The TRPV2 channel mediates Ca^{2+} influx and the Δ9-THC-dependent decrease in osmotic fragility in red blood cells

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Running head: TRPV2 channel in mouse and human RBCs

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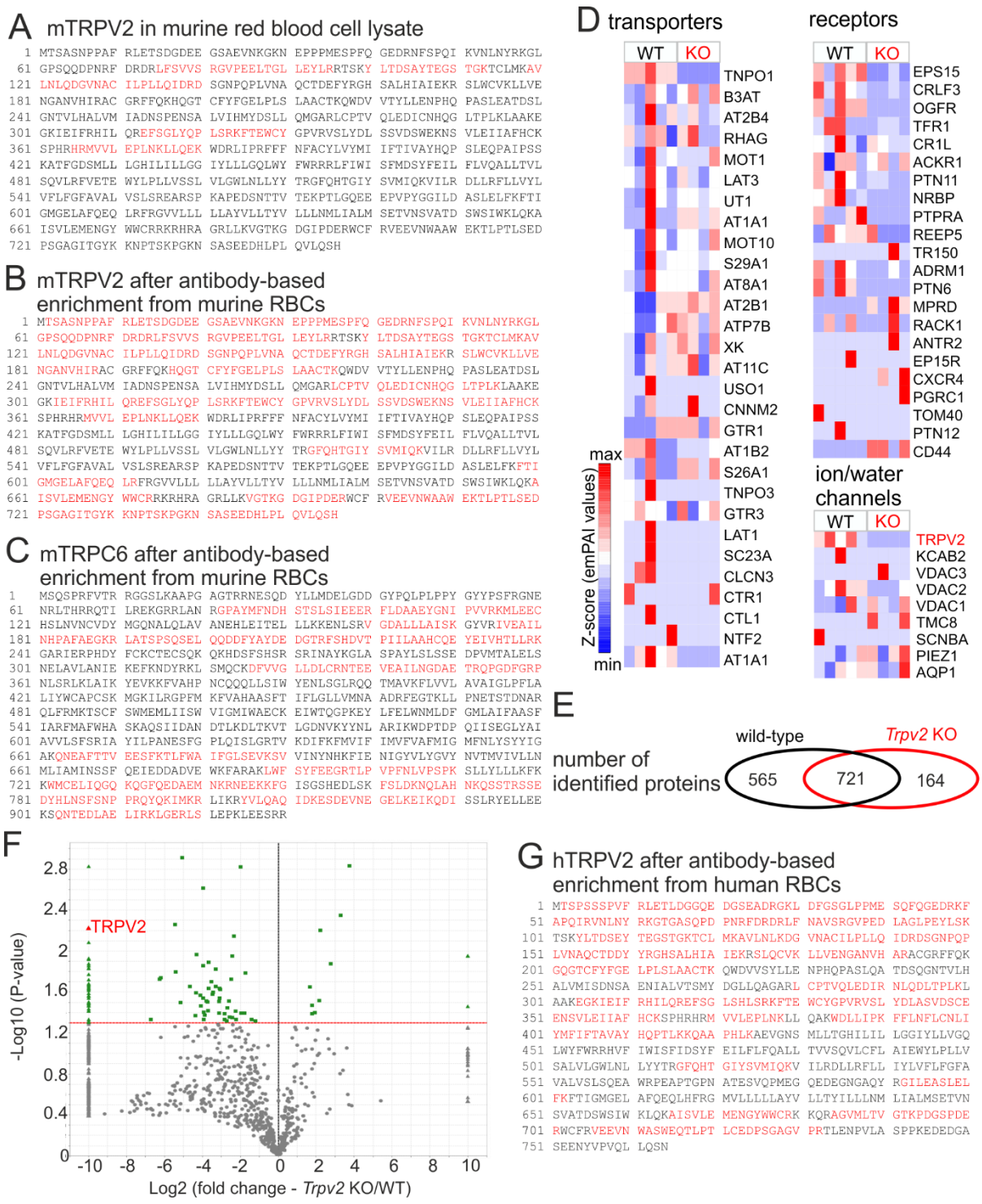
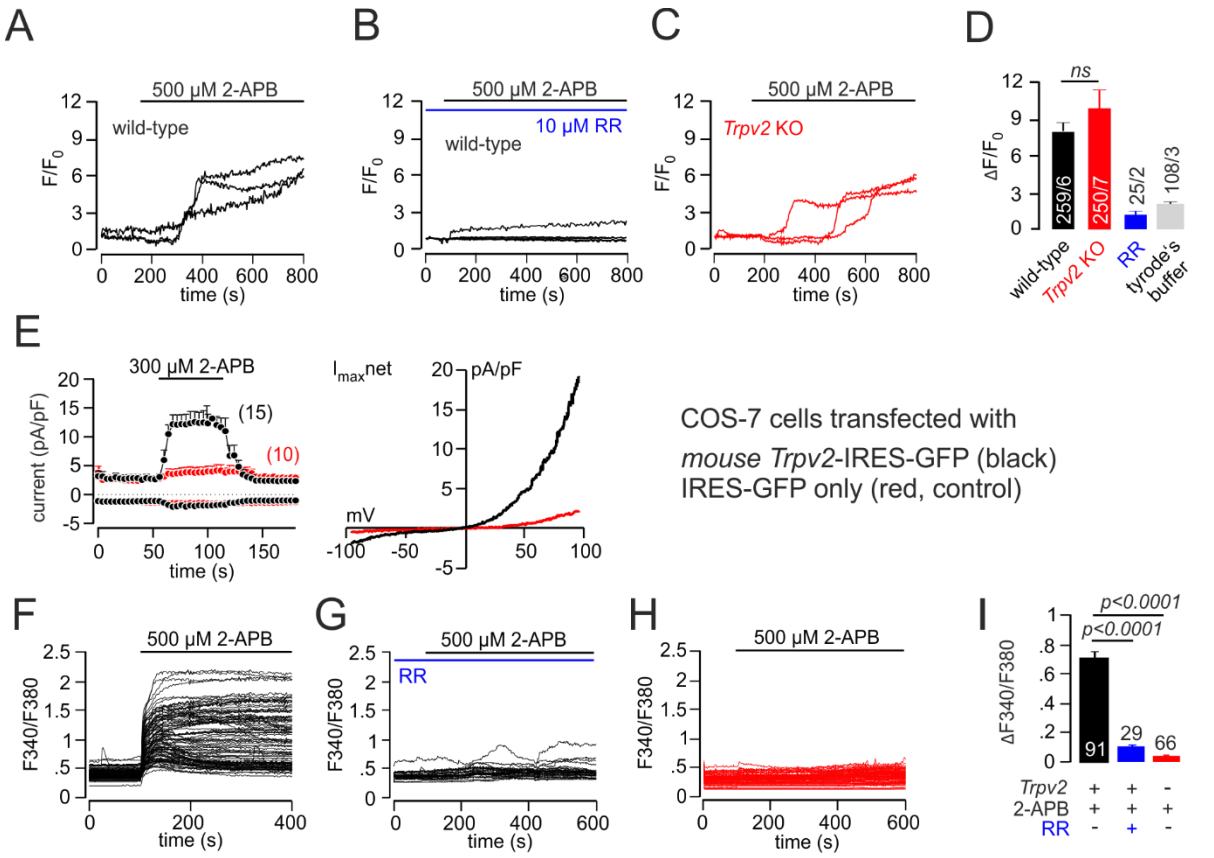


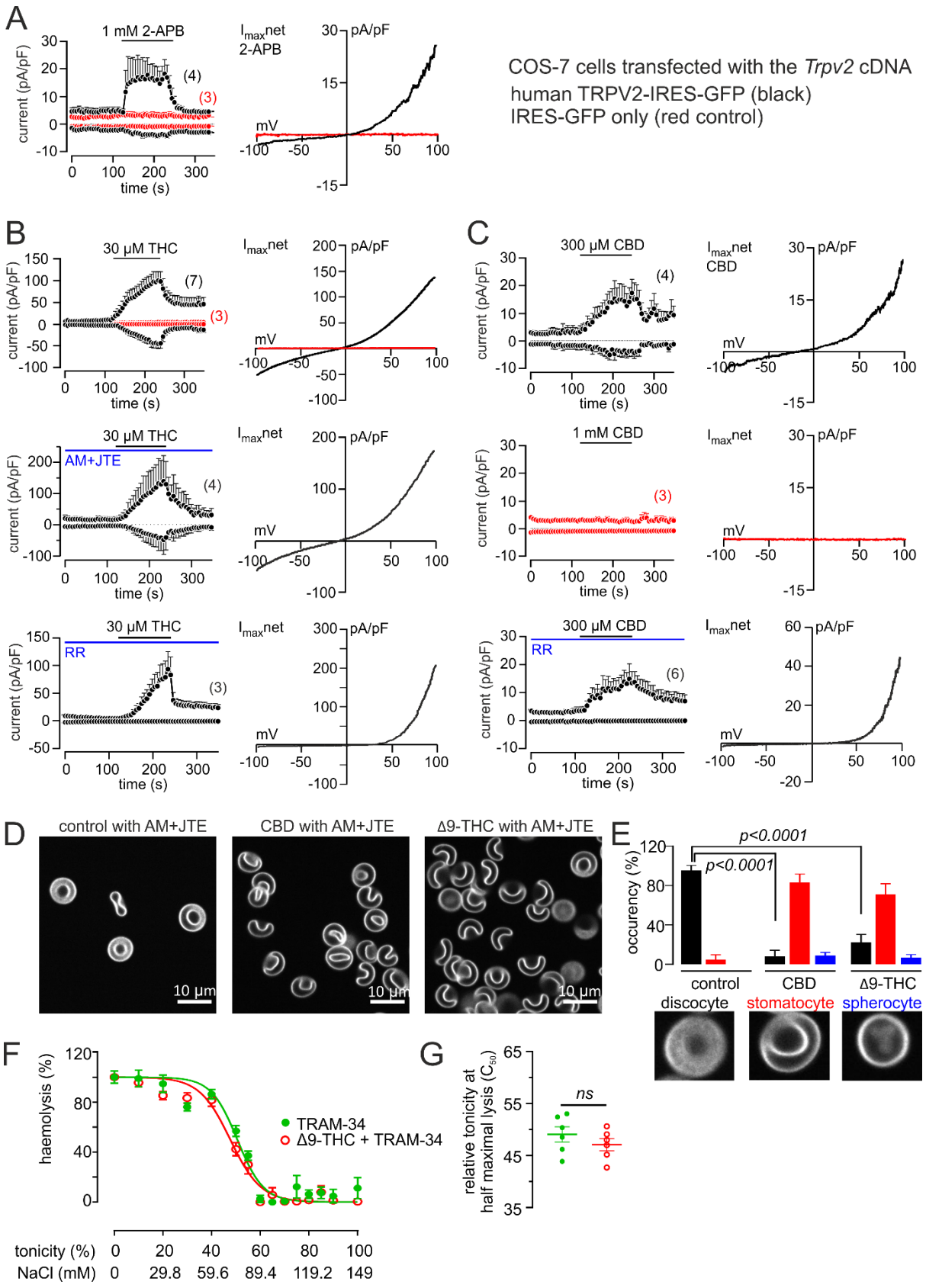
Supplemental Figure 1. Identification of TRPV2 protein in RBCs and proteome profiling by mass spectrometry (nano-LC-MS/MS). (A) Mouse TRPV2 (SwissProt: Q9WTR1) amino acid sequence coverage (red: identified tryptic peptides) as detected in RBCs lysate with nano-LC MS/MS mass spectrometry covering 13.2% of the protein sequence (black). **(B)** Mouse TRPV2 amino acid sequence coverage of 54% (red: identified tryptic peptides) as detected after antibody-based enrichment with mouse TRPV2 antibodies from mouse RBCs lysates by nano-LC MS/MS mass spectrometry (n=4). **(C)** The mouse TRPC6 protein (SwissProt: Q61143) is identified by MS/MS fragmentation covering 33.9% of the protein sequence, (red: identified tryptic peptides). **(D)** Heatmaps reporting semi-quantitative analysis of transporter,

receptor and ion channel (water channel) proteins identified by mass spectrometry in wild-type and *Trpv2* KO RBCs lysate. Heatmap shows the Z-scores of the Exponentially Modified Protein Abundance Index (emPAI) values from independent mass spectrometrical measurements from five wild-type and four *Trpv2* KO samples. Heatmaps were prepared by the "heatmap2" function from the gplots R package (https://cran.r-project.org/web/packages/gplots/). **(E)** Venn diagram showing the number of MS identified proteins in RBC lysate fractions from wild-type (n=5 experiments) and *Trpv2* KO (n=4 experiments) animals. Each experiment was done with the pooled RBCs from 2-3 individual mice. 1450 proteins were identified in both genotypes using the thresholds settings; 2 peptides/protein, protein false discovery rate FDR:0.05, peptide FDR:0.004. **(F)** The volcano plot of P-value vs. x-fold change (emPAI values, semi quantitative) summarizes the proteins differentially expressed in *Trpv2*-deficient erythrocytes. Proteins highlighted in green are significantly (unpaired two tailed Student´s t-test, P-value <0.05) upregulated (upper-right) or downregulated (upper-left) in *Trpv2* KO compared with wild-type. The TRPV2 protein was exclusively identified in each WT RBCs lysate (n=5) and not detectable in lysates from *Trpv2* KO RBCs (n=4). **(G)** Human TRPV2 amino acid sequence coverage of 54.8% (red, identified tryptic peptides) after antibody-based enrichment with the human TRPV2 antibody (n=8) by nano-LC MS/MS mass spectrometry.



Supplemental Figure 2. 2-APB-activated TRPV2 currents and Ca^{2+} entry in mouse RBCs and COS-7 cells transfected with the murine *Trpv2* cDNA. (A-C) Representative traces of cytosolic Ca^{2+} changes, detected as Fluo-4 fluorescence (F/F_0), in RBCs isolated from wild-type **(A, B)** and *Trpv2* KO mice (**C**) challenged by the application of 500 µM 2-APB in the absence **(A, C)** and presence of 10 µM ruthenium red **(**RR, **B)**. Calcium imaging experiments has been performed at 21°C in the presence of a Tyrode´s solution containing (in mM): 135 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 HEPES, 10 Glucose, and 1.8 $CaCl_2$, pH7.35. Fluo-4 fluorescence in RBCs was excited at 488 nm every 3 seconds with the emitted fluorescence detected at >515 nm. **(D)** Bar graphs summarizing the peak amplitudes ($\Delta F/F_0$) from experiments in **A-C** shown as means ± SEM. **(E)** In- and outward currents at -80 and 80 mV, respectively, measured from COS-7 cells transiently expressing mouse *Trpv2* cDNA (black traces) or GFP cDNA as control (red traces) plotted versus time (left panel). COS-7 cells were challenged by the application of 300 µM 2-APB as indicated by the bar. The corresponding current-voltage relationships (IVs) at the peak net current (I_{max} net) are depicted in the right panel. Data are shown as means (right panel) or means ± SEM (left panel). Numbers in parenthesis indicate the numbers of measured cells. **(F-H)** Cytosolic Ca^{2+} changes, detected with Fura-2 (F340/F380), in COS-7 cells (loaded with 5 µM Fura-2) expressing mouse *Trpv2* cDNA **(F**, **G)** and GFP cDNA as control **(H)** in the absence (**F**, **H)** or presence of 30 µM ruthenium red **(G)**, challenged by the application of 500 µM 2-APB (black bars). **(I)** Bar graph showing the means ± SEM of the peak amplitudes ($\Delta F340/F380$) from experiments as in **F-H**. The P-values are calculated using one-way ANOVA, followed by a Bonferroni's multiple comparison test. Numbers in the bars indicate the numbers of measured cells. Note: 2-APB blocks TRPC6[1] and KCa3.1[2] present in RBCs

and acts on additional targets[3-6]. Thereby it may affect the RBC membrane potential and Ca^{2+}-signalling pathways independent of TRPV2 during monitoring cytoplasmic Ca^{2+}. As shown in COS-7 cells (*Figure S2E-I and S3A)*, which do not endogenously express TRPC6 and KCa3.1, the 2-APB-induced increase in cytosolic Ca^{2+} and plasma membrane currents required the presence of over-expressed mouse or human TRPV2. (Other targets of 2-APB including TRPV1, TRPV3, TRPV4, TRPV6, TRPC2, TRPC3, TRPC5, TRPM2, TRPM3, TRPM7.)



Supplemental Figure 3. CBD- and Δ9-THC-mediated currents in COS-7 cells transfected with the human *Trpv2* cDNA, and shape and haemolysis of human RBCs. (A, B, C, left panels**)** In- and outward currents at -80 and 80 mV, respectively, measured from COS-7 cells

transiently expressing human TRPV2 cDNA (black traces) or GFP cDNA (red traces) as control plotted versus time. Cells were challenged by the application of 1 mM 2-APB **(A)**, 30 µM Δ9-tetrahydrocannabinol **(**Δ9-THC, **B)** in the absence **(B**, top**)** or presence of either CB1/CB2-receptor antagonists AM251 and JTE907 **(**100 nM each, **B**, middle**)** or 10 µM ruthenium red **(B**, bottom**)**, 300 µM cannabidiol **(**CBD, **C)** in the absence **(C**, top**)** or presence of 10 µM ruthenium red **(C**, bottom**). (A, B, C,** right panels**)** Corresponding current-voltage relationships (IVs) at the peak net currents (I_{max} net) from the left panels. Data are shown as means (right panels) and means ± SEM (left panels). Numbers in parenthesis indicate the numbers of measured cells. **(D, E)** Confocal microscopic images of human RBCs in the presence of the CB1- and CB2-receptor antagonists AM251 and JTE907 (both 100 nM) either non-treated (control, left) or treated with 100 µM CBD (middle) or 30 µM Δ9-THC (right). RBCs were stained with CellMask deep red at a concentration of 5 µg/ml in Tyrode buffer for 30 min and placed into polymeric coverslips. **(E)** Bar graphs showing the percentage of discocytes (black), stomatocytes (red) and spherocytes (blue) in RBCs as means + SEM, from three independent healthy donors. P-values were calculated using one-way ANOVA, followed by Bonferroni's multiple comparison. The classification was done with 3-D stacks of confocal images. **(F)** Haemolysis (%) of human RBCs in buffer A (149 mM NaCl, 2 mM $CaCl_2$, 4 mM KCl, 2 mM HEPES, pH7.4), treated with 2 µM TRAM-34 (green) or 30 µM Δ9-THC plus TRAM-34 (red) for 30 min, after 26-fold dilution in buffer B (0-149 mM NaCl, 2 mM HEPES, pH 7.4) as indicated; extracellular [Ca^{2+}] was kept at 76 µM.**(G)** Tonicity at which 50% lysis occurred (C_{50}), calculated by sigmoidal fitting of the individual experiments in **F**. Single values are shown and the mean ± SEM from 2 independent experiments performed in triplicates with P-value calculated by unpaired two-tailed Student's t-test. Note: Cannabinoids found in plants such as cannabidiol (CBD) and Δ9-tetrahydrocannabinol (Δ9-THC) have been described as activators of TRPV2.[7-9]

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