

## Supplement Figure 1:

### **Kcv<sub>wt</sub>**

ATGTTAGTGTTTAGTAAATTTCTAACGCGAACTGAACCATTTCATGATACATCTCTTTATTCTCGCAATGTTTCGTGATGA  
TCTATAAAATTTCTTCCCGGGAGGGTTGAAAAATAAATTCTCTGTTGCAAACCCGGACAAAAAGGCATCATGGATAGATTG  
TATATACTTCGGAGTAACGACACACTCTACTGTCGGATTTCGGAGATATACTGCCAAAGACGACCGGCGCAAAGCTTTGT  
ACGATAGCACATATAGTAACAGTGTTCTTCATCGTCTCTAACTTTA

### **Kcv<sub>op</sub>**

ATGCTGGTGTTCTCTAAGTTCTTGACTAGAACTGAACCATTTCATGATCCACTTGTTTCATTTTGGCCATGTTTCGTGATGA  
TCTACAAGTTTTTTCCAGGTGGTTTTCGAGAACAATTCTCTGTTGCTAATCCAGATAAGAAGGCTTCTTGATTGATTG  
CATCTACTTCGGTGTTACTACTCATTCTACTGTTGGTTTTCGGTGATATTTTGCCAAAACTACTGGTGCTAAGTTGTGC  
ACTATTGCTCATATCGTTACCGTTTTCTTCATCGTCTTGACCTTG

### **Kesv<sub>wt</sub>**

ATGTCCCGGCGACTGTTTGCGACTTGCGGCATCGCTATCGCGCTCAGGGGACTGGTGGTGAGCGGGGGCGTAAAAGAGA  
TTGTATCGTTCAGGCCACTGATTGATACTTCGCTCGTCGGCGGAATATTGTCTAATCTGATTTTGCTCGTCGTTTTTCGC  
TGAACTTTATTTGGCAGCTGGACCAAGGGGATGATCACACACACTTCGGCTTCTCGTCCGCGATCGACGCTTACTACTTC  
AGTGCGGTACAGTCTTCTCTGTGCGATACGGCGATTTGTTGCCGAAAACCTCCGAAGGCAAAAATTGCTTACCATCGCAC  
ACATTTTGCCATGTTCTTCGTGATGCTCCCCGTGTGCGCAAGGCTCTCGAGAAG

### **Kesv<sub>op</sub>**

ATGAGCAGACGGCTGTTTCGCCACCTGTGGAATCGCCATTGCCCTGCGGGGCCCTGGTGGTGCTCTGGCGGCGTGAAAGAAA  
TCGTGTCCTTCCGGCCCCCTGATCGACACCAGCCTCGTGGGAGGCATCCTGAGCAACCTGATCCTGCTGGTGGTGTTCGC  
CGAGCTGTACTGGCAGCTGGACCAGGGCGACGACCACACCCACTTCGGCTTCAGCAGCGCCATCGACGCCTACTACTTC  
AGCGCCGTGACCAGCAGCAGCGTGGGCTACGGCGACCTGCTGCCCAAGACCCCAAGGCCAAGCTGCTGACAATCGCCC  
ACATCCTGGCCATGTTCTTCGTGATGCTGCCCCTGGTGGCCAAGGCCCTGGAAAAG

### **>Kesv<sub>ran</sub>**

ATGTCGCGGCGGCTCTTTGCGACGTGCGGCATTGCTATAGCCTTAAGGGGCCCTTGTCGTTTCCGGAGGCGTCAAGGAGA  
TTGTCTCTTTTAGACCCCTCATTGACACCTCGTTGGTAGGTGGTATACTTTTGAATCTGATTCTGCTAGTCGTTTTTGC  
AGAAGTGTACTGGCAATTGGACCAGGGAGACGATCACACTCACTTCGGATTTCAGTTCAGCCATAGATGCCTACTACTTC  
TCAGCAGTGACGTCATCTAGTGTAGGTTACGGCGACTTGTTGCCCAAGACCCCAAGGCAAAAATTACTGACAATCGCGC  
ACATCTTGCTATGTTCTTCGTAATGCTTCCGGTGTAGCGAAGGCTCTTGAAAAG

### **Linker 1**

TTGTTGCCGAAAACCTCCGAAGGCAAAATTGCTTACCATCGCACACATTTTGCCATGTTCTTCGTGATGCTC  
CCCGTTGTGCGGAAGGCTCTCGAGAAG

### **Linker 2**

TTGTTGACTATTGCTCATATTTTGCTATGTTTTTTGTTATGTTGCCAGTTGTTGCTAAGGCTTTTGAAAAG

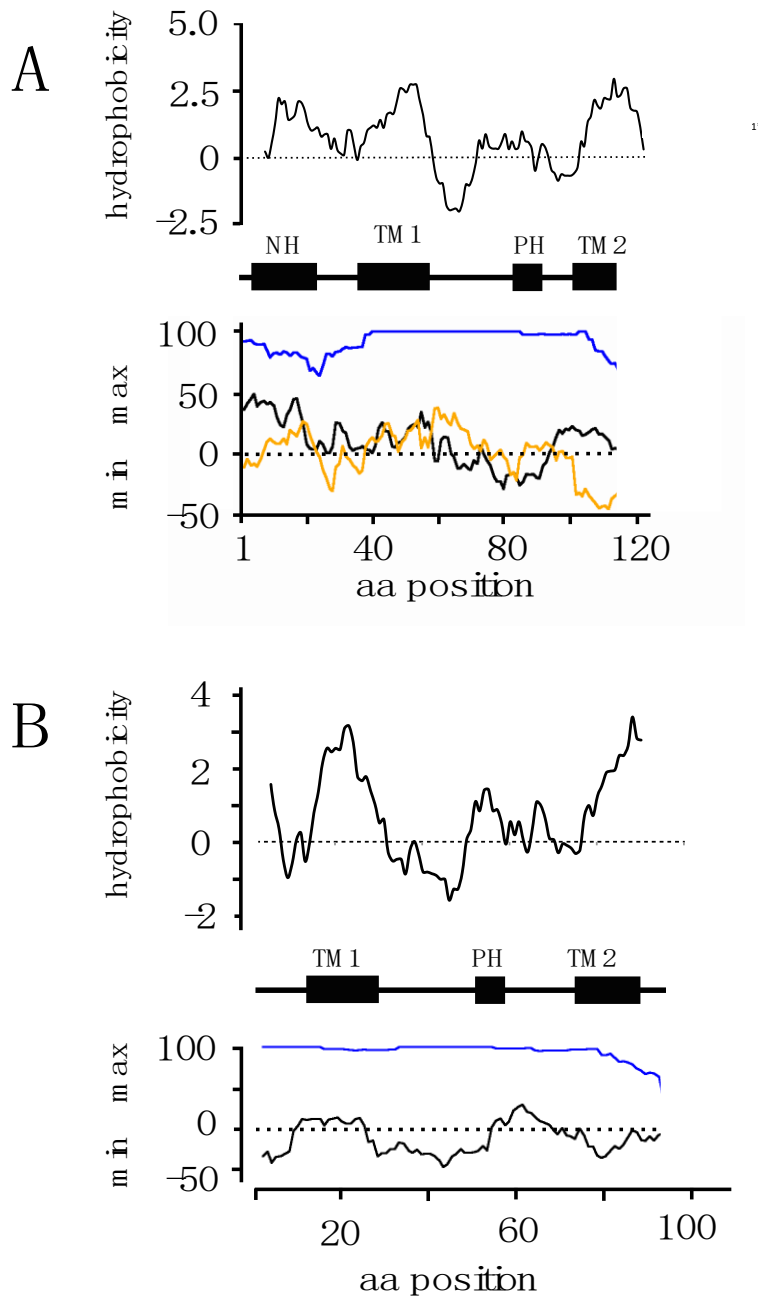
### **eGFP**

GGGATCCACCGGCCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTG  
GTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCGGGCGAGGGCGAGGGCGATGCCACCTAC  
GGCAAGCTGACCCTGAAGTTCATCTGCACCACCGCAAGCTGCCCGTGCCCTGGCCACCCCTCGTGACCACC  
CTGACCTACGCGCTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCC  
ATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAG  
GTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAAC  
ATCTTGGGGCACAAGCTGGAGTACAACACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAAC  
GGCATCAAGGTGAAGTTCAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAG  
CAGAACACCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCAGTCCGCCCTG  
AGCAAAGACCCCAACGAGAAGCGGATCACATGGTCCTGCTGGAGTTTCGTGACCGCCCGGGGATCACTCTC  
GGCATGGACGAGCTGTACAAGTAA

## Figure S1: Components of channel-linker-GFP constructs

Gene sequences of wt (wt), codon optimized (op) and a random mix of rare and non-rare codons (ran) for Kcv and Kesv channels as well as gene sequence of linkers (1 and 2) and eGFP.

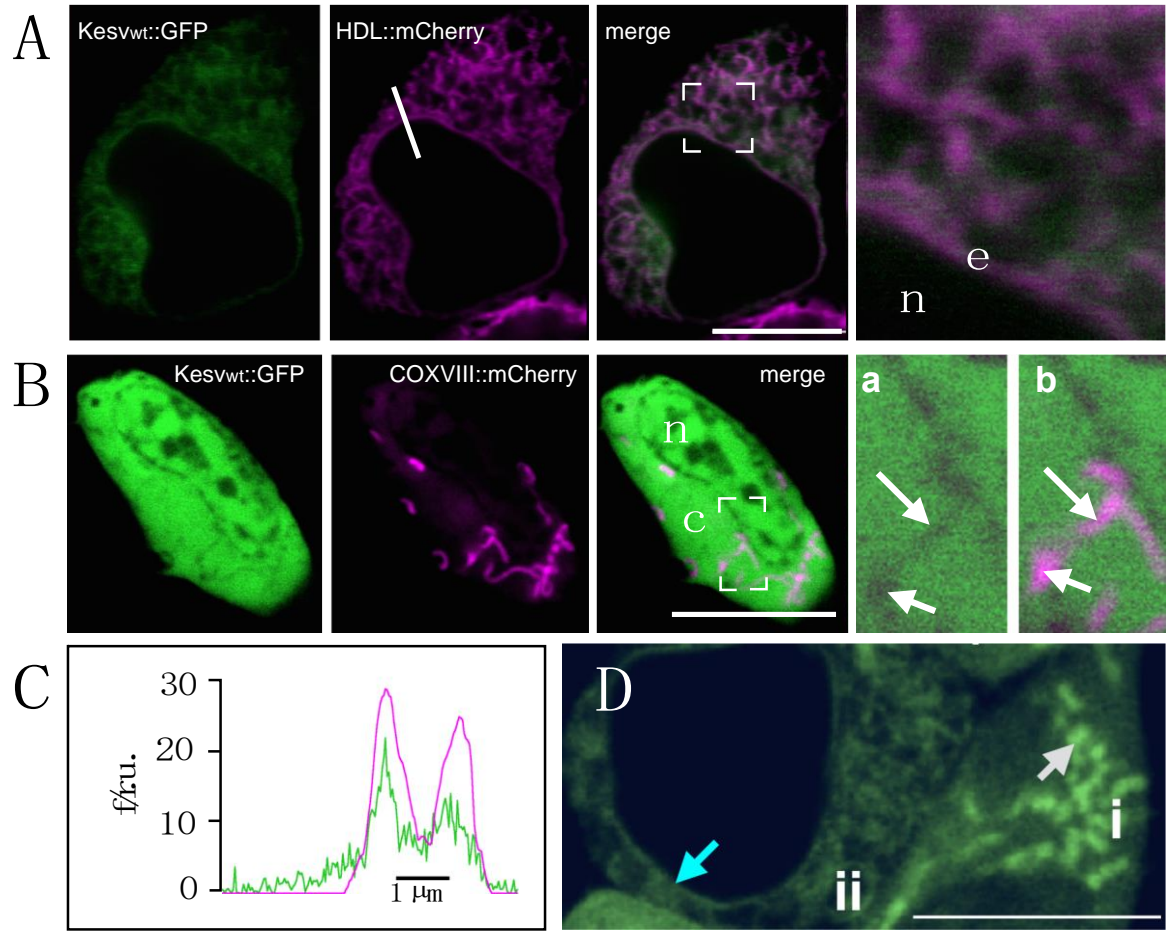
## Supplement Figure 2:



**Fig. S2: Schematic domain architectures and codon usage plots of Kesv and Kcv.**

Kyte-Doolittle plots of Kesv (**A**) and Kcv (**B**) channels (top) and predicted location of  $\alpha$ -helices including the N-terminal helix (NH), outer (TM1) and inner (TM2) transmembrane domain and pore helix (PH) (central panel). Lower panel shows calculation of mean distribution of most common (max) and least common codons (min) in human cells for wt gene (Kesv<sub>wt</sub> and Kcv<sub>wt</sub>: black) codon optimized gene (Kesv<sub>op</sub>, Kcv<sub>op</sub>: blue) and randomized gene (Kesv<sub>ran</sub>: orange) calculated with %MinMax algorithm (<http://www.codons.org/Info.html>). The plot provides information on whether the entire gene sequence for channel plus linker and GFP is composed of most common codons possible (=100%) or least common codons possible (~100%).

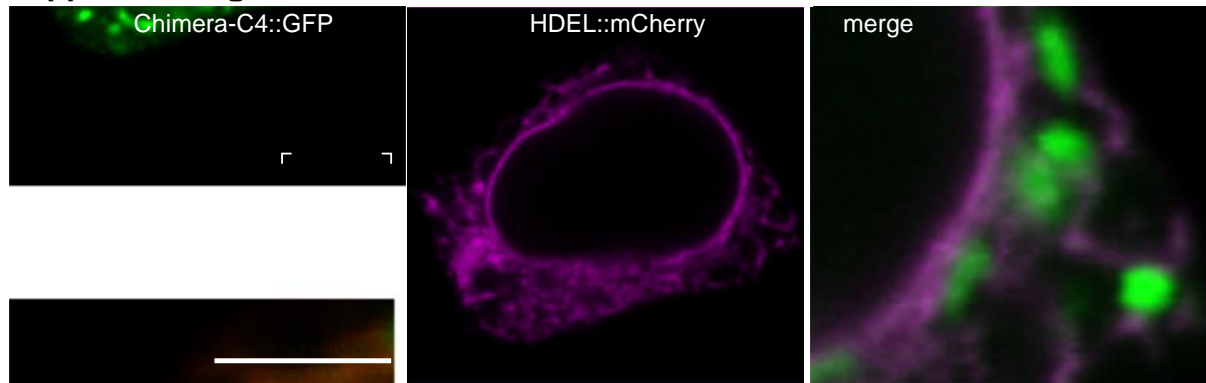
### Supplement Figure 3:



**Figure 3S. Sorting pattern of Kesv channel.**

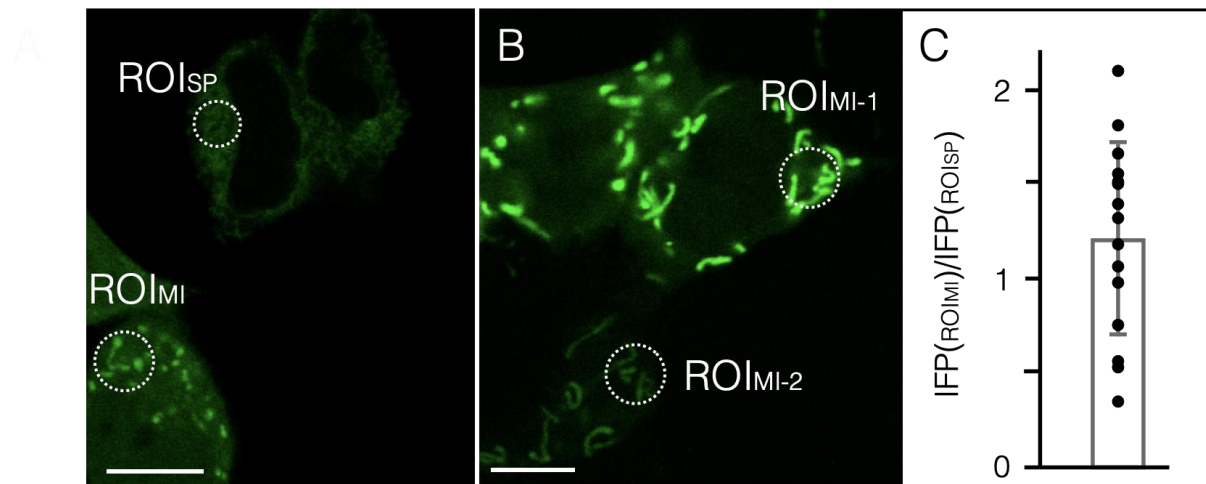
**A,B** Fluorescent images of HEK293 cells transfected with Kesv<sub>wt</sub>. Images show: fluorescence of GFP tagged Kesv channel (green, first column), fluorescence from mitochondrial marker COXVIII::mCherry or ER marker HDL::mCherry (magenta, second column) and overlay of magenta and green channels (third column). A magnification from areas marked in overlay images is shown in the fourth column. Letters in the images refer to cytosol (c), nucleus (n), mitochondria (m), and ER (e). Magnified images in 2<sup>nd</sup> row show the same section from the GFP channel only (a) and from overlay of green and magenta channel (b). Arrows indicate areas which are spared from green fluorescence (a) and which contain a signal from the magenta signal in the overlay (b). **C** reports the co-localization of green and magenta channels at position along the line shown in magenta channel in **A**. **D**. Fluorescence image of two adjacent HEK293 cells transfected with Kesv<sub>wt</sub> gene. In one cell (i) the protein is sorted to the mitochondria indicated by the white arrow. In the second cell (ii) the channel appears in the SP, which is characterized by typical net-like structure and the peri-nuclear ring (blue arrow). Scale bars: 10 μm.

#### Supplement Figure 4:



**Figure S4: Dual sorting of Chimera C4 in the same HEK293 cell into SP and mitochondria.** Fluorescent image of HEK293 cell with fluorescence from GFP-tagged channel (green, left panel) and fluorescence of ER marker HDEL::mCherry (magenta, central image). Magnification of overlay of green and magenta channels with colocalization between ER and GFP fluorescence as well as bright GFP fluorescence in mitochondria. Scale bar 10  $\mu\text{m}$ .

#### Supplement Figure 5:



**Figure S5: Comparative GFP fluorescence intensity from Kesv in adjacent HEK293 with either mitochondria or SP sorting.** **A** Fluorescent image of HEK293 cells with fluorescence from GFP-tagged Kesv channel. In one cell GFP is present in mitochondria (down) in two other cells in SP (up). Dashed circles indicate regions of interest (ROI) covering mitochondria (ROI<sub>Mi</sub>) or SP (ROI<sub>SP</sub>). **B** Same as in (A) but with cells showing Kesv in mitochondria with high (MI-1) or low (MI-2) fluorescence. **C** Ratios of integrated fluorescence density per ROI area from ROI<sub>SP</sub> and ROI<sub>Mi</sub> (IFD(ROI<sub>Mi</sub>)/IFD(ROI<sub>SP</sub>)). Mean value  $\pm$  SD and individual data from 15 cell pairs as in A (circles). Scale bars: 10  $\mu\text{m}$ .