Expanded View Figures

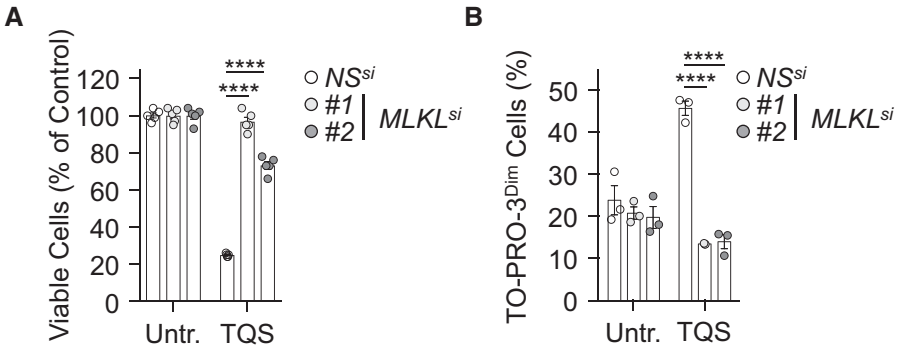

Figure EV1. Impact of MLKL silencing on cell survival and TO-PRO-3 uptake.

HT-29 cells were transfected with two individual siRNA for MLKL, or scramble non-specific (NS) siRNA for 72 h. Cells were pre-treated with 10 μM QVD-OPh (Q) together with 5 μM Birinapant (S), prior stimulation with 10 ng ml^{-1} of TNFα (T), as indicated.

A Cell survival was evaluated by CellTiter-Glo after 24 h of treatment. Data are means ± SEM, $n = 4$ biological replicates, ****$P < 0.0001$ (ANOVA).

B TO-PRO-3 uptake was analyzed by flow cytometry in cells treated with TQS for 4 h. Data are means ± SEM of three independent experiments. ****$P < 0.0001$ (ANOVA).

Figure EV2. Uptake of TO-PRO-3 via Pannexin-1 during necroptosis.

A HT-29 cells were transfected with a siRNA for PANX1 (#1), or scramble non-specific (NS) siRNA for 72 h. Cells were pre-treated with 10 μM QVD-OPh (Q) together with 5 μM Birinapant (S), prior stimulation with 10 ng ml^{-1} of TNFα (T), as indicated. Cell lysates were analyzed by Western blotting, as indicated. Molecular weight markers (M_r) are shown.

B Schematical representation of MLKL. The position of the epitopes recognized by the two antibodies used is shown. The star indicates the S358 position.

C HT-29 cells were treated as indicated. Necrostatin-1 (Nec-1s, 20 μM) was also used. Cell lysates were prepared and analyzed by Western blotting. White arrowheads show intact MLKL, and green or red arrowheads show cleaved MLKL.

D Cells as in (A) were exposed to TQS for 5 h. Necrostatin-1 (Nec-1s, 20 μM) was also used. MLKL oligomers ($MLKL_n$) were resolved by non-reducing SDS–PAGE after cross-linking.

E Cell viability assay by CellTiter-Glo in cells treated with TQS for 24 h (mean ± SEM, $n = 9$ biological replicates, *$P < 0.1$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ANOVA).

F, G Cells were treated with TQS for 4 h, stained with TO-PRO-3 and Annexin V (A5), and analyzed by flow cytometry. Dead cells (TO-PRO-3^{high}) were not analyzed. Data are means ± SEM of three independent experiments. *$P < 0.1$, ****$P < 0.0001$ (ANOVA).

H, I HT-29 cells were infected with a lentivirus containing a shRNA targeting *PANX1* in the 5′-UTR region. PANX1 was reintroduced after an infection with a lentivirus containing a *PANX1* cDNA. Cell lysates were analyzed by Western blotting (H). Flow cytometric analysis of cells treated with TQS and cycloheximide for 4 h and stained with TO-PRO-3 (I). Data are means ± SEM of three independent experiments. *$P < 0.1$, **$P < 0.01$, ****$P < 0.0001$ (ANOVA).

J, K TO-PRO-3 uptake was analyzed by flow cytometry (mean ± SEM, $n = 3$ biological replicates, ****$P < 0.0001$, ANOVA). Gap19 (20 μM) and $LaCl_3$ (1.5 mM) were used.

L Schematical representation of PANX1. The position of the epitopes recognized by the antibodies used and the cleavage sites by caspases are shown.

M Western blotting analysis as indicated. PGNase F was used to remove glycosylation. Open and close symbols represent full-length and cleaved proteins, respectively.

N Western blotting analysis as indicated. The presented data are representative of at least three independent experiments.

Data information: (A, D) The arrowhead indicates MLKL cleaved fragment.
Source data are available online for this figure.

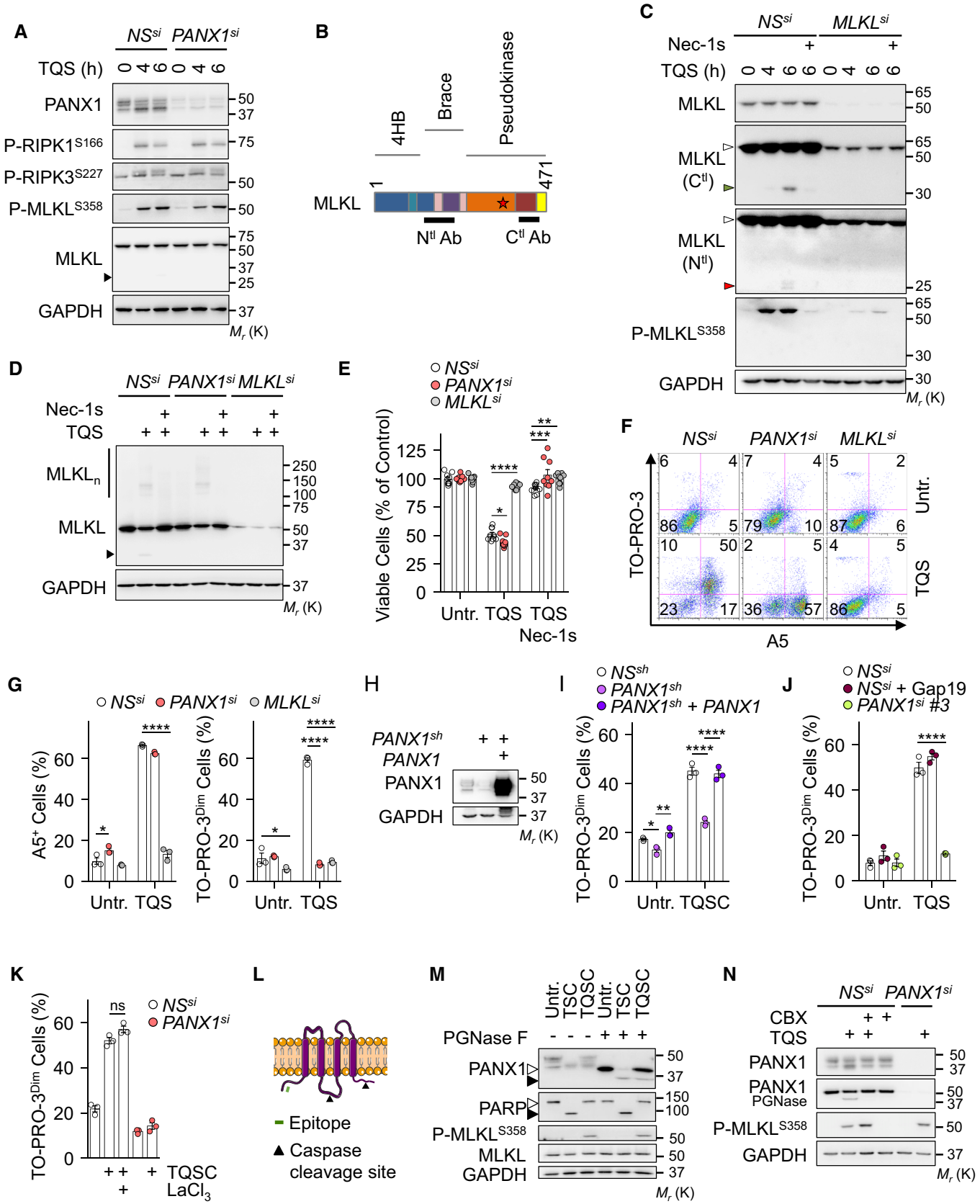

Figure EV2.

Figure EV3. MLKL controls intracellular vesicles trafficking.

A HT-29 cells were transfected with the indicated siRNA for 72 h. Cells were pre-treated with 10 μM QVD-OPh (Q) together with 5 μM Birinapant (S), prior stimulation with 10 ng ml^{-1} of TNFα (T) for 4 h, and TO-PRO-3 uptake was analyzed by flow cytometry. Data are means ± SEM of three independent experiments. *$P < 0.1$, ***$P < 0.001$, ****$P < 0.0001$ (ANOVA).

B–D Confocal microscopic analysis of CD63 and GM130 in HeLa cells, as indicated. The staining was analyzed by Structure Illumination microscopy (SIM). Nuclei were counterstained with DAPI. Scale bar, 10 μm. The volumes of CD63-positive structures, or scatter plots of numbers and mean volumes of CD63-positive structures analyzed by SIM ($n = 10$ cells per condition; ****$P < 0.0001$; ANOVA), were quantified. Ellipses show 95% confidence. On each box, the central mark indicates the median, and the bottom and top edges of the box indicate the 25^{th} and 75^{th} percentiles, respectively. The whiskers extend to the most extreme data points not considered outliers.

E Density plots showing dispersion of the volume of CD63-positive structures for each cells.

F Flow cytometric analysis of cells treated as in (A) and stained with TO-PRO-3. Shown is mean ± SEM ($n = 3$ biological replicates, ****$P < 0.0001$, ANOVA).

G HT-29 cells were transfected with siRNA for MLKL, or scramble non-specific (NS) siRNA for 72 h. Cells were pre-treated with 10 μM QVD-OPh (Q) together with 5 μM Birinapant (S), and with 100 μM CBX, prior stimulation with 10 ng ml^{-1} of TNFα (T) for 6 h. Cell lysates were analyzed by Western blotting as indicated. Molecular weight markers (Mr) are shown.

H HT-29 cells were treated as in (G), and 5 μM NSA was also used. Western blotting analysis was performed with the indicated antibodies.

I HT-29 cells were transfected with siRNA for MLKL, or scramble non-specific (NS) siRNA for 72 h. Cells were pre-treated with 10 μM QVD-OPh (Q) together with 5 μM Birinapant (S) and KCl, prior stimulation with 10 ng ml^{-1} of TNFα (T) for 4 h. Flow cytometric analysis of cells stained with TO-PRO-3 (mean ± SEM, $n = 5$ biological replicates).

J Western blotting of cell lysates as in (A). MCC-950 (1 μM) was also used. The arrowhead indicates MLKL cleaved fragment.

Source data are available online for this figure.

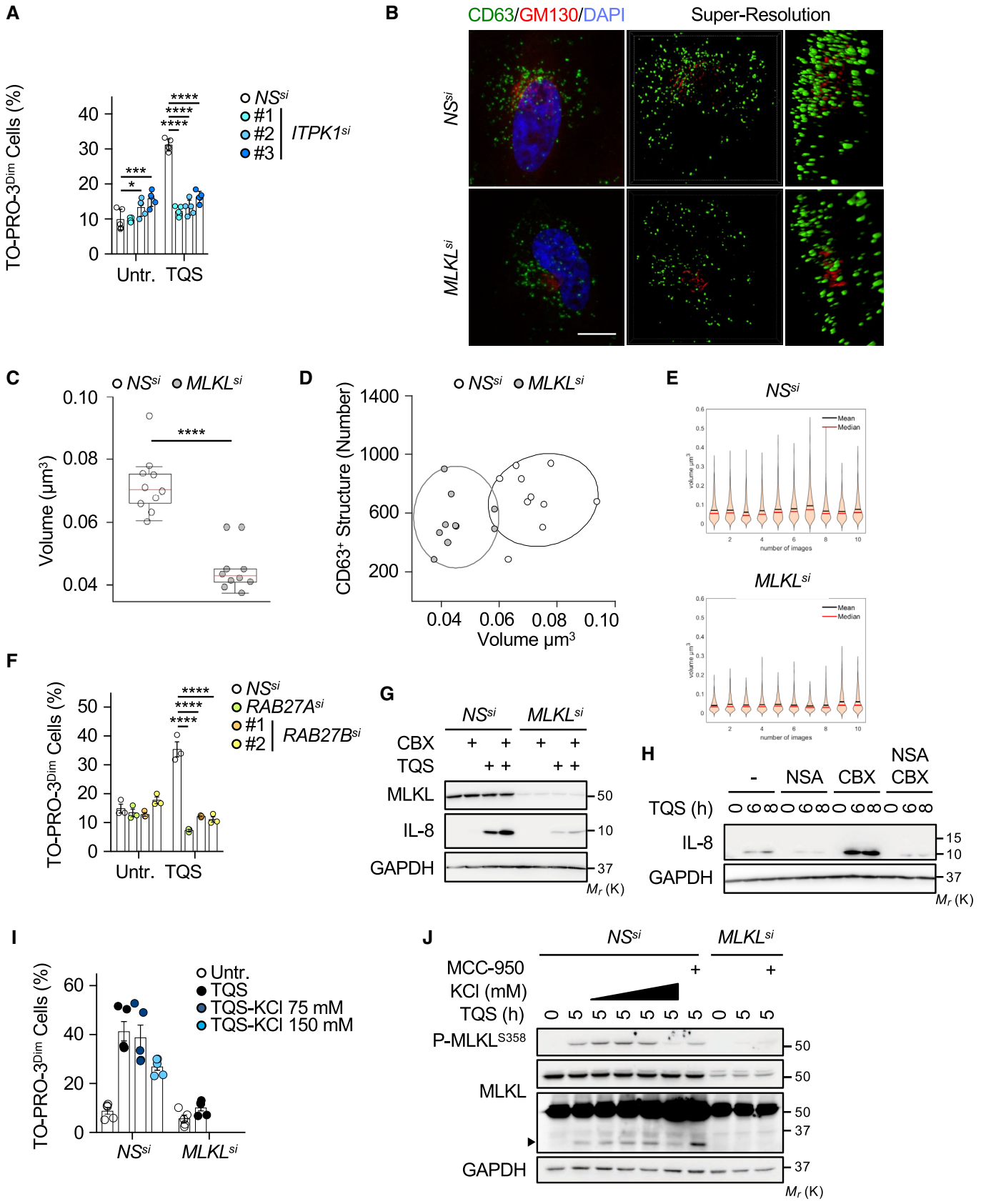

Figure EV3.