Supplementary Materials and Methods

Cell cycle and cell division analyses

To investigate cell cycle, carboxyfluorescein diacetate succinimidyl ester (CFSE) was utilized as indicators of cell division (Dengler et al., 2001). Cells were resuspended in PBS with 2.5 mM CFSE. After 8 min at RT, cells were washed and cultured in medium with FCS. The first FACS measurement was performed 12 h after labeling. To measure intracellular μ chain content, cells were washed twice in PBS, fixed, permeabilized and incubated for 30 min with 200 μl of the 2.4G2 hybridoma supernatant and 10% rat serum to quench FcRs. Next, cells were incubated with fluorescent anti-μ antibodies and analyzed by FACS.

For DNA content analyses, $1x10^6$ cells were washed, fixed in 70% ethanol at 4°C, and resuspended in 0.5 ml of PBS. 0.5 ml of 0.2 M Na_2HPO_4, 250 mM citric acid, pH 7.8 were added, and cells incubated for 5 min at RT. After centrifugation, pellets were resuspended in PBS supplemented with 20 mg/ml propidium iodide and 0.2 mg/ml RNase A, incubated 30 min at RT and analyzed by FACS.

For nuclear visualization, cells on poly-L-lysine coated multi-slides were fixed in 3.8% paraformaldehyde, permeabilized with 0.1% NP-40 for 10 min and stained with Hoechst.

Caspase activity assays

I.29μ+ cells were sonicated in ice cold buffer (25 mM Hepes pH 7.5, 5 mM EGTA, 1 mM EDTA, 5 mM MgCl2, 1% CHAPS, 5 mM DTT and protease inhibitors), and extracts centrifuged for 5 min at 3000 rpm in microfuge to remove insoluble material and cell debris. Ac-DEVD-AMC (for caspase III activity), Z-IETD-AFC (for caspase VIII) and Ac-LEHAD-AFC (for caspase IX) were used at a final concentration of 0.4 μM in 25 mM Hepes pH 7.5, 10%, sucrose, 10% CHAPS and 10 mM DTT. Reactions were started by adding an aliquot of cellular extract and the fluorescence of released fluorogenic peptides (excitation, 380, 400 and 400 nm; emission, 460, 505, and 505 nm, for caspases III, VIII and IX, respectively) was monitored continuously at 37°C with a Carry Eclipse spectrofluorometer.

Proteasome activity assays and immunoblot analyses

Proteasome activity was assessed as described (Cascio et al., 2002).
Cellular extracts were prepared from I.29μ+ cells as described (Mo et al., 1999) with minor modifications. Briefly, cells were sonicated in ice-cold buffer (50 mM Tris/HCl pH 7.5, 1 mM DTT, 0.25 M sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA, 2 mM ATP) and extracts prepared by centrifugation for 30 min at 10,000 g and 15 min at 100,000 g. Peptidase activities of proteasome were assayed by monitoring the production of 7-amino-4-methylcoumarin (amc) from fluorogenic peptides (Bachem, Bubendorf, CH) (Kisselev et al., 1999). Suc-LLVY-amc (for the chymotrypsin-like activity), Boc-LRR-amc (for the trypsin-like activity) and Ac-YVAD-amc (for the caspase-like activity) were used at a final concentration of 100 μM in 20 mM Tris-HCl pH 7.5, 1 mM ATP, 2 mM $MgCl_2$, 0.1% BSA. Reactions were started by adding an aliquot of cellular extract and the fluorescence of released amc (excitation, 380 nm; emission, 460 nm) was monitored continuously at 37°C with a Carry Eclipse spectrofluorometer. Background activity (caused by nonproteasomal degradation) was determined by addition of the proteasome inhibitors MG132 (for the chymotrypsin and the caspase-like activities) and β-lactone (for the trypsin-like activity) at a final concentration of 10 μM and 20 μM respectively. Assays were calibrated using standard solutions of free fluorophores and the reaction velocities were calculated from the slopes of the initial linear portions of the curves. Substrate consumption at the end of incubation never exceeded 1%.

Extracts from I.29μ+ cells were prepared in 10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 M Sucrose, 0.1 mM EDTA, 0.5% Triton X-100. Nuclei were isolated by centrifugation and nuclear extracts prepared by sonication in10 mM HEPES pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 1 mM DTT. 30 μg of proteins from total or nuclear I.29μ+ extracts were resolved by electrophoresis, blotted, probed with antibodies against Ub, Bcl2, IkBα, CHOP/GADD153, Xbp1 (St.Cruz sc-8017, sc-492-G, sc-1643, sc-793, sc-7160, respectively) and Bax (MDL, JM-3032), and revealed by ECL. Immunoblot analyses of proteasomal subunits were performed as described (Cascio et al., 2001). Densitometric analysis was performed by a VersaDoc 1000 Imaging System using the Quantity One software (Bio-Rad).

RT-PCR analyses

For RT-PCR analysis, total cellular RNA was reverse-transcribed by the Superscript II RT (Invitrogen, Milan, I). Splicing of XBP1 mRNA [RefSeq: NM013842] was analysed by PCR with primers flanking the 26b intron (5'-GGAGTGGAGTAAGGCTGGTG and 5'-CCAGAATGCCCAAAAGGATA). PCR products deriving from the spliced (s) and unspliced (u) XBP1 mRNAs were resolved by electrophoresis on a 2.5% agarose gel and visualised by ethidium bromide staining. For real-time quantification of EDEM1 [RefSeq: NM_138677] and Bip [RefSeq: NM_022310] mRNAs, we used the iCycler apparatus (Bio-Rad, Milan, I) and the iQ SYBR Green Supermix (Bio-Rad) under conditions recommended by the supplier. Expression was normalised to β-actin [RefSeq: NM_007393] by the Q-gene software application (Muller et al., 2002). Primers were 5'-CCATGCAGTTGTTACTGTACCAG and 5'-TCTTCTCTCCCTCTCTCTTATCCA for Bip, 5'-ACTGATTCCAAACAGCCCTT and 5'-GGATCCCTGTCTTGGTGTTT for EDEM1 and 5'-TGCTATGTTGCTCTAGACTTCGAG and 5'-TGCCACAGGATTCCATACCCA for β-actin.

Vector production and infection.

Lentiviral vectors encoding unstable GFPs (Ub-R-GFP, a N-end rule substrate, and Ub^{G76V}-GFP, containing a mutated uncleavable ubiquitin moiety) (Dantuma et al., 2000) were generated by cloning the sequences encoding the two unstable GFPs in the vector pCRI 2.1 TOPO (Invitrogen) (Not I/ Eco RI) and then in the lentiviral transfer vector pCCLsin.PPT.hPGK.deltaNGFR.Wpre (Cavalieri et al., 2003) (Sma/Xba I for the lentivirus and Sac-blunted/Xba I for Ub-GFP sequences) (Dantuma et al., 2000). Vector stocks were prepared as previously described (Dull et al., 1998). Briefly, 293T cells were transfected with the transfer vector plasmids pCCLsin.cPPT.hPGK.UbG76V-GFP.Wpre or pCCLsin.cPPT.hPGK.Ub-R-GFP.Wpre, the packaging plasmids pMDLg/pRRE and pRSV-Rev, and the VSV-G envelope-encoding plasmid pMD2.VSV-G (Follenzi et al., 2000). Conditioned medium was harvested and frozen at -80° C. Hela μs Tet off cells were transduced using different dilutions of the vector stocks. Cells expressing optimal amounts of the transgene were sorted by FACS (FACSCalibur, BD Biosciences) upon reversible proteasomal inhibition 7 days after infection.

Supplementary References

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