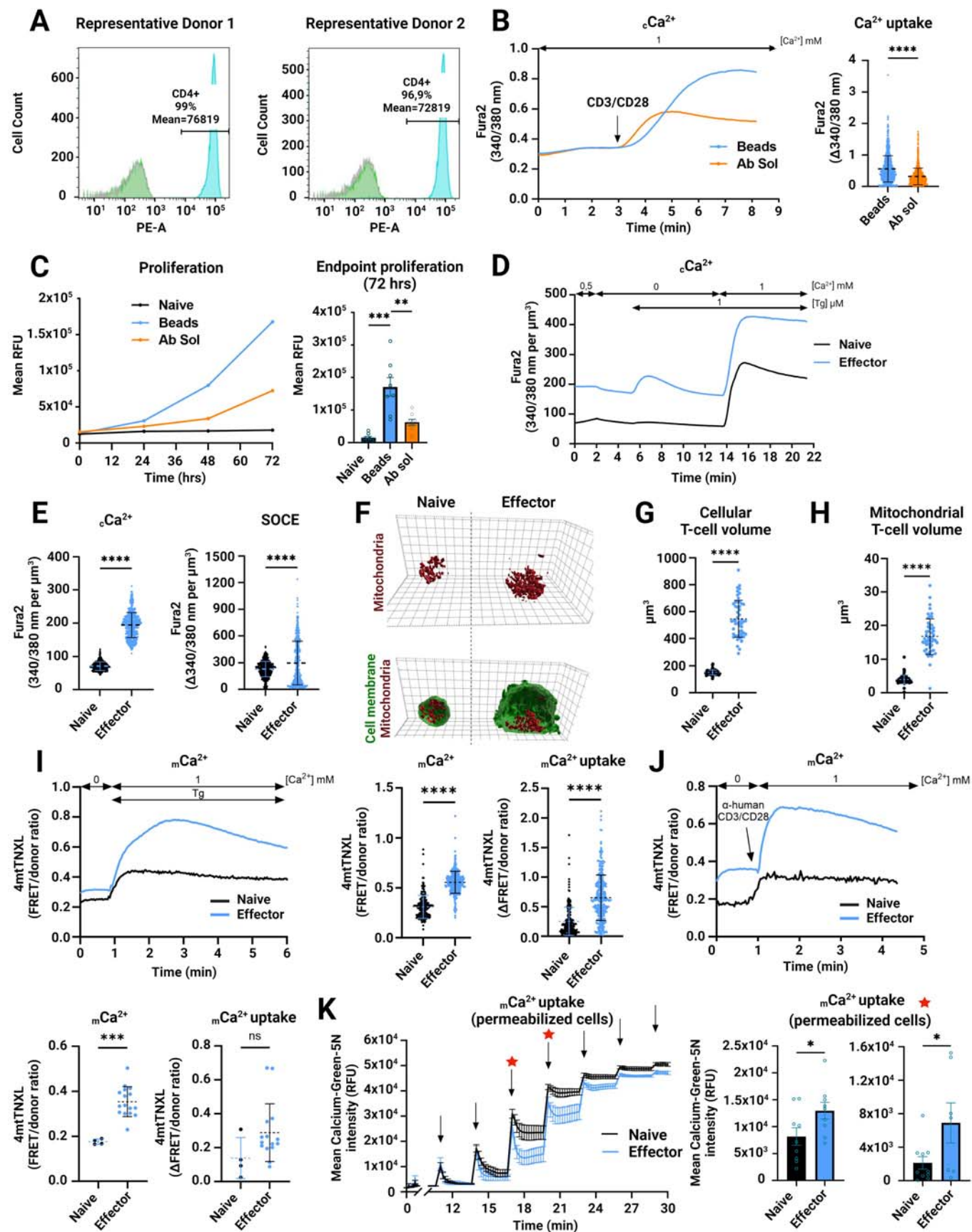


Expanded View Figures

Figure EV1. Cytosolic and mitochondrial Ca^{2+} levels are increased upon T-cell activation.

(A) Pure CD4^{+} naive T-cell population isolated from blood of healthy human donors. Naive T-cells mainly exhibited positive CD4 (PE-A) staining, and not CD8. 99% and 96.9% of cells of from donor 1 (left) and donor 2 (right) were CD4^{+} , respectively. The histograms were generated from 10,000 events after gating. The gating strategy for flow cytometry is shown in Appendix Fig. S1. Legend: Grey=unstained control; Green=CD8 staining; Blue = CD4 staining. (B) Fura-2 measurements of Ca^{2+} in naive CD4^{+} T-cells treated with anti-CD3/CD28-coated magnetic beads (1:5 bead:cell ratio) or antibody solution (1:1000 working solution). Quantification shown as mean $\pm$ SD of 1479 bead-activated and 1,656 antibody solution-activated T-cells/biological replicates from 5 healthy donors. **** $p < 0.0001$ ($p = 1.34872\text{E}-74$), assessed by two-tailed unpaired Student's t-test. (C) Naive T-cell proliferation following beads or antibody solution stimulation. Quantification shown as mean $\pm$ SEM of 8 healthy donors/biological replicates. *** $p < 0.001$ ($p = 0.0006$); ** $p < 0.01$ ($p = 0.0039$), assessed by two-tailed paired Student's t-test. RFU = relative fluorescence units. (D) Fura-2 measurements of Ca^{2+} in naive and effector T-cells. The SERCA-blocker thapsigargin (Tg; 1 μM) was used to deplete ER Ca^{2+} stores and activate SOCE. (E) Quantification of data given in (D). Data show mean $\pm$ SD of 768 naive T-cells and 577 effector T-cells/biological replicates from 3 healthy donors, normalised to their respective cell volume (in μm^3) as shown in (G). **** $p < 0.0001$ ($p = 2.73994\text{E}-12$), assessed by two-tailed unpaired Student's t-test. (F) Representative 3D images of a naive (left) and an effector (right) T-cell. Using pixel classification in Imaris v10.1.0, the cell surface (green) and the mitochondria (red) were created (refer to methods for more details). Quantification of cellular (G) and mitochondrial (H) volume in naive and effector T-cells showing a mean $\pm$ SEM of 66 naive and 52 effector T-cells/biological replicates from 3 healthy donors. Cell volume was quantified by using Machine Learning-based segmentation. **** $p < 0.0001$ ($p = 2.40289\text{E}-45$) and ($p = 2.3739\text{E}-37$), assessed by two-tailed unpaired Student's t-test. (I) Measurements of mCa^{2+} levels with the 4mtTNXL biosensor in human naive and effector T-cells. Quantification plots show an average of 118 naive and 331 effector cells/biological replicates from 2 different donors. Cell stimulation was achieved by addition of 1 μM Tg at the indicated time point. The quantification/violin plots show mean $\pm$ SD of basal mCa^{2+} levels and maximal mCa^{2+} uptake. **** $p < 0.0001$ (Basal $p = 1.6523\text{E}-33$; Uptake $p = 8.3596\text{E}-16$), assessed by two-tailed unpaired Student's t-test. (J) Measurements of mCa^{2+} levels with the 4mtTNXL biosensor in human naive and effector T-cells. Traces show an average of 4 naive and 16 effector cells/biological replicates from 1 healthy donor. Cells were stimulated with anti-human CD3/CD28-coated beads at the indicated time point. The quantification/violin plots show mean $\pm$ SD for both the basal mCa^{2+} levels and mCa^{2+} uptake. *** $p < 0.001$ ($p = 0.0004$). (K) Calcium Retention Capacity (CRC) assay measuring mCa^{2+} uptake in digitonin-permeabilized naive and effector T-cells using Calcium-Green-5N. Traces show mean $\pm$ SEM relative fluorescence units (RFU) of 10 naive and 10 effector T-cells/biological replicates from 3 healthy donors (assays always performed at least in duplicate). mCa^{2+} uptake was induced by subsequent additions of a bolus of Ca^{2+} at the indicated time points (black arrows). The quantification plots show mean $\pm$ SEM for basal mCa^{2+} uptake at the time points indicated by a red star. mCa^{2+} uptake was quantified as the delta decrease in measured RFU upon Ca^{2+} addition. * $p \leq 0.05$ ($p = 0.0481$ -left; $p = 0.0443$ -right panel), assessed by two-tailed unpaired Student's t-test.



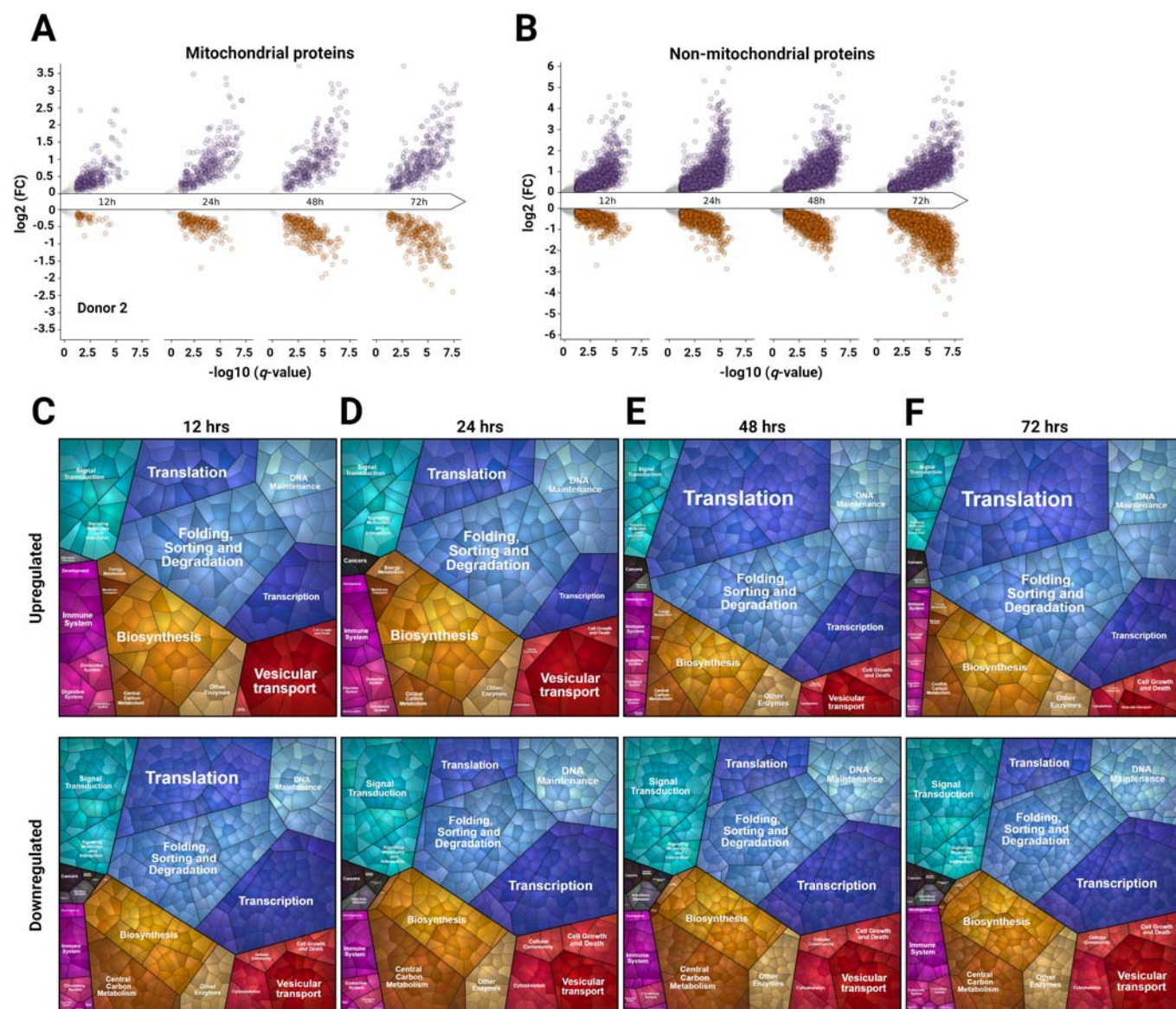


Figure EV2. Activation of naive human T-cells causes time-dependent proteome alterations.

Proteomics and bioinformatic analyses of bead-activated T-cells for 12, 24, 48, and 72 h. The volcano plots (A, B) show significantly up- (purple) and downregulated (orange) proteins as compared to naive T-cells. (C–F) Proteomap-based evaluation of signalling pathways and cellular functions regulated by significantly up- (upper panels) and downregulated (lower panels) proteins following bead-induced T-cell activation (from 12 to 72 h). Proteomaps are based on differentially-expressed proteins (DEPs) depicted in Fig. 3F.

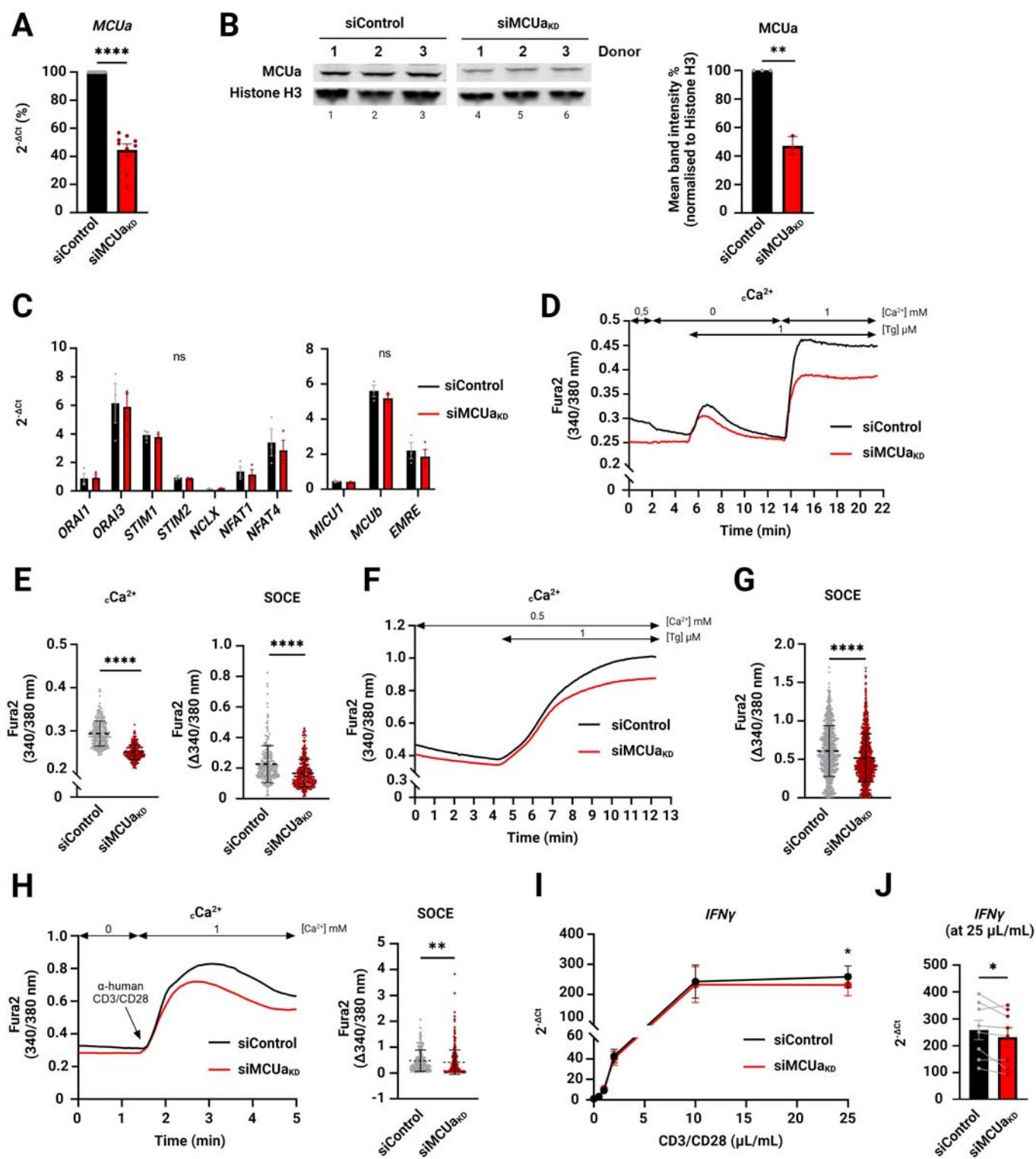


Figure EV3. MCUa_{KD} affects human effector T-cell function.

(A) RT-qPCR-based evaluation of transient MCUa knockdown efficiency in human CD4⁺ effector T-cells. The quantified graph shows mean $\pm$ SEM of 9 healthy donors/biological replicates, as compared to control transfection. **** $p < 0.0001$ ($p = 3.89251E-13$), assessed by two-tailed paired Student's t-test. (B) Immunoblot-based evaluation of transient MCUa knockdown efficiency in human CD4⁺ effector T-cells. Quantification shows mean $\pm$ SEM from 3 healthy donors/biological replicates as compared to control transfection, normalised to Histone H3. ** $p < 0.01$ ($p = 0.0033$), assessed by two-tailed paired Student's t-test. (C) RT-qPCR-based evaluation of Ca²⁺ signalling-related genes and MCU complex components in effector T-cells with transient knockdown of *MCUa*. Quantifications show mean $\pm$ SEM from 3 healthy donors/biological replicates for all assessed genes. ns - not significant, assessed by two-tailed paired Student's t-test. (D, E) Measurements of Ca^{2+} levels using Fura2 in control and siMCUa_{KD} effector T-cells. Quantification shows mean $\pm$ SD of 648 control and 739 siMCUa_{KD} T-cells/biological replicates from 3 healthy donors. Tg = 1 μM . **** $p < 0.0001$ (Basal $p = 8.86452E-78$; SOCE $p = 3.26694E-11$), assessed by two-tailed unpaired Student's t-test. (F, G) Measurements of Ca^{2+} levels using Fura2 in siMCUa_{KD} effector T-cells. Quantification shows mean $\pm$ SD of 848 control and 930 siMCUa_{KD} T-cells/biological replicates from 2 healthy donors. Tg = 1 μM . **** $p < 0.0001$ ($p = 5.75699E-46$), assessed by two-tailed unpaired Student's t-test. (H) Measurements of Ca^{2+} levels using Fura2 in control and siMCUa_{KD} effector T-cells stimulated with anti-human CD3/CD28-coated beads. Quantification shows mean $\pm$ SD of 169 control and 365 siMCUa_{KD} T-cells/biological replicates from 2 healthy donors. ** $p < 0.01$ ($p = 0.0033$), assessed by Student's t-test. (I, J) *IFN γ* mRNA levels in re-stimulated siMCUa_{KD} T-cells. The quantification (at 25 $\mu\text{L/mL}$ Ab solution) shows mean $\pm$ SEM of 8 healthy donors/biological replicates. * $p \leq 0.05$ ($p = 0.0457$), assessed by two-tailed paired Wilcoxon test. Source data are available online for this figure.

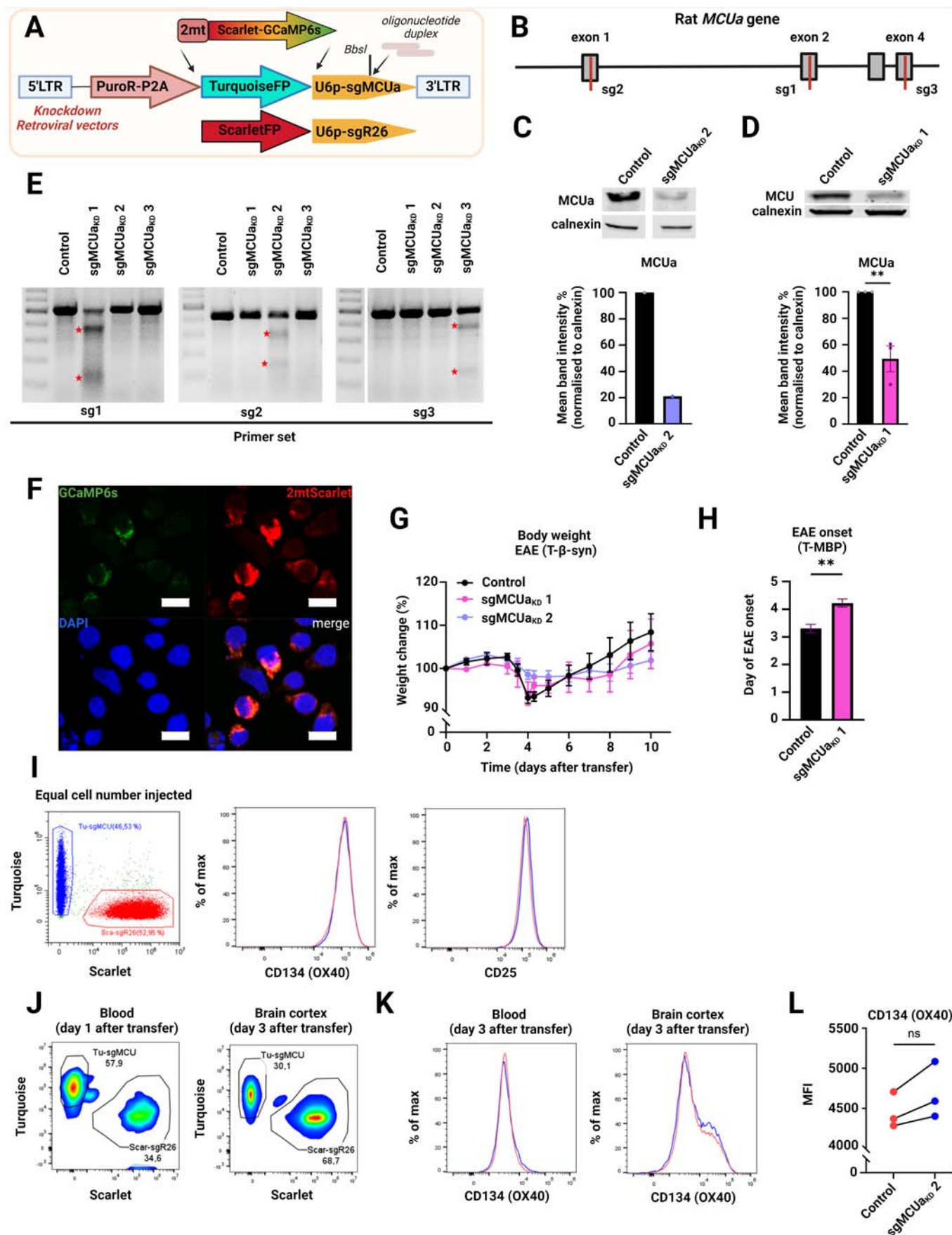


Figure EV4. Establishment of MCU_{aKD} models in rat effector T-cells.

(A) Schematic representation of retroviral constructs used to establish control and $sgMCU_{aKD}$ rat effector T-cell lines. LTR – long terminal repeats; PuroR – puromycin resistance gene; U6p – U6 promoter; 2mt – tandem mitochondrial localisation signals. (B) Schematic representation of the rat $MCUa$ gene and the position of protospacers targeted by single guide RNAs (sg; the numbering of exons starts from the first coding exon) (Images (A) and (B) created with BioRender). (C, D) Immunoblot-based evaluation of knockdown efficiency in $sgMCU_{aKD}$ 2 (C) and $sgMCU_{aKD}$ 1 (D) cell lines. Protein expression data are normalised to the loading control calnexin. Quantification shows mean $\pm$ SEM of 3 experiments/biological replicates. $**p < 0.01$ ($p = 0.0065$), assessed by two-tailed unpaired Student's t-test, in (D). (E) PCR-based T7 endonuclease cleavage confirmed the intended editing of the respective sequence in the different $sgMCU_{aKD}$ cell lines. T7 cleavage fragments, marked by a star, revealed sequence alterations (indels) introduced by Cas9 at a genomic level. (F) Confocal images of DAPI-stained $sgMCU_{aKD}$ 1 T-cells transduced by Puro2A-2mtScarlet-GCaMP6s retrovirus show proper mitochondrial localisation of the biosensor 2mtScarlet-GCaMP6s. Scale bar: 10 μ m. (G) Body weight change curves in animals during transfer EAE experiments (related to Fig. 6H). Data show mean $\pm$ SEM, as percentage of initial weight on day 0 ($n = 4$ animals for the control group and $n = 3$ animals for the $sgMCU_{aKD}$ 1 and 2 groups). (H) Inactivation of $MCUa$ results in a delayed EAE onset in rats injected with MBP-specific effector T-cells. Data are represented as mean $\pm$ SEM of 3 animals/biological replicates per condition. $**p < 0.01$ ($p = 0.0018$), assessed by Mann-Whitney U test. (I) (related to Fig. 7). An equal number of different fluorescently-labelled control (Scarlet fluorescent protein) and $sgMCU_{aKD}$ (Turquoise fluorescent protein) T-cells were co-transferred in Lewis rats. Both cell lines that were injected showed a similar activation state assessed by CD134 (OX-40) and CD25 expression using flow cytometry. (J) Ex vivo-isolated T-cells were analysed at different time points after co-transfer using flow cytometry. At day 1 after co-transfer, $sgMCU_{aKD}$ T-cells (57.9%) outnumbered the control cells (34.6%) in the blood. At day 3 after co-transfer, the control T-cells (68.7%) outnumbered the $sgMCU_{aKD}$ cells (30.1%) in the brain. (K) $sgMCU_{aKD}$ T-cells that have succeeded to enter the CNS show comparable activation as those cells in the blood, determined by CD134 (OX-40) expression. (L) OX-40 expression of control and $sgMCU_{aKD}$ T-cells in the brain at day 3 after co-transfer. Quantification shows mean fluorescence intensity (MFI) of CD134 from 3 rats/biological replicates. ns – not significant, assessed by unpaired Student's t-test. Source data are available online for this figure.

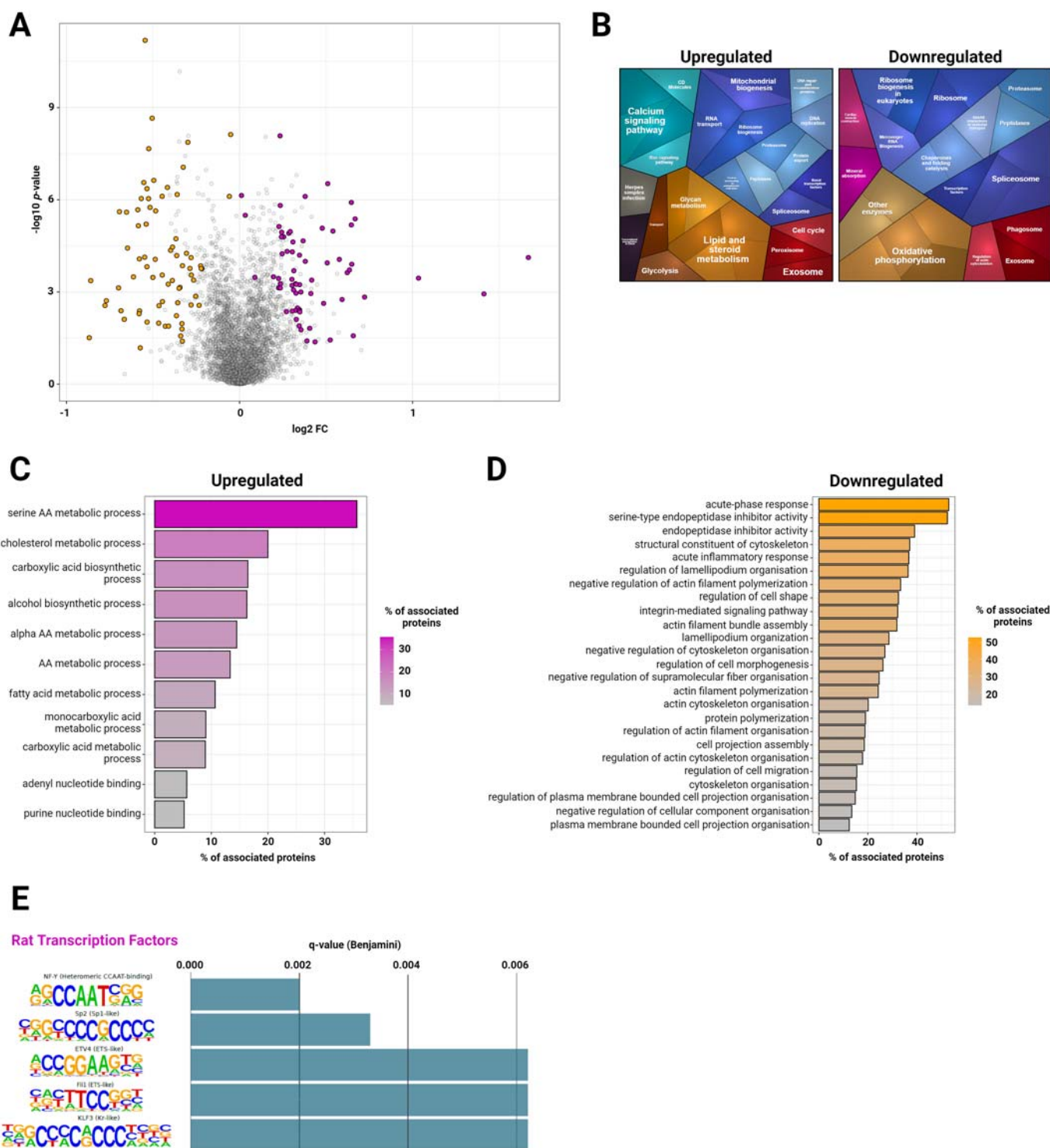


Figure EV5. MCu_aKD in rat effector T-cells alters their proteome.

Proteomics data generated from the two sgMCu_aKD cell lines and the control rat T-cell line. (A) The volcano plot shows multiple significant up- (magenta) and downregulated (orange) proteins in the two sgMCu_aKD cell lines, compared to control, analysed by ANOVA (5% FDR). (B) Proteomaps showing the quantitative composition of proteomes with a high probability to be significantly up- (left panel) and downregulated (right panel) upon MCu_a knockdown in rat effector T-cells. Proteomaps are based on DEPs shown in (A). (C, D) Significant up- (C) and downregulated (D) cellular functions and signalling pathways in sgMCu_aKD cells ordered by percentage of associated proteins, based on DEPs shown in (A). (E) HOMER analysis of the proteomic data showing the top five most enriched motifs (TFs). Analysis is based on *q*-values (Benjamini-Hochberg-C).