Supplementary figure legends

Supplementary Figure 1. *Exposure of CRT occurs independently from the apoptosis-associated loss of the mitochondrial membrane potential* (MMP). (A) HeLa cells treated with MTX were microscopically analyzed for CRT exposure, MOMP and nuclear fragmentation by means of immunofluoresecence detection, staining with the mitochondrial membrane potential-sensitive dye CMXROS and with the chromatin dye Hoechst33342, respectively. Representative cells for CRT localization are depicted. (B, C, D) HeLa cells treated with MTX were analyzed at the indicated time points by fluorescence microscopy. The percentage of cells showing CRT surface exposure, MMP loss and nuclear fragmentation was evaluated. Columns represent means ± SD of triplicate determinations in one experiment representative of three. A minimum of 200 cells were analyzed for each point.

Supplementary Figure 2. MTX *treatment does not result in a general redistribution of ER proteins to the cell surface.* Surface exposure of the indicated ER resident proteins was analyzed in untreated and MTX-treated CT26 cells using flow cytometry (left panel, representative histograms are shown) and confocal microscopy (middle and right panels, FITC signal in green). Shown micrographs represent merged fluorescent and differential interference contrast (DIC) images. Note that all antibodies have been subjected to a quality control and were found to reveal an ER-specific staining pattern when tested on permeabilized cells.

Supplementary Figure 3. *Effect of PERK, ATF6 and IRE1 on CRT/ERp57 exposure.* (A) HeLa cells were transiently transfected with siRNA against PERK or PKR and the expression of these proteins was analyzed by immunoblotting. CT26 cells were transiently transfected

with ATF6 siRNA, and the ATF6 protein levels were examined by immunoblot. Actin was used as a loading control. (B) Quantitative analysis of CRT/ERp57 exposure in HeLa cells transfected with siRNA against PERK and PKR and in CT26 transfected with ATF siRNA. In addition, WT and *ire1-/-* MEF were treated with MTX for 4h and the percentage of ecto-CRT/ERp57 positive cells was measured by flow cytometry. Columns represent means ± SD of triplicate determinations in one experiment representative of three (ERp57 measurements, $^{*}P < 0.05$, $^{**}P < 0.01$).

Supplementary Figure 4. *Effect of PERK on CRT/ERp57 exposure and cell death induced by MTX*. (A) Representative flow cytometry histograms showing the cell surface exposure of ERp57 among viable (propidium iodide-negative) CT26 cells as described in Materials and Methods. CT26 clones transfected with either control shRNA (shCO) or PERK shRNA (shPERK) were treated with MTX and stained with an isotype control or an anti-CRT antibody. (B) Quantitative analysis of annexin V-FITC and PI staining of shCO and shPERK CT26 cell clones 1 and 3, treated with the indicated MTX concentrations for 48 h. (C) CT26 cells stably expressing shCO, or shPERK-1 or -3, were treated with the indicated MTX concentrations, washed and plated to determine the frequency of surviving clones. The frequency of clones from untreated shCO cells was defined as 100%. Results are shown as means ± SD of triplicates of one experiment that was repeated twice, yielding similar results.

Supplementary Figure 5. *Involvement of caspases in CRT/ERp57 exposure.* (A) CRT/ERp57 surface exposure of WT, *casp3*$^{-/-}$*/casp7*$^{-/-}$ and *casp12*$^{-/-}$ MEF, which were left untreated or pre-incubated with zVAD-fmk followed by MTX treatment, was measured by flow cytometry (means ± SD, n=3 of ERp57 measurements). (B) CT26 cells were pre-incubated with either biotinylated bVAD-fmk or Z-VAD-fmk followed by treatment with MTX. The percentage of

cells with ecto-CRT/ERp57 was measured by flow cytometry (means ± SD, n=3 of ERp57 measurements, $^{*}P < 0.05$, $^{**}P < 0.01$). (C) HCT116 stably transfected with an empty vector or a vector expressing p35 were treated with MTX and the percentage (means±SD, n=3 of ERp57 measurements, $^{*}P < 0.05$) of cells with ecto-CRT/ERp57 was measured by flow cytometry. (D) Representative flow cytometry histograms showing the cell surface exposure of ERp57 among viable (propidium iodide-negative) CT26 cells. (E) Quantitative analysis of annexin V-FITC and PI staining of control shCO and shCasp8 CT26 cell clones 1 and 2, treated with the indicated MTX doses for 48 h. (F) CT26 cells stably expressing shCO, or shCasp8-1 or -2, were treated with the indicated MTX concentrations, washed and plated to determine the frequency of surviving clones. The frequency of clones from untreated control shRNA cells was defined as 100%. Results are means ± SD of triplicate determinations. This experiment has been repeated twice, with similar results.

Supplementary Figure 6. *TRAIL induces CRT/ERp57 exposure.* (A) Hela Cells were pre-treated with 5 mM NAC followed by treatment with the indicated concentrations of TRAIL for 4, 8 or 24h. The percentage of cells with exto-CRT/ERp57 was analyzzed by flow cytometry (means ± SD, n=3, $^{*}P < 0.05$). (B) Quantitative analysis of annexin V-FITC and PI staining of HeLa cells treated for the indicated time points with 50 or 100 ng/ml TRAIL in the presence or absence of NAC; (C) CT26 cells were treated with MTX for 4h in the absence or the presence of 10 µg/ml anti-TRAIL blocking antibody. The percentage of ecto-CRT/ERp57 positive cells was measured by flow cytometry. Columns represent means ± SD of triplicate determinations in one experiment representative of three.

Supplementary Figure 7. *Bap31 is required for CRT/ERp57 exposure*. (A) Involvement of Bap31 in CRT/ERP exposure. HeLa cells transfected with either an empty vector or the

caspase resistant Bap31 mutant (CR-Bap31), were treated with MTX and the levels of cleaved Bap31 were analyzed by immunoblot. Actin was used a loading control. (B) Quantitative analysis of the percent of ecto-CRT/ERp57 positive HeLa cells transfected with a control vector or CR-Bap31 and treated with MTX. Results are means ± SD of triplicates and are representative for three independent experiments of ERp57 measurements, $^{*}P < 0.05$. (C) HeLa cells were transfected with either siCO or siBAP31 and the percent of cells positive for ecto-CRT/ERp57 upon treatment with MTX was measured by flow cytometry. (D) Wild type HeLa cells or stably transfected with Bcl-2 or vMIA were treated with or without MTX and the percent of cells with ecto-CRT/ERp57 was measured by flow cytometry. Results are means ± SD of triplicates and are representative for three independent experiments of ERp57 measurements, $^{*}P < 0.05$. (E) Bax activation upon MTX treatment. Active Bax (green), the ER marker Bap31 (red,) and nucleic marker DAPI (blue) were visualized in untreated and MTX-treated HeLa cells by immunofluorescence microscopy.

Supplementary Figure 8. *Effects of Bax and Bak on CRT/ERp57 exposure.* (A) Representative flow cytometry histograms showing the cell surface exposure of ERp57 among viable (propidium iodide-negative) MEF cells as described in Materials and Methods. WT, *bak*$^{-/-}$, *bax*$^{-/-}$ and *bak*$^{-/-}$*bax*$^{-/-}$ MEF cells were treated with or without MTX and stained with an isotype control or an anti-ERp57 antibody. (B) HCT116 *bax*$^{-/-}$ cells transiently transfected with a vector expressing Bax, were treated with MTX and the percentage of cells with ecto-CRT/ERp57 was measure by flow cytometry.

Supplementary Figure 9. *Schematic illustration of the introduced glycosylation sites on calreticulin.* Shown is the crystal structure of the ER luminal domain of calnexin which, based on modeling studies, the NMR structure of the CRT arm domain and functional similarities,

resembles that of CRT (with the arm domain of CRT being ~25% shorter). Lectin site residues are depicted in red and the locations of the two introduced glycosylation sites in yellow.

Supplementary Figure 10. *Effects of brefeldin A on CRT/ERp57 exposure.* (A) Representative flow cytometry histograms showing the cell surface exposure of ERp57 among viable (Propidium iodide-negative) HeLa cells as described in Materials and Methods. HeLa cells were pre-incubated with brefeldin A followed by treatment with MTX and stained with an isotype control or an anti-ERp57 antibody. (B) Immunofluorescence detection of CRT (appearing in green, FITC) and ERp57 (appearing in red, Alexa 568) in HeLa cells pre-incubated with brefeldin A followed by treatment with MTX. The nuclei were visualized with DAPI.

Supplementary Figure 11. *Alterations in the actin cytoskeleton and ER organization induced by MTX*. (A) Effects of MTX on the actin cytoskeleton. HeLa cells were treated with MTX and then either examined by electron microscopy (upper panels) or stained with Phalloidin (Alexa 568) (lower panels). (B) Electron micrographs were acquired from untreated and MTX-treated HeLa cells visualizing alterations in the subcellular ultrastructure (ER: endoplasmic reticulum; N: nucleus).

Supplementary Figure 12. *Effects of MTX on the ER to Golgi transport kinetics of class I molecules*. CT26 cells were incubated in the absence or presence of MTX for 4 h, radiolabeled with [^{35}S]Met for 15 min and then chased for the times indicated. H-2K^{d} molecules were immunoisolated, digested with endo H and analyzed by SDS-PAGE. The conversion of H-2K^{d} oligosaccharides from endo H-sensitive to endo H-resistant forms

provides a measure of the rate of export of this molecule from the ER to the Golgi. The half-times for acquisition of resistance to endo H digestion in the absence or presence of MTX were 27 min and 29 min, respectively. ER to Golgi transport rates for H-2D^d and H-2L^d molecules were also unaffected by MTX treatment (not shown).

Supplementary Figure 13. *Relationship between SNAREs and CRT/ERp57 exposure.* (A) Immunofluorescence detection of SNAP23 (appearing in green, FITC) and CRT (appearing in red, Alexa 568) in CT26 cells treated with MTX. The nuclei were visualized with DAPI. (B) Representative flow cytometry histograms showing the cell surface exposure of CRT among viable (Propidium iodide-negative) CT26 cells as described in Materials and Methods. CT26 cells transfected with either siCO or against siSNAP23-1or siVAMP1-1, were treated with or without MTX and stained with an isotype control or an anti-CRT antibody.

Supplementary Figure 14. *Contribution of SNAP25 to CRT/ERP57 exposure in HeLa cells.* (A) HeLa cells were transiently transfected with siCO or siSNAP25 1-3 and its expression was analysed by immunobloting. Actin was used as a loading control. (B) Quantitative analysis of ecto-CRT/ERp57, measured by flow cytometry, in HeLa cells transfected with siCO or siSNAP25-1 and treated with MTX. Results are means ± SD of triplicates and are representative for three independent experiments of ERp57 measurements, $^{**}P < 0.01$.

Supplementary Figure 15. *Effect of SNAP23 on cell death induced by MTX.* (A) Quantitative analysis of annexin V-FITC and PI staining of CT26 cells transfected with either siCO or siSNAP23-1, were treated with the indicated MTX concentrations for 48 h. (B) CT26 cells transfected with either siCO or siSNAP23-1, were treated with the indicated MTX

concentrations, washed and plated to determine the frequency of surviving clones. The frequency of clones from untreated shCO cells was defined as 100%.