RF-Prestin groups, respectively. (C) Peak R-GECO fluorescence amplitude in response to FUS stimulation in cells expressing the indicated constructs. Data (in red) represent mean ± S.E.M. from more than three independent experiments. Student's *t*-test was used to compare values with Venus alone (green), MM-Prestin (orange), or MM-Prestin(N7T, N308S) (red), respectively.

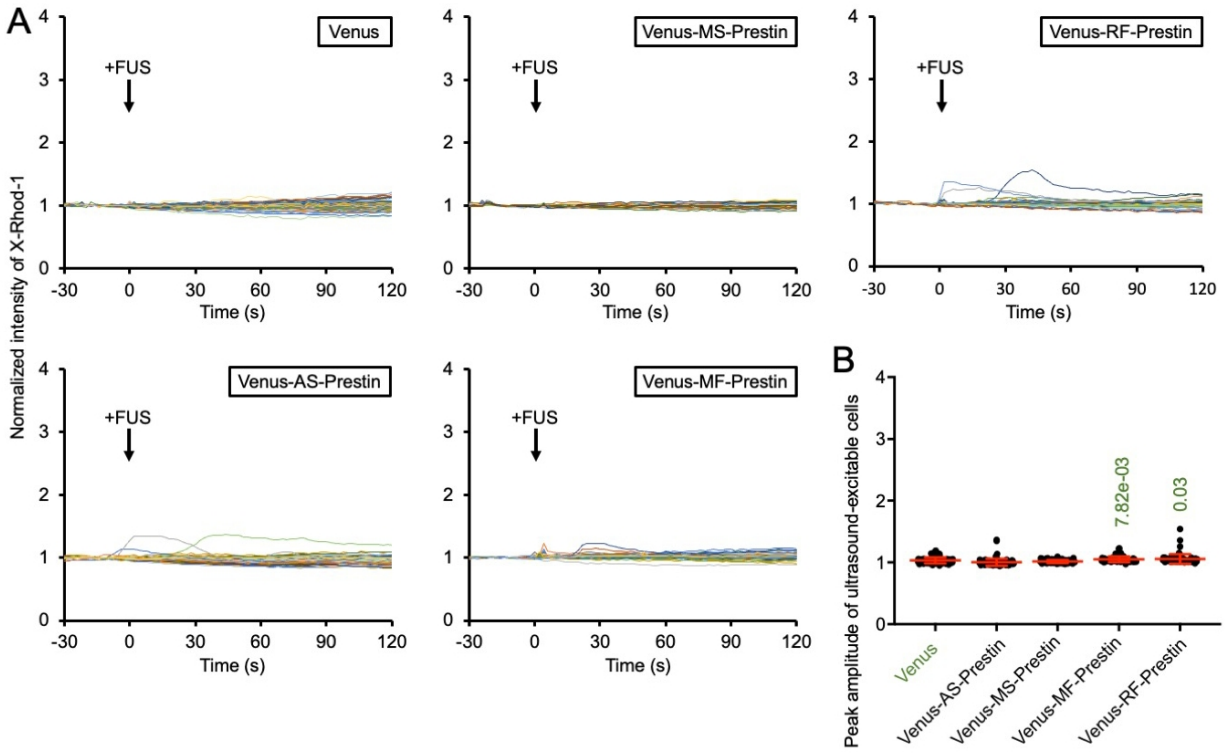

Figure S3. Prestins from four echolocating bats do not, or only weakly, confer US sensitivity to primary neurons. (A) Rat primary cortical neurons were transfected with plasmids encoding either Venus or Venus-tagged Prestins from four echolocating bat species. Transfected neurons were stained with the calcium indicator X-Rhod-1. Time course of X-Rhod-1 fluorescence intensity following FUS stimulation (0.5 MHz, 0.5 MPa, 10 Hz PRF, 2000 cycles, 3-sec duration) was monitored by live-cell imaging. Data were collected from three independent experiments: n = 129, 112, 88, 79, and 75 cells for the Venus, Venus-AS-Prestin, Venus-MS-Prestin, Venus-MF-Prestin, and Venus-RF-Prestin groups, respectively. (B) Peak X-Rhod-1 fluorescence intensity quantified from A. Data (in red) present mean ± S.E.M. Student's *t*-test was used to compare Venus with each indicated conditions.

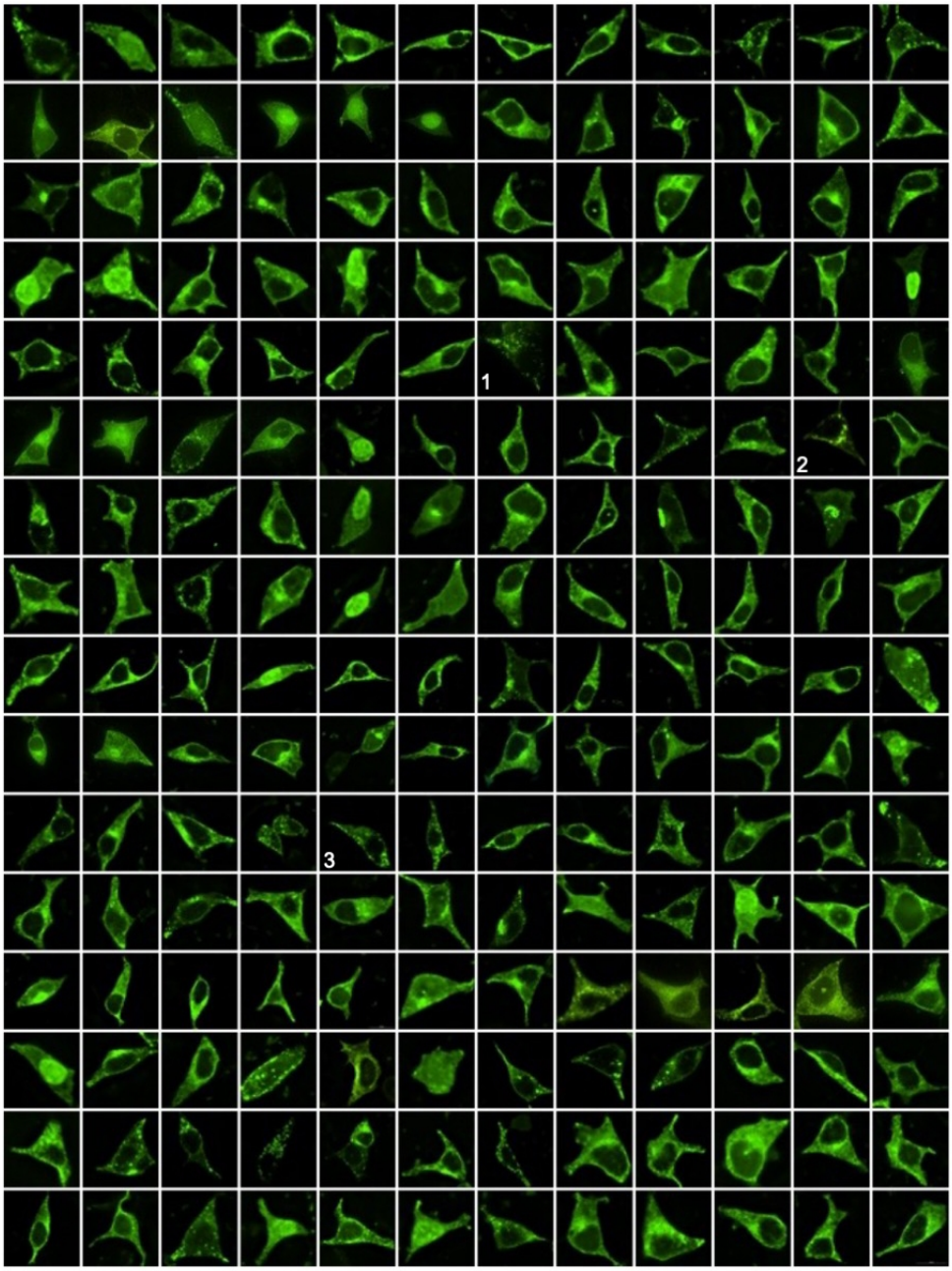

Figure S4. Representative images of 192 Venus-tagged MM-Prestin variants generated by random mutagenesis. HEK293T cells were transfected with Venus-tagged MM-Prestin variants. Several variants exhibiting strong protein clustering were selected for sequencing. Scale bar, 10 μm.

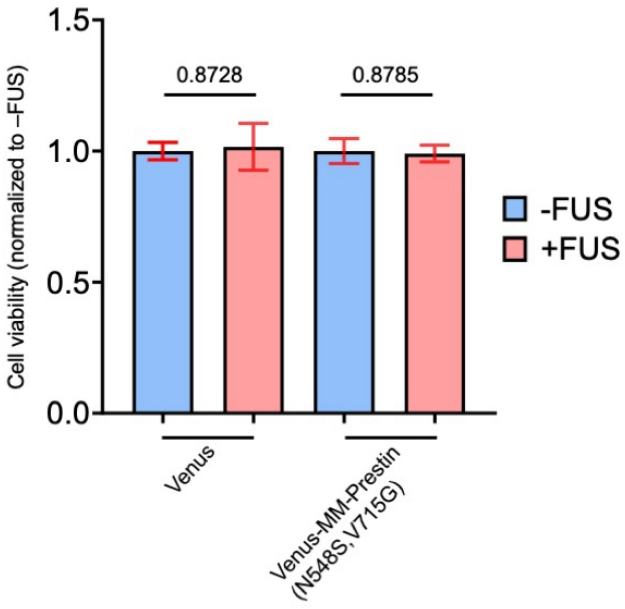

Figure S5. Assessment of cell viability following FUS stimulation using CCK-8 assay. HEK293T cells expressing either Venus or Venus-MM-Prestin(N548S, V715G) were exposed to FUS (0.5 MHz, 0.5 MPa, 10 Hz PRF, 2000 cycles, 3-s duration). Cell viability was assessed 3 hours post-stimulation using the CCK-8 assay, with absorbance at 450 nm. Data represent mean ± S.E.M. from three independent experiments.

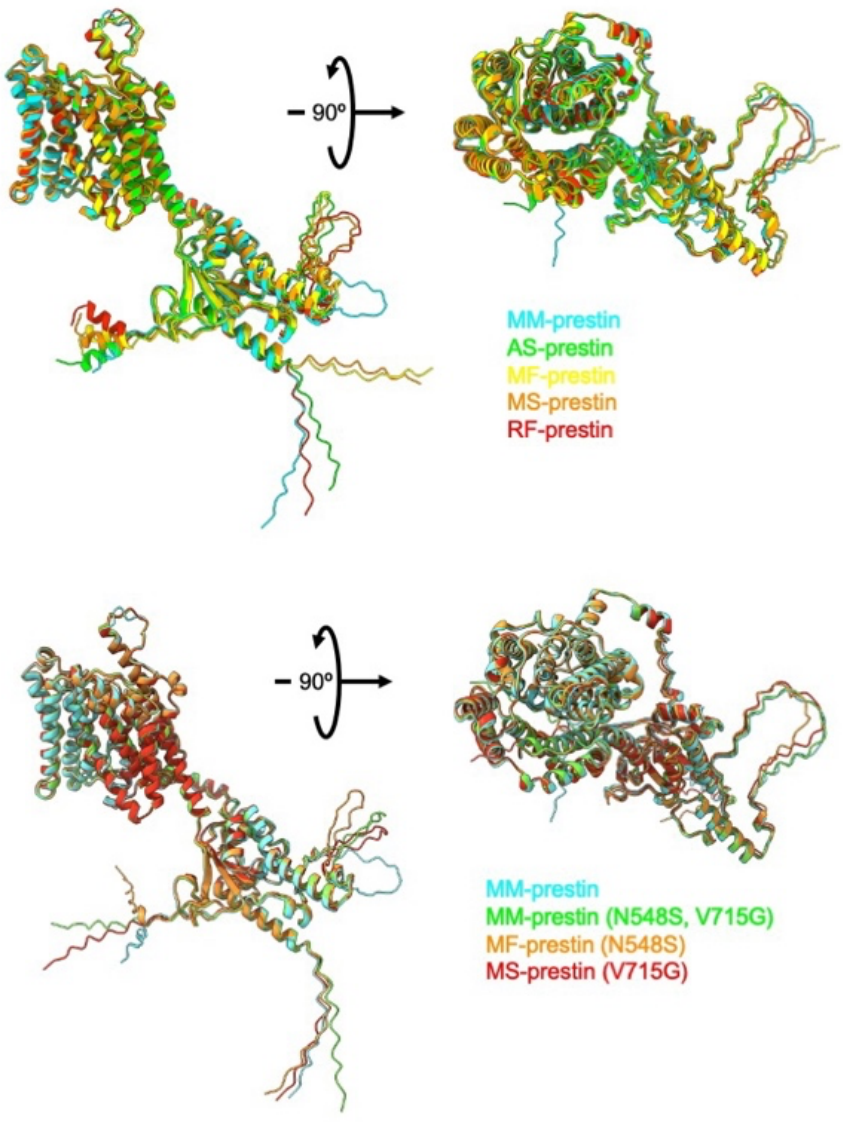

Figure S6. Alignment of different prestin variants. Protein structures of the indicated prestin variants were predicted using AlphaFold and aligned using ChimeraX software.

Protein sequence of different prestin variants

MM-Prestin

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KIRNIIYMFLPITKWLPAYKFKEYVLGDLVSGISTGVLQLPQGLAFAMLAAVPPVF
GLYSSFYPVIMYCFFGTSRHISIGPFAVISLMIGGVAVRLVPDDIVIPGGVNATNGTE
ARDALRVKVAMSVTLLSGIIQFCLGVCRFGFVAIYLTEPLVRGFTTAAAVHVFTSM
LKYLFGVKTKRYSGIFSVVYSTVAVLQNVKNLNVCSLGVGLMVFGLLLGGKEFN
ERFKEKLPAPIPLEFFAVVMGTGISAGFNLHESYSVDVVGTLPLGLLPPANPDTSLF
HLVYVDAIAIAIVGFSVTISMAKTLANKHGYQVDGNQELIALGICNSIGSLFQTFSI
SCSLSRSLVQEGTGGKTQLAGCLASLMILLVILATGFLFESLPQAVLSAIVIVNLKG
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QEDMEPNATPTTPEA

MM-Prestin(N7T, N308S)

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QEDMEPNATPTTPEA

AS-Prestin

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SEPNATPEV

MS-Prestin

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PQEDSEPNATPEP

MF-Prestin

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RF-Prestin

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PQEDSEPNATPDV

MM-Prestin(N548S, V715G)

MDHAEENEIPAETQRYYVERPIFSHPVLQERLHVKDKVTESIGDKLKQAFTCTPK
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MM-Prestin(N548S)

MDHAEENEIPAETQRYYVERPIFSHPVLQERLHVKDKVTESIGDKLKQAFTCTPK
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QEDMEPNATPTTPEA

MM-Prestin(V715G)

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