Supplementary Materials and Methods

Construction and expression of HA-tagged, glycosylated mouse calreticulin

To introduce two glycosylation sites into mouse CRT cDNA, a modified Quick Change™ (QC) protocol was performed (Zheng et al, 2004): In a first QC-PCR, the sequon NNT was generated at position 162-164 using primer pair F1-mut/ R1-mut and template plasmid pcDNA 3.1/zeo containing wild type CRT (Ireland et al, 2008). The resulting construct was used as the template in a second round of QC-PCR using primer F2-mut and R2-mut to introduce the sequon NAT at position 195-197 (supplementary table 1). To insert an HA-tag after position E389, upstream of the C-terminal KDEL motif, an overlap extension PCR (Ho et al, 1989) was performed. In the first round of PCR reactions, a 1.2 kb and a 0.1 kb DNA fragment were generated with primer pairs 5-NotI/3-HA and 3-BamHI/5-HA, respectively (supplementary table 1). After purification, they were used in equimolar amounts as the template in a PCR using primer pair 5-NotI/3-BamHI to amplify the complete CRT cDNA including the HA-tag. Subsequently, it was subcloned into the retroviral expression plasmid pQCXIH (Clontech) using NotI/BamHI sites (supplementary table 1). The final construct was named pQCXIH-glycCrt and its integrity was confirmed by sequencing (supplementary table 1). For the generation of a stable cell line expressing glycosylated mouse CRT, recombinant Moloney virus packaged with plasmid pQCXIH-glycCrt was used to infect CT26 cells as described previously (Ireland et al, 2008). Stable transformants were selected in 200 µg/mL of hygromycin B (Invitrogen).

Cell fractionation

CT26 cells were cultured for 4 h with 1 µM mitoxantrone. All following steps were performed at 4ºC. After trypsinization, 1 x 10^7 cells were washed once in sucrose buffer (0.25 mM sucrose, 5 mM Hepes, pH 7), resuspended in 1 mL sucrose buffer supplemented with 5

μg/mL digitonin and incubated for 5 min under gentle agitation. Following centrifugation, the supernatant was removed and was designated the cytoplasmic fraction. The pellet was washed several times with 1 mL of digitonin-free sucrose buffer and was used as the organelle fraction. The organelle fraction was solubilized in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2 and protease inhibitors) and both fractions were subjected to SDS-PAGE and immunoblotting.

Biotinylation of cell surface proteins

In a 6-well plate, 1 x 10^6 cells were cultured for 12 h in the presence or absence of 1 μM mitoxantrone and then washed three times in PBS. Subsequently, cell surface proteins were biotinylated for 30 min in borate buffer (2.5 mM boric acid, pH 9.0, 40 mM NaCl, 0.5 mM $CaCl_2$, 2 mM KCl) supplemented with 5 mg/mL NHS-SS-biotin (Pierce). After washing 3 times with quenching buffer (100 mM glycine, pH 8.3), the cells were further incubated in quenching buffer for 30 min. Cells were then washed three times with PBS and then lysed for 30 min on ice in 1 mL RIPA buffer. Following centrifugation (12,000 x g, 15 min) the lysate was incubated with 100 μL of packed streptavidin-agarose beads (Pierce) for 2 h at 4ºC. Beads were pelleted by centrifugation and the supernatant was used as the non-biotinylated, intracellular protein fraction. Biotinylated cell surface proteins bound to the beads were washed three times with lysis buffer. Both biotinylated and non-biotinylated fractions were then incubated in the presence or absence of endoglycosidase H (endo H; New England Biolabs) for 6 h at 37ºC, following the manufacturer's protocol. Digests were separated on a reducing 10% SDS-PAGE gel containing concanavalin A, which was copolymerized in a top layer of the separating gel to enhance the resolution of glycoproteins from nonglycosylated proteins (Popov et al, 2000).

Pulse-chase radiolabeling

CT26 cells (1 x 10^6 per 60 mm dish) were cultured in the presence or absence of 1 μM mitoxantrone for 4 h and then incubated for an additional 30 min in Met-free RPMI 1640 medium in the presence or absence of drug. Cells were radiolabeled for 15 min with 50 μCi [^{35}S]Met in 0.5 mL Met-free RPMI 1640 and then chased in complete RPMI medium containing 1 mM Met for various times. At each time point, cells were washed twice with cold PBS and then lysed in 1 mL of cold 10 mM Hepes, pH 7.4, 150 mM NaCl, 1% NP-40, 10 mM iodoacetamide, and protease inhibitors. Following centrifugation, H-2K^d, H-2D^d and H-2L^d molecules were isolated sequentially from the lysates by incubation for 2 h with mAbs 20-8-4S, 34-5-8S and 30-5-7S, respectively, followed by a 1 h incubation with protein A-agarose beads. Beads were washed 4 times in lysis buffer lacking iodoacetamide and inhibitors and then subjected to digestion with endo H and analysis by SDS-PAGE (10% gel) and fluorography.

Western blot analyses

Cells were harvested and homogenized in RIPA lysis buffer (10 mM Tris, pH 7.2, 150 mM NaCl, 1% deoxycholate, 1% Triton, 0.1% SDS, 5 mM EDTA) containing complete protease inhibitor cocktail (Roche Diagnostics, Meylan, France). After 1 h on ice, samples were sonicated and protein quantification was carried out using a Bio-Rad protein assay. Equal amounts of soluble proteins (15-25 μg) were denatured by boiling and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. After blocking in 5% non-fat dry milk in PBS for 1 h and probing with a specific primary antibody and a horseradish peroxidase-conjugated secondary antibody, the protein bands were detected by chemiluminescence (Supersignal, Pierce) and X-

ray film exposure (Kodak) or CCD camera (Ozyme). Protein loading was normalized by using anti-actin antibodies.

Immunoprecipitation

Immunoprecipitation was carried out as described in Kepp et al. (Kepp et al, 2007). Briefly, cells were lysed in CHAPS buffer (150 mM NaCl; 10 mM HEPES 1% CHAPS, pH 7.4 supplemented with complete protease inhibitors (Roche)). The cleared lysates were incubated overnight with 2 µg of anti-Bak (clone AB-1) or anti-Bax (clone 6A7) followed by the addition of protein G-Sepharose (Amersham). After extensive washing the precipitates were analysed by immunoblotting.

Clonogenic assays

CT26 cells (800 cells/well) were seeded in duplicate or triplicate into six-well plates. Once the cells were attached, they were treated with 20 and 200 nM of mitoxantrone for 24 h, followed by replacement of the medium with new, drug-free medium. Cells were cultured for up to 14 days. Colonies were fixed and stained with crystal violet. All colonies of 50 cells or more were then counted.

Electron microscopy

For electron microscopy, cells (fixed for 1 h at 4°C in 2.5% glutaraldehyde in phosphate buffer, pH 7.4) were washed and fixed again in 2% osmium tetroxide, then embedded in Epon, and ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate and examined with a Leo 902 electron microscope, at 80 kV.