

Supplementary Material

Figure S1. Rotenone treatment does not affect cellular viability during Th cell differentiation.

We used 7-Aminoactinomycin (7-AAD) staining to determine cellular viability of CD4 T cells left untreated or treated with 20 μ M rotenone for 2 hours and then stimulated with plate-bound anti-CD3 ϵ plus anti-CD28 for 24, 48, 72, and 96 hours in the presence of specific Th cell polarization conditions. Viability is shown for (A) Th1 cells, (B) Th2 cells, (C) Th17 cells, and (D) iTreg cells. Data represent the mean + SEM of three independent replicates.

Figure S2. Rotenone treatment does not affect Notch1 expression in Th1 and Th2 cells. Notch1 levels were measured in Th cells, using flow cytometric approaches. CD4 T cells were left untreated or treated with 20 μ M rotenone for 2 hours and then stimulated with plate-bound anti-CD3 ϵ plus anti-CD28 for 24, 48, 72, and 96 hours in the presence of specific Th cell polarization conditions. At the indicated timepoints, cells were harvested and we determined the (A) percent Notch1-positive and (B) Notch1 MFI in Th1-polarized cells, and the (C) percent Notch1-positive, and (D) Notch1 MFI in Th2-polarized cells.

Figure S3. Rotenone reduces mitochondrial mass in Th1 and Th2 cells and alter mitochondrial Notch1 localization only in Th2 and iTreg cells. CD4 T cells were left untreated or treated with 20 μ M rotenone for 2 hours and then stimulated with plate-bound anti-CD3 ϵ plus anti-CD28 for 24, 48, 72, and 96 hours in the presence of specific Th cell polarization conditions. We stained mitochondria for each cell type by using Mitotracker Red CMXRos and measured the intensity by imaging flow cytometry. Mitochondrial mass was determined by the median fluorescent intensity of Red CMXRos for DMSO control and rotenone-treated cells under (A) Th1-polarized conditions at 72 hours after stimulation, (B) Th2-polarized conditions at 48 hours after stimulation, (C) Th1 and Th2 cell frequency graphs and representative histograms that have colocalization of Notch1 at the mitochondria with their corresponding colocalization scores determined by AMNIS software. (D) Representative cell images of Notch1-mitochondrial colocalization in Th1 and Th2 cells. (E) Notch1 distribution in subcellular compartments in four different Th cell subsets calculated by AMNIS software. Data represent the mean + SEM of three independent experiments. * p <0.05; ** p <0.01, *** p <0.001 calculated using an unpaired, two-tailed Student's t -test.

Figure S4. Expression of master transcription factors for Th1 and Th2 cell differentiated in the absence or presence of rotenone. CD4 T cells were left untreated or treated with 20 μ M rotenone for 2 hours and then stimulated with plate-bound anti-CD3 ϵ plus anti-CD28 for 24, 48, 72, and 96 hours in the presence of specific Th cell polarization conditions. (A) Percent T-bet-positive and (B) MFI of T-bet expression in Th1-polarized cells. and (C) percent GATA3-positive and (D) MFI of GATA3 expression in Th2-polarized cells was measured by flow cytometry. Data are the mean + SEM of three independent replicates.

Figure S5. Protein distribution profiles of Th-specific master transcription factors after rotenone treatment. CD4 T cells were left untreated or treated with 20 μ M rotenone for 2 hours and then stimulated with plate-bound anti-CD3 ϵ plus anti-CD28 for 24, 48, 72, and 96 hours in the presence of specific Th1 or Th2 cell polarization conditions. We determined the median fluorescent intensity of specific master transcription factors both within (Nuclear MFI) and outside of the nucleus (Non-Nuclear MFI) by Imaging Flow Cytometry using AMNIS IDEAS software. We calculated the percent of total T-bet protein within and outside of the nucleus using the following equations, respectively: [Nuclear MFI] / [Total MFI] x 100 and [Non-Nuclear MFI] / [Total MFI] x 100. (A) Percent of T-bet protein with representative histograms and images for T-bet nuclear localization in Th1-polarized cells and for (B) GATA-3 nuclear localization in Th2-polarized cells. (C) Th1 and Th2 cell frequency graphs and representative histograms that have localization of Notch1 in the nucleus. (D) Representative cell images of Notch1 nuclear colocalization in Th1 and Th2 cells. Data represent the mean + SEM of three independent experiments. * p <0.05; ** p <0.01, *** p <0.001 calculated using an unpaired, two-tailed Student's t -test.

Figure S6. Rotenone treatment alters colocalization of Notch1 with Th master transcription factors. CD4 T cells were left untreated or treated with 20 μ M rotenone for 2 hours and then stimulated with plate-bound anti-CD3 ϵ plus anti-CD28 for 24, 48, 72, and 96 hours in the presence of specific Th1, Th2, Th17, or iTreg cell polarization conditions. We determined the median fluorescent intensity of Notch1 or of specific master transcription factors both within (Nuclear MFI) and outside of the nucleus (Non-Nuclear MFI) by Imaging Flow Cytometry using AMNIS IDEAS software. Cell frequency data, cumulative histograms, and colocalization scores with representative images for (A) Notch1-Tbet colocalization in Th1 cells after 72 hours of differentiation, for (B) Notch1-GATA3 colocalization in Th2 cells after 48 hours of differentiation, for (C) Notch1-ROR γ t colocalization in Th17 cells after 72 hours of differentiation, and for (D) Notch-FOXP3 colocalization in iTreg cells after 48 hours of differentiation. Data represent the mean + SEM of three independent experiments. * p <0.05; ** p <0.01, *** p <0.001 calculated using an unpaired, two-tailed Student's t -test.

Figure S7. Effects of rotenone in PDHK1-ROR γ t-Notch1 axis in Th17-polarized cells. CD4 T cells were treated either with 20 μ M rotenone for 2 hours or 1 mM DCA (left in the cell suspension throughout) and then stimulated with plate-bound anti-CD3 ϵ plus anti-CD28 for 72 hours in the presence of Th17 polarization conditions. Cells were stained for PDHK1, total PDH-E1 α , pPDH-E1 α (Ser232), ROR γ t, and Notch1 to determine their localization in the cytosol (Tubulin AF647 staining) and mitochondria (Mitotracker CMXRos). Data were acquired using an AMNIS ImageStream X Mark Imaging Flow Cytometer. Frequency histograms, along with their localization scores, are shown for cytosolic and mitochondrial (A) PDHK1, (B) Total PDH-E1 α , and (C) pPDH-E1 α (Ser232) localization. For colocalization, frequency histograms, along with their localization scores, are shown for (D) Notch1 + pPDH-E1 α (Ser232), (E) ROR γ t + pPDH-E1 α (Ser232), and (F) ROR γ t + Total PDH-E1 α colocalization. (G) Percent of ROR γ t + Total-PDH-E1 α colocalizing Th17-polarized cells for each treatment condition and representative images. Data represent the mean + SEM of three independent experiments.

Figure S8. Graphical representation of PDHK1-ROR γ t-Notch1 localization in Th17-polarized cells under different treatment conditions. (A) Notch1 and ROR γ t colocalize in the nucleus during Th17 differentiation. The metabolic enzyme, PDHK1, and its non-phosphorylated substrate, PDH-E1 α , are found primarily in the mitochondria in Th17 cells. (B) Rotenone treated Th17 cells show, decreased nuclear Notch1 and ROR γ t that is redistributed to non-nuclear compartments, where it can be found in association with pPDH-E1 α . (C) When Th17 cells are treated with the PDHK1 inhibitor, DCA, Notch1 shows increased association with pPDH-E1 α in the cytosol and at the mitochondria, however, this same effect is not seen with ROR γ t.

Figure S1

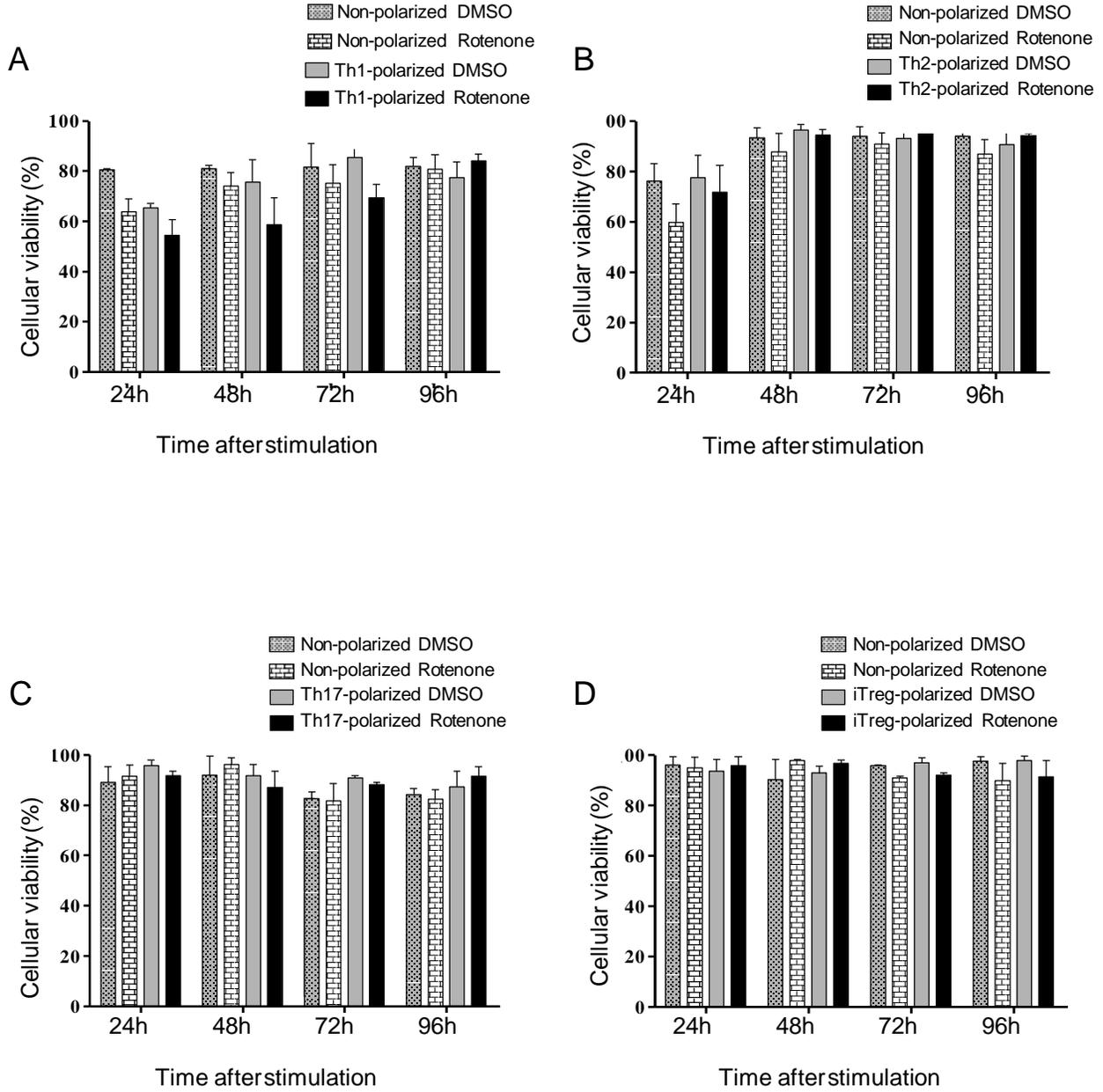
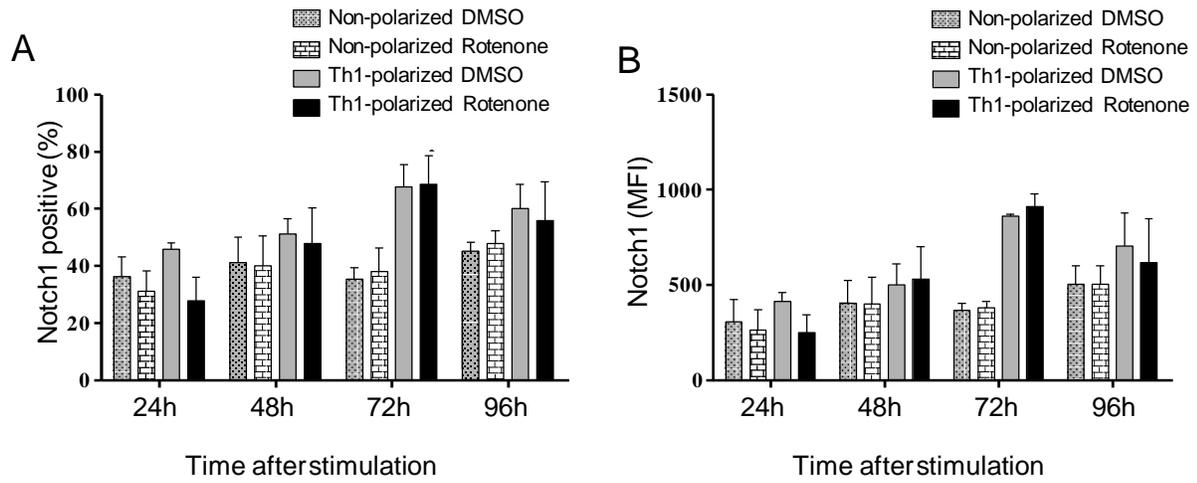


Figure S2

Th1 Cells



Th2 Cells

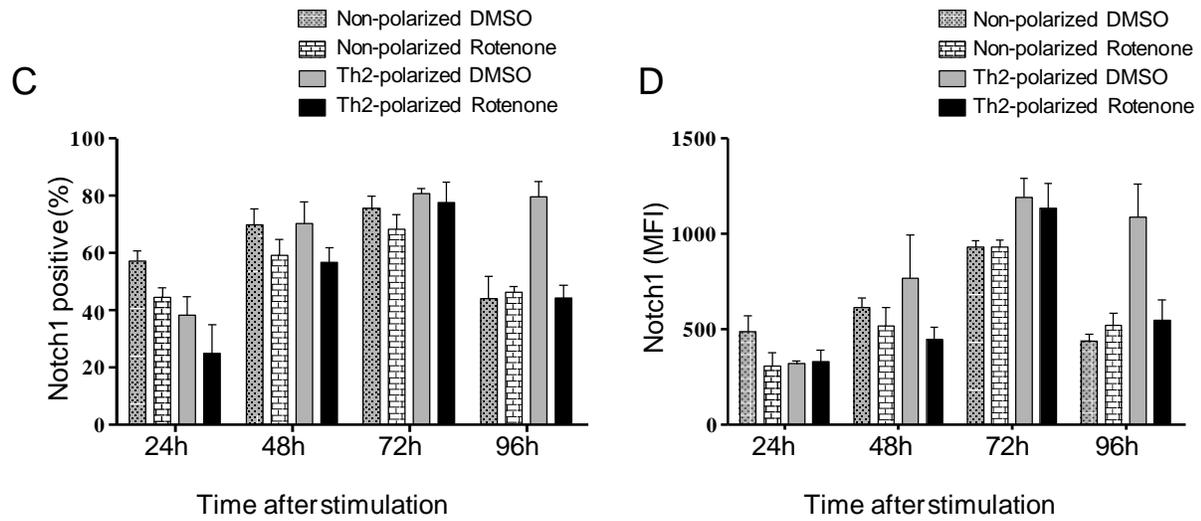


Figure S3

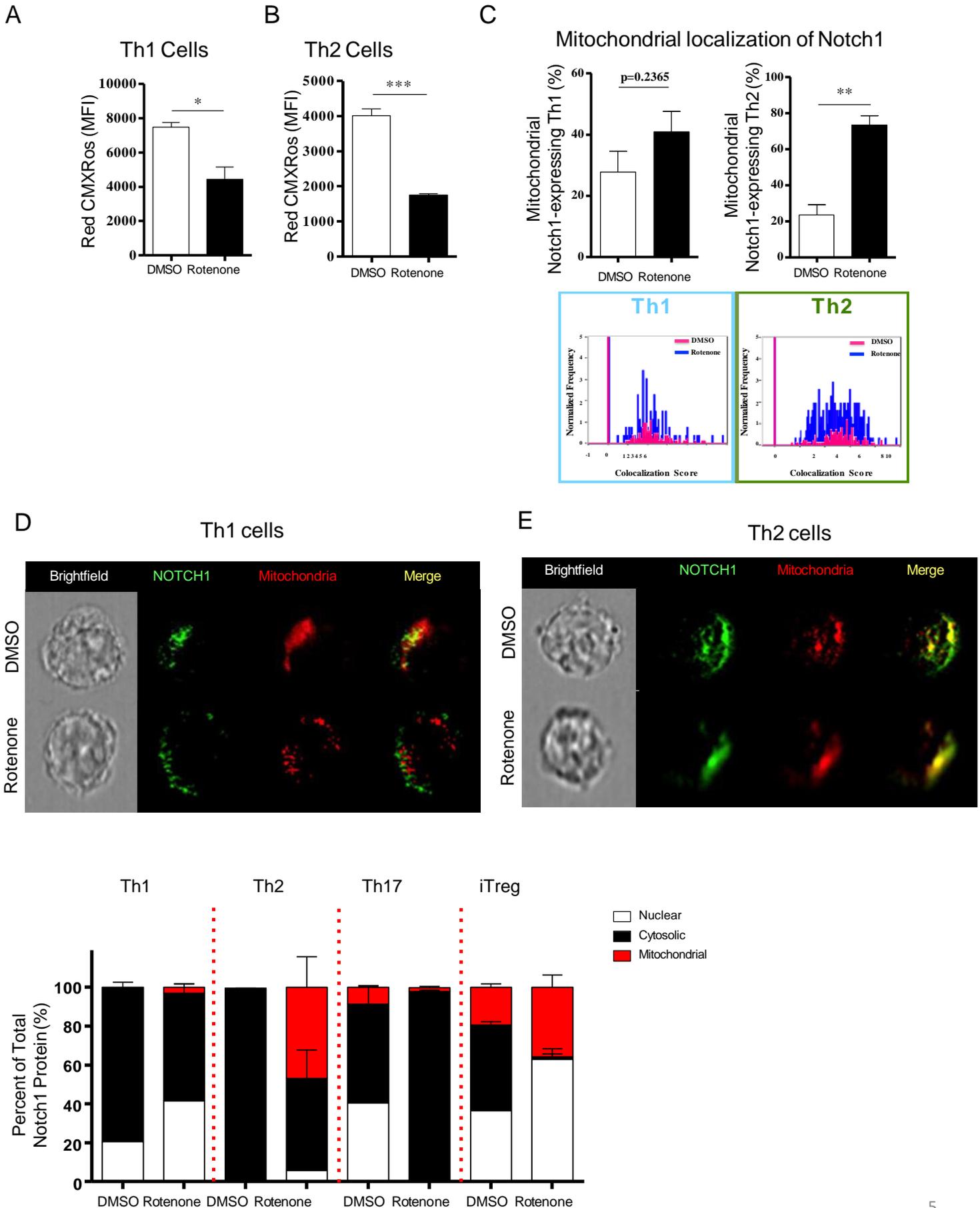


Figure S4

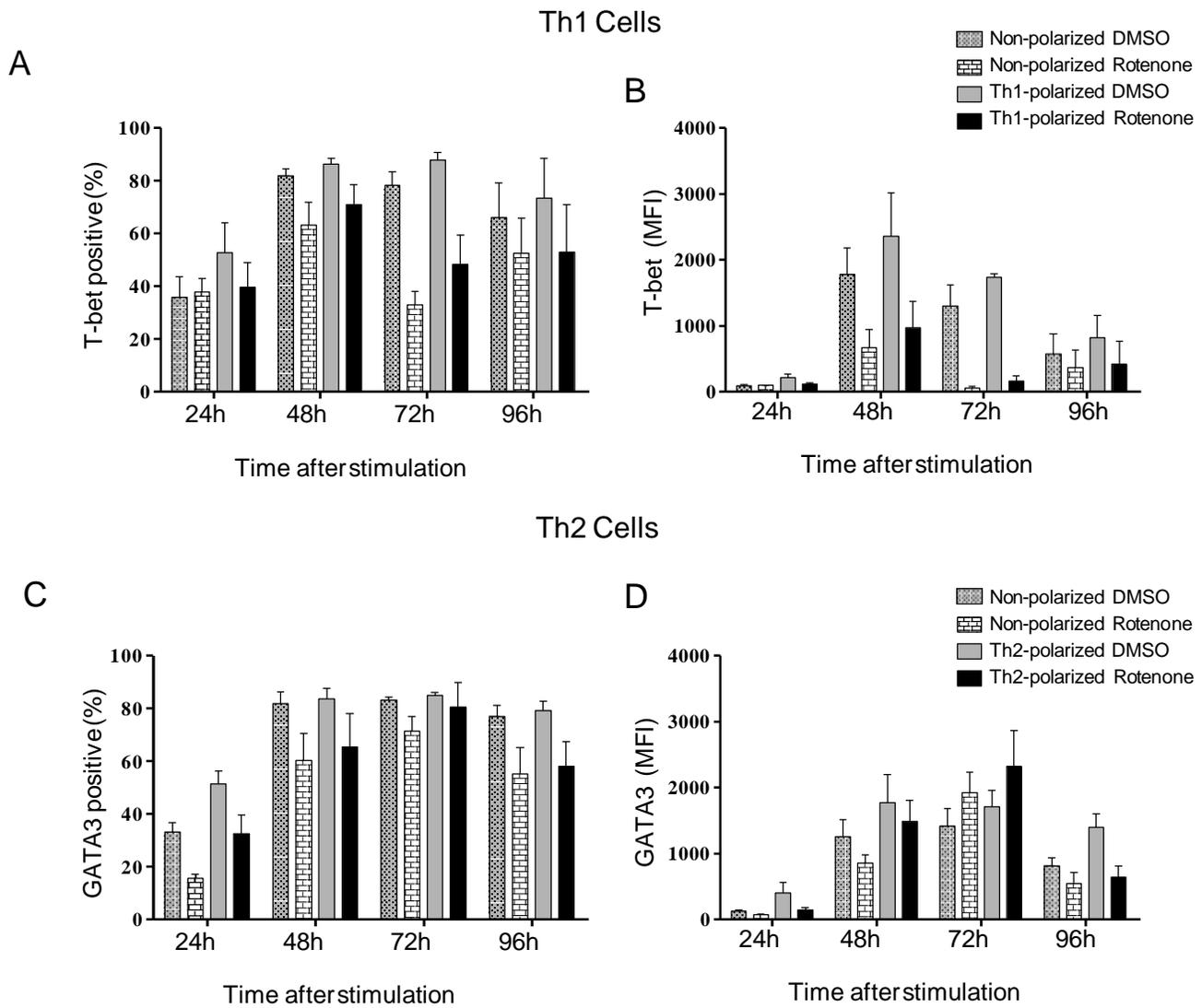


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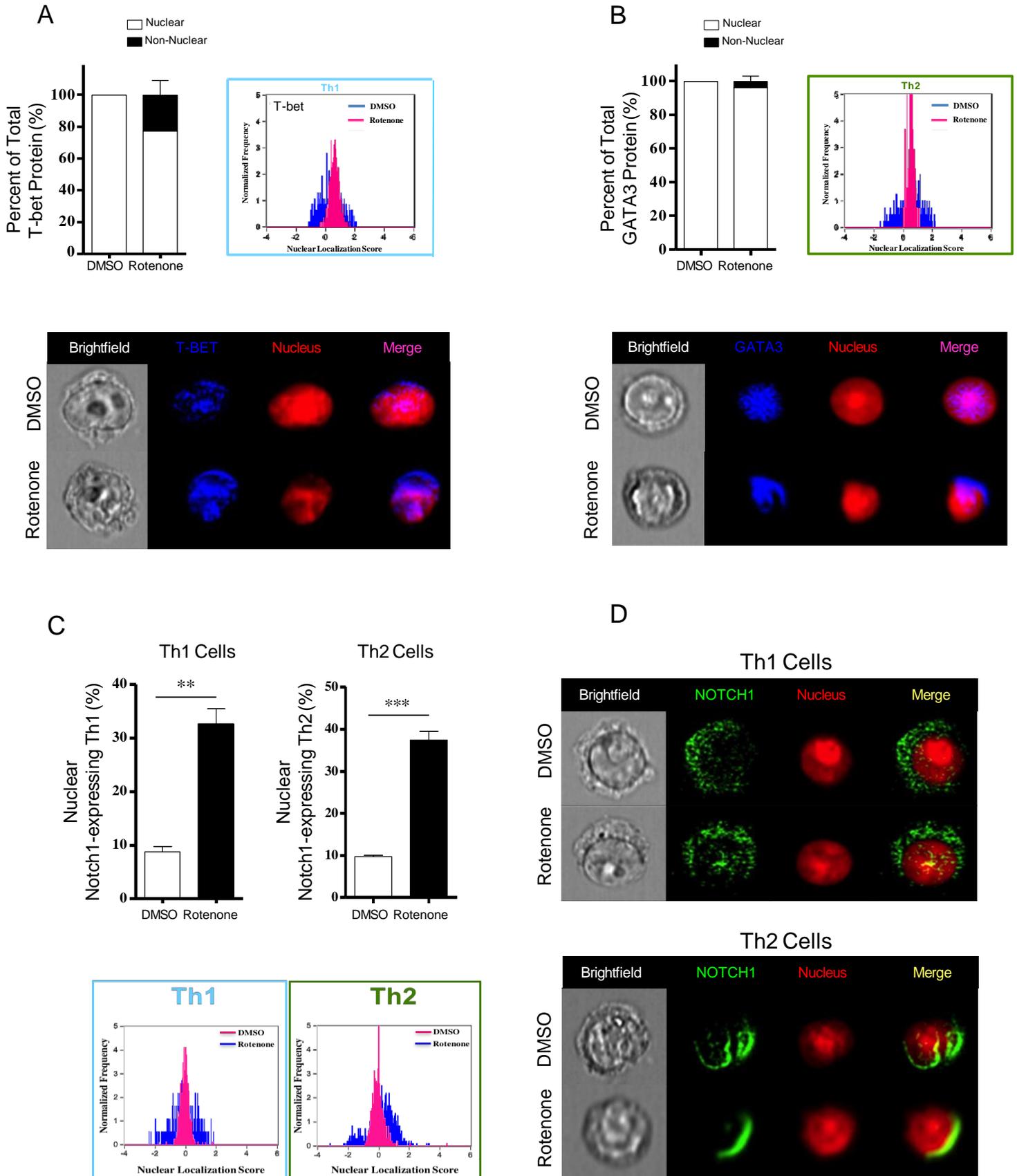
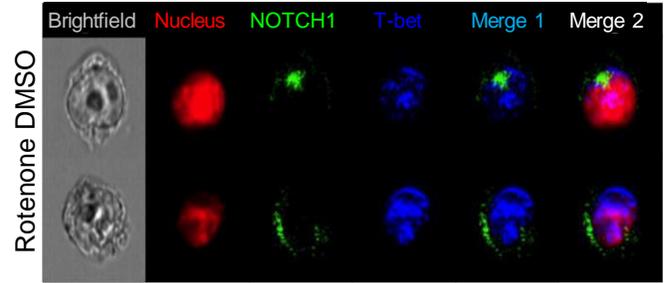
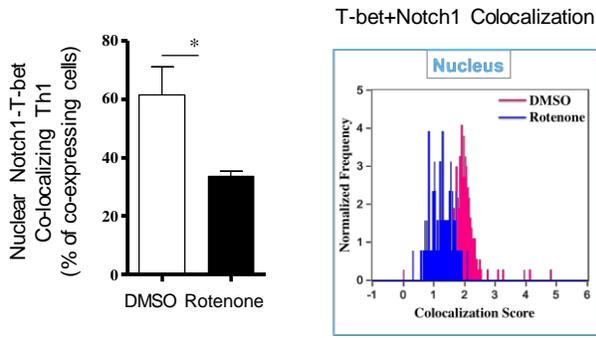
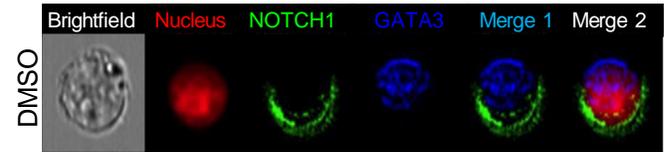
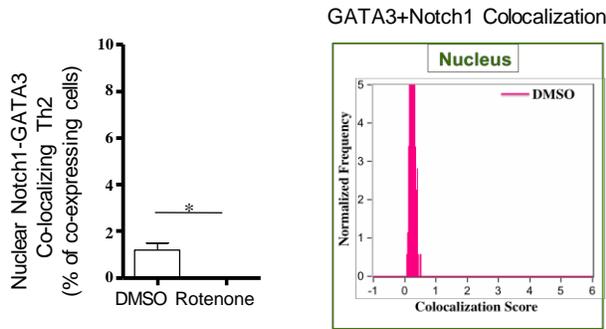


Figure S6

A

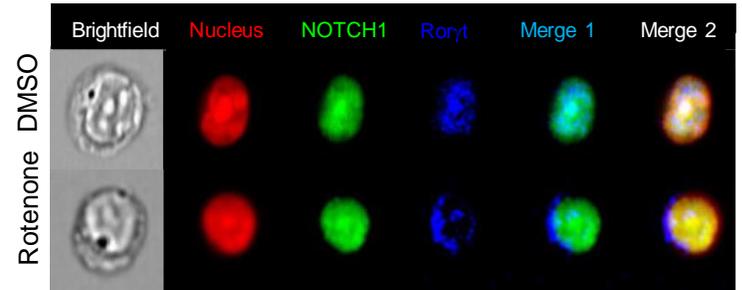
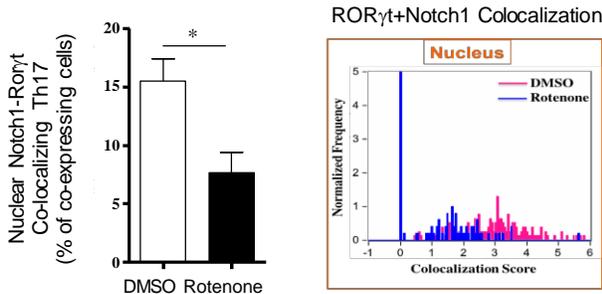


B



No double positive cells for NOTCH1 and GATA3 with rotenone treatment

C



D

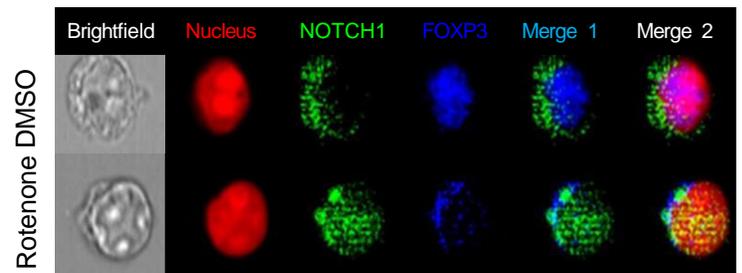
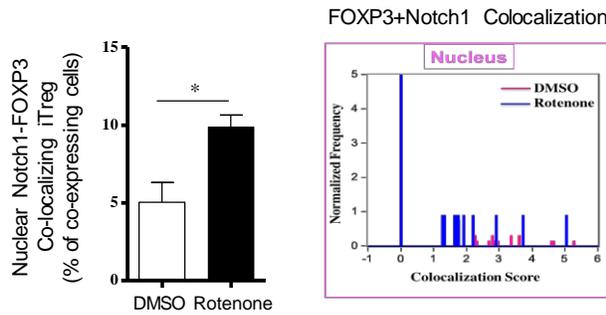


Figure S7

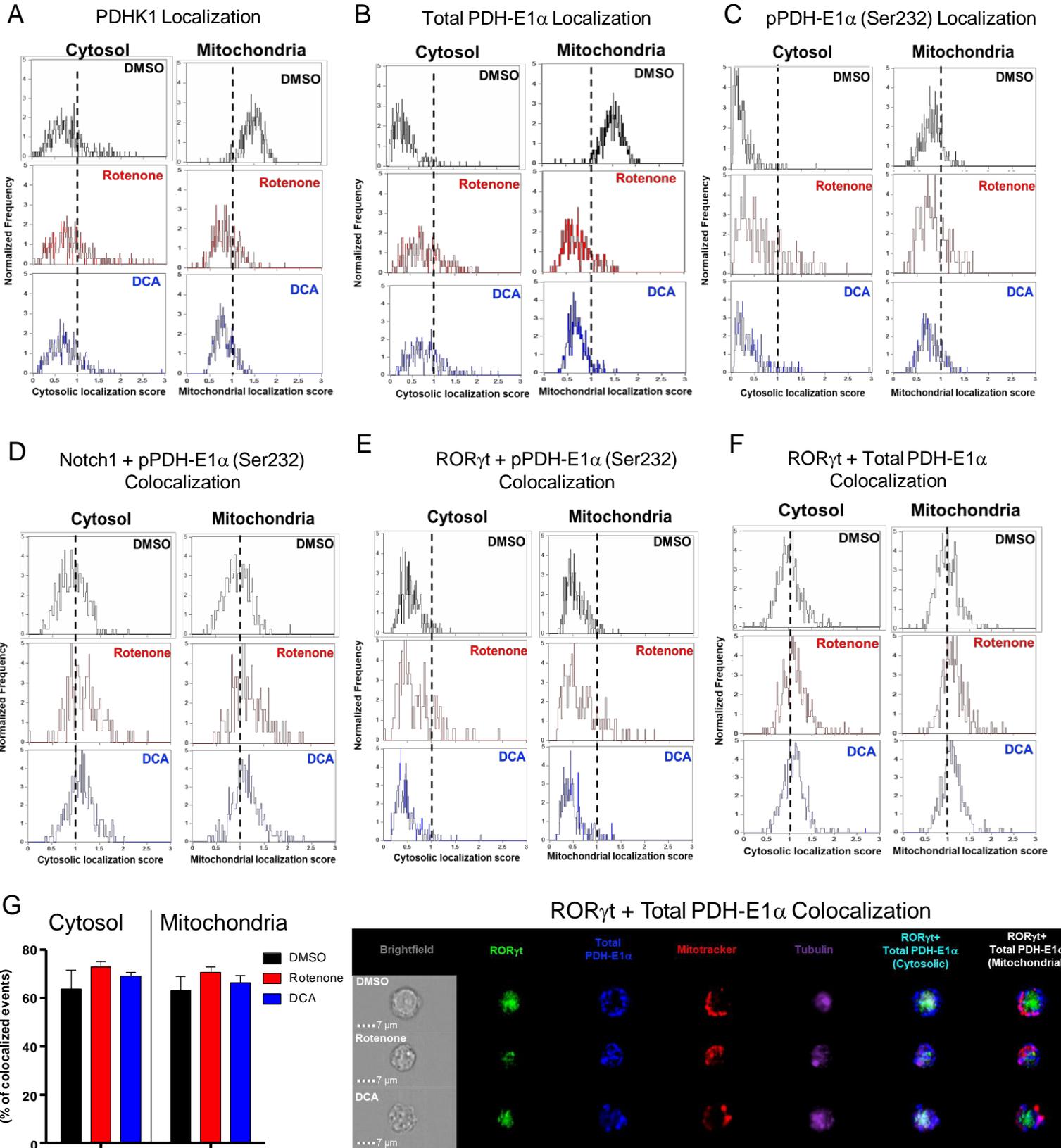


Figure S8

