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Supplementary material

Methods

Antibodies and reagents

The ERp44 specific monoclonal 2D5, 36C9 (specific for human molecules) and 39E5 (recognising also mouse ERp44) were raised against GST-ERp44 (Anelli et al., 2003); rabbit anti ERp44 (B68) was previously described (Anelli et al., 2002).

Rabbit anti-mouse J chain and rabbit anti-PDI were kind gifts from Drs. R. M. E. Parkhouse (Pirbright Laboratory, Surrey, UK) and I. Braakman (Utrecht, NL); rabbit anti-p115 and rabbit anti-giantin were kindly provided by Dr. M. A. De Matteis (Chieti, IT); monoclonal anti-ERGIC-53 (G1/93) was described elsewhere (Schweizer et al., 1988); polyclonal anti ERGIC-53 (Spatuzza et al., 2004) and anti-ERp58 were generously provided by Drs. S. Bonatti (Naples, IT) , J. Saraste (Bergen, NO), E. Neve and R. Petterson (Stockholm, Sweden) (Neve et al., 2005).

Rabbit anti-calreticulin was from Stressgen (Victoria, BC Canada); rabbit polyclonal antibody anti-EEA1 from Abcam (Cambridge, UK); Rabbit anti-BiP from Santa Cruz Biotechnology (Santa Cruz, USA); mouse monoclonal anti-actin (clone AC-40) from Sigma Chemical Co (St. Louis, MO); goat anti-mouse λ and horseradish peroxidase (HRP) goat anti-mouse k from Southern Biotechnology Associates, Inc. (Birmingham, AL); HRP rabbit anti-mouse IgG (H+L) was from DakoCytomation (Glostrup, DK). HRP goat anti-rabbit IgG from Jackson ImmunoResearch (Baltimore, USA); polyclonal rabbit anti-mouse IgM from ZYMED (San Francisco, CA); Hoechst, Alexa Fluor 488 Goat anti-mouse IgG (H+L), Alexa Fluor 564 Goat anti-rabbit IgG (H+L) from Molecular Probes (Oregon, USA).

Mouse monoclonal antibodies specific for myc (9E10) and HA (12CA5) were immobilized by cross-linking to Protein G and Protein A beads respectively (Reddy et al., 1996).

Unless otherwise indicated, chemicals were from Sigma Chemical Co (St. Louis, MO). Dithiobis succinimidyl propionate (DSP) was from Pierce; fetal calf serum and culture media from Gibco BRL (Milan, IT); Lipofectin, Lipofectamine and Plus reagents, Lipofectamine RNAi max from Invitrogen (Carlsbad, USA); Sepharose-conjugated proteins A and G, ECL reagents and [^{35}S] aminoacids (PROMIX) from Amersham-Biosciences (Milan, IT).

Cell lines

HeLa, Ramos and 3T3 were obtained from ATCC. HeLa tet-off ERGIC-53 KKAA (Vollenweider et al., 1998) were a kind gift of Dr. H. P. Hauri (Basel Switzerland). I.29μ+ cells were cultured and activated with LPS as described (van Anken et al., 2003). CD19+ B lymphocytes were enriched from C57Bl/6 splenocytes by immunomagnetic selection and stimulated with LPS in vitro as described (Cenci et al., 2006)

Plasmids and vectors

The vectors for the expression of HA-ERp44, HA-ERp44 C29S and λ1chain were described previously (Fagioli et al., 2001; Anelli et al., 2002; Anelli et al., 2003).

The vectors for expression of wild type ERGIC-53 (GM ERGIC-53 in pcDNA 3.1), and the mutants KKAA(GMAA ERGIC-53 in pECE) and the N156A (GM N156A ERGIC-53 in pECE) were kind gifts of Dr. H.P.Hauri (Basel, Switzerland).

The vector encoding no-tag ERp44 was obtained by PCR with the primers TA2S (5' CAACTCGAGCGTTACCATGCATCCTGCC 3') and TA4R (5' TTTGGTACCTTAAAGCTCATCTCGATCCCTCAATAG 3') using pBS SK+II KIAA0573 (Kazusa DNA Research Institute (Anelli et al., 2002)) as a template. The PCR product was cleaved using the XhoI and Acc65I sites and inserted into pcDNA 3.1 (-) (Invitrogen, Life Technologies).

The vector for μs expression was prepared by RT-PCR from RNA isolated from N[μ1] cells (Benedetti et al., 2000; Sitia et al., 1987; Valetti et al., 1991). PCR amplification was performed on RT product from 2μg RNA, with primers containing unique restriction sites, BamHI for the forward primer (5' GGAATTCGCACACAGGACCTCACC 3') and XbaI for the reverse primer (5' AAAAAATCTAGACTGGTTGAGCGCTAGCATGG 3'). PCR product were digested and cloned in pcDNA3.1(+).

MCFD2 6his was amplified by PCR on the plasmid pDONR221 MCFD2 6his (a kind gift of Dr. Y.Lindqvist, Karolinska Inst., Stockholm, SE) with the primers forward 5' GAAGATATCACCATGACCATGAGATCCCT 3' and reverse 5' TACCTGGGTGTCAATGATGATGATG 3'. The PCR product, inserted in pGEMTeasy (Promega, Charbonnières, France), was then cloned in pcDNA 3.1 (-) using the restriction sites for EcoRI (orientation was checked by sequencing, Primm Milano, Italy).

RNAi

Silencing was performed with the following duplexes: ERp44 forward 5' CAACUCUGGCAAACACUAC 3', ERp44 reverse 3' UUGAGACCGUUUGUGAUG 5'; ERGIC-53 forward 5' GGACAGAAUCGUAUUCAUC 3', ERGIC-53 reverse 3' CCUGUCUUAGCAUAAGUAG 5' (Nyfeler et al., 2006). Nonsilencing control siRNA was purchased from MWG-Biotech AG (siRNA specific for Luciferase2).

Immunoflorescence

Lymphoid cells were incubated on poly-L-lysine-coated cover-slips for 20 min at 37°C whereas HeLa cells were grown directly (and, when indicated, transiently transfected) on 10-mm^2 cover-slips. Cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% TX100; for ERGIC-53 staining, HeLa cells were permeabilized in 0.5% saponin. After decoration with the indicated antibodies, cells were stained with Hoechst, and then samples prepared in Mowiol.

Secretion assay an determination of total IgM secretion and polymerization

For secretion assays, cells were washed 3 times and then cultured in OPTIMEM. After 4 hours, 10 mM NEM was added to block disulphide interchange, supernatants harvested and cells lysed in RIPA buffer + NEM (Anelli et al., 2002). Aliquots of supernatants (corresponding to 2 x 10^6 cells) were precipitated with Sepharose-bound Concanavalin A to concentrate glycoproteins, and resolved under non-reducing conditions (2-14% acrylamide gradient SDS-PAGE). Western Blots images were acquired with the Chemidoc-it Imaging System (UVP, Upland, CA); densitometric analysis was performed using Image Quant 5.2. Total IgM secretion was determined as the ratio between the total signal revealed with anti-μ antibody in the supernatant and the signal of intracellular μ for each transfectant. Polymerization was calculated as the amount of polymers present in the secreted material as a percentage of the total signal detected (on the secreted material) with anti-μ antibody. In order to compare different experiments, each value obtained both for total secretion and or polymerization was then expressed as a ratio with the correspondent value obtained in μλ transfectant.

Immunoprecipitation

For isolation of μ/ERGIC-53 complexes, cells were washed with PBS + 2 mM $CaCl_2$ and lysed in 10 mM Tris HCl pH 7.4, 150 mM NaCl, 0.25% NP40, 2 mM $CaCl_2$, 10 mM NEM, and EDTA-free protease inhibitor cocktail. All the subsequent reactions were performed in 2 mM $CaCl_2$.

For isolation of μ/BiP complexes, 20 U/ml of apyrase (Sigma) were added to cell lysates. When indicated, aliquots of the lysates were IP with anti-ERp44, anti-ERGIC-53, anti-BiP, anti-HA or anti-μ antibodies immobilized on Protein A beads (Reddy et al., 1996).

PCR and Real Time PCR

RNA extraction was performed as previously described (Benedetti et al., 2000; Sitia et al., 1987; Valetti et al., 1991). RT was performed on 2μg of RNA using AMV reverse trasnscriptase (Promega, Charbonnières, France), following the protocol suggested by the supplier.

PCR to analyze Xbp1 splicing was performed with the primers: forward 5' CCTTGTGGTTGAGAACCAGG 3' and reverse 5' GGGGCTTGGTATATATGTGG 3'. For murine MCFD2 expression analysis, the following primers were used: forward 5' AGAGCTCCCTTGCTGTGTGT 3', reverse 5' CAGTTCCTGTGGGGACATCT 3'.

The sequences of the primers used for Real Time PCR are: ERp44 FP - TGTGCCTTCCTTTCTGCTTT RP- CGGACAAGAGGGACACATTT; ERGICp53 FP- TGGTTATCCCCACACAAGGT RP- GCTGGAAGTGCTCAAACTCC; MCFD2 FP- AGAGCTCCCTTGCTGTGTGT RP- CAGTTCCTGTGGGGACATCT; μ chain FP – TGTGACAGAGGAGGAATGGA RP- TCAGACATGATCAGGGAGACA; J chain FP – TCCGAATTGTTGTCCCTTTG RP- ATCTTCCAGCTCCACTTCCA; BiP FP - TATTGGAGGTGGGCAAACCAAG RP- CGCTGGGCATCATTGAAGTAAG; H3 FP- GTGAAGAAACCTCATCGTTACAGGCCTGGT RP - CTGCAAAGCACCAATAGCTGCACTCTGGAA.

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Legends to Supplementary Figures

Figure S1: *The different localization of endogenous or over-expressed ERp44 in 3T3 and HeLa cells does not depend on the presence of a HA tag.*

3T3 cells transiently transfected with no-tag ERp44 (**A** and **B**) or non transfected controls (**C**) were co-stained with antibodies specific for ERp44, and markers of the ER (CRT), ERGIC (p115) or Golgi (giantin), as indicated. HeLa cells over-expressing ERp44 with or without a HA tag (**D** and **E**) were co-stained with antibodies specific for the HA tag (**D**) or for ERp44 (**E**) and the ER marker calreticulin (CRT). Images were taken with a fluorescence microscope and analyzed with deconvolution techniques. Magnifications of the boxed areas are shown in the inserts; arrows indicate examples of co-localizing structures; bar =10μm. Independently from the HA tag, over-expressed ERp44 shows a high level of co-localization with calreticulin (CRT) both in HeLa (**D-E**) and in 3T3 cells (**A**). Nonetheless, some over-expressed ERp44 can reach the ERGIC compartment (**B**). Also in 3T3 cells, endogenous ERp44 is instead mainly localized in ERGIC and vesicles very close to the Golgi apparatus, some showing co-localization with giantin (**C**).

Figure S2: *Ectopic accumulation of endogenous ERp44 in cells over-expressing the ER-localized ERGIC-53 KKAA mutant.*

HeLa cells expressing the tetracycline repressible ERGIC-53 wt or the ER-localized ERGIC-53 KKAA mutant (kind gifts of Dr. H. P. Hauri, Basel,CH) were cultured without the antibiotic to induce the over-expression of the transgene. Cells were co-stained with antibodies specific for ERp44 and the ER marker CRT. Images were taken with a fluorescence microscope and analyzed with deconvolution techniques. Magnifications are shown in the inserts; bar =10μm. Two images are shown for each transfectant. While in wt ERGIC-53 over-expressing cells a very low level of co-localization is detectable between ERp44 and CRT (as shown in non-transfected cells, see Figure 1), the appearance of numerous yellow spots in KKAA expressing cells indicates that part of endogenous ERp44 is ectopically localized in the ER, in association with the KKAA mutant.

Figure S3: *Ectopic accumulation in the ER of endogenous ERp44 following silencing of ERGIC-53.*

HeLa cells subjected to RNAi for ERp44 or ERGIC-53, or treated with control duplexes (ctrl), were stained with antibodies specific for ERp44, ERGIC-53 and the ER marker CRT or the ERGIC marker p115, as indicated. Images were taken with a fluorescence microscope and analyzed with deconvolution techniques. Magnifications are shown in the inserts; arrows indicate examples of co-localizing structures; bar =10μm. As shown previously, in control cells a very low level of co-localization between ERp44 and CRT is detectable (**a**), while a high level of overlapping is present between the two ERGIC markers ERGIC-53 and p115, as expected (**b**). Silencing of ERp44 (see panel **f** for a control) does not modify ERGIC-53 localization (**e-g**). On the contrary, silencing of ERGIC-53 (see panel **l** for a control) significantly affects the subcellular localization of ERp44, as shown by the more diffuse staining (**i**) and by the increased co-localization with CRT (**h**). As a control of the efficiency of RNAi, see Figure 5B.

Figure S4: *Neither over-expression of ERGIC-53 KKAA nor ERp44 or ERGIC-53 silencing induce ER stress.*

A) RNA from HeLa cells transiently transfected with ERGIC-53 (wt or KKAA mutant) or with an empty vector, or cells treated for 4 hours with 10μg/ml tunicamycin to induce pharmacological ER stress (tuni, lane 4), were subjected to RT-PCR to detect the presence of spliced Xbp1 as a marker of UPR induction. No Xbp1 splicing is present when ERGIC-53 (wt or KKAA) is over-expressed.

B) RNA from HeLa cells subjected to ERGIC-53 or ERp44 silencing or treated with control duplexes (ctrl) were subjected to RT-PCR as in A. No Xbp1 splicing can be detected in RNAi treated cells.

Figure S5: *The presence of μ chain in transfected HeLa culture medium is due to active secretion and not to cell death.*

Aliquots of the cell lysates (lys) or supernatants (SN) from HeLa transfectants expressing μ and λ chains were resolved electrophoretically under reducing conditions and blots decorated with anti-μ (top panel) or anti-LDH (bottom). Note the presence of

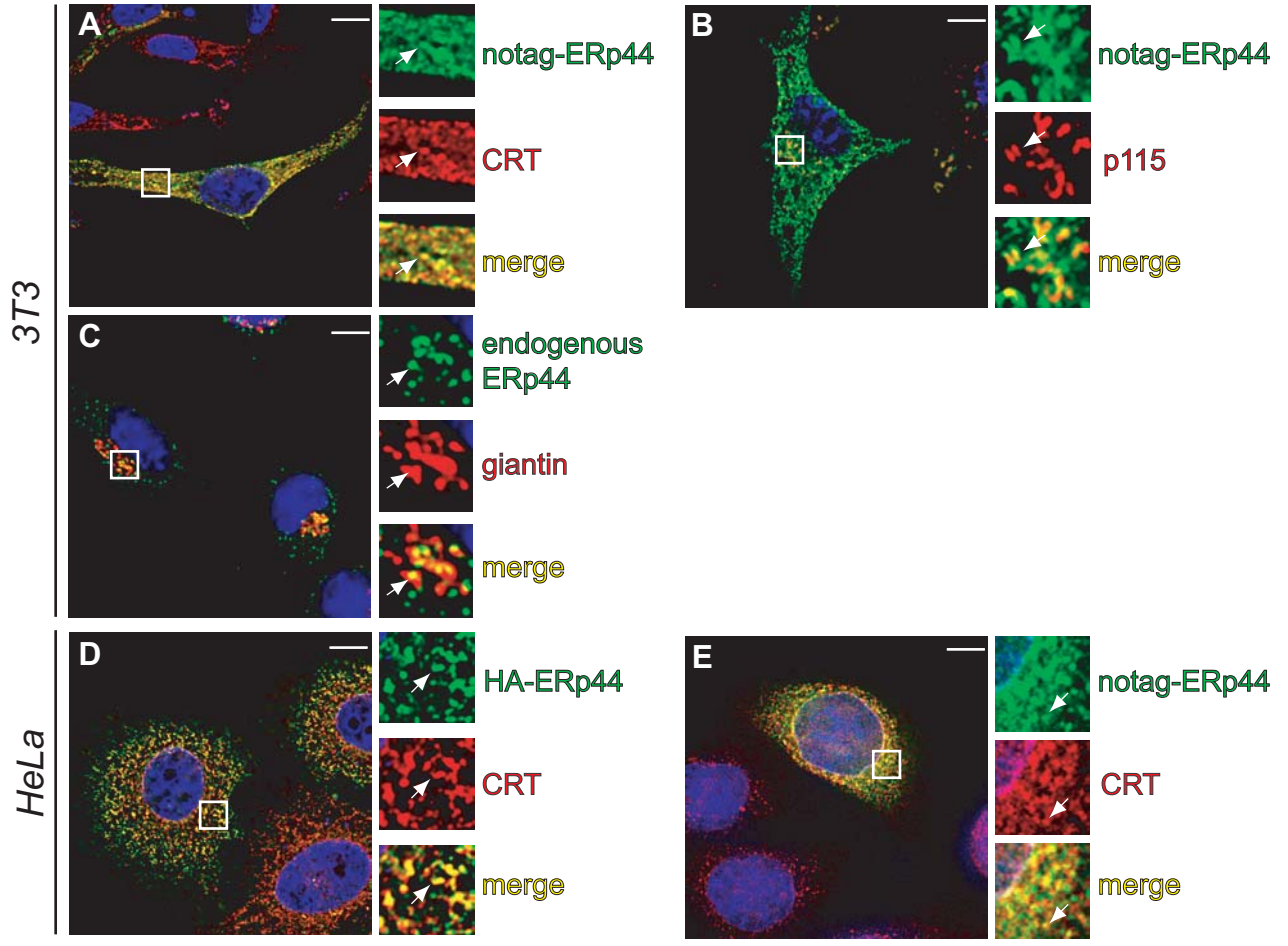
slower migrating μ chains, but no LDH, in the supernatants. No μ chains were secreted by HeLa cells expressing μ chains alone (not shown).

Figure S6: *ERp44 covalently interacts with $\mu_2\lambda_2$ and with $\mu\lambda$.*

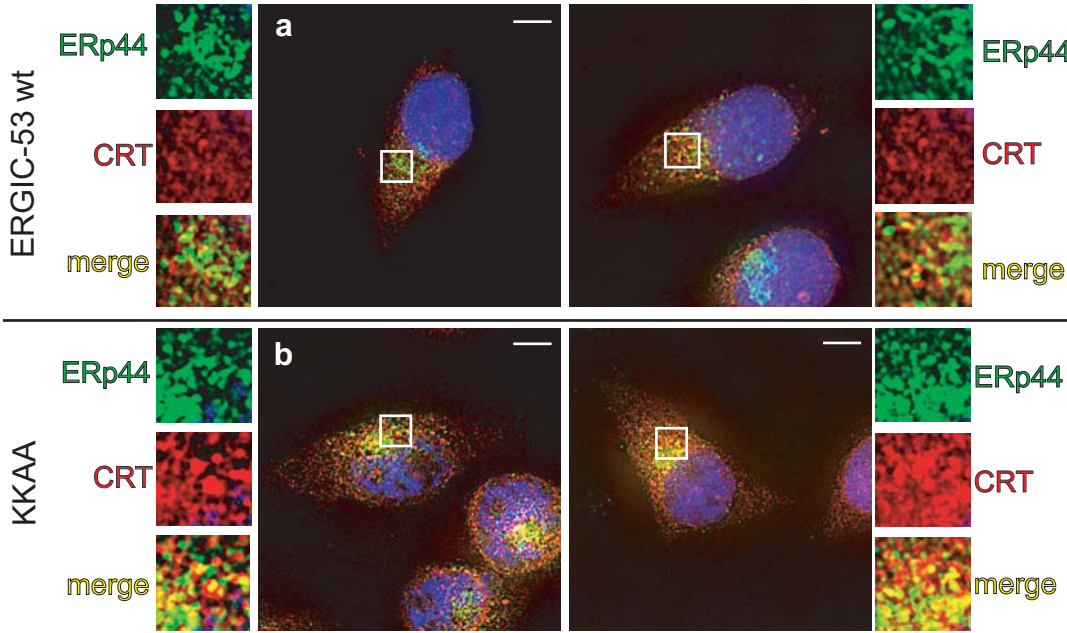
HeLa cells were transiently transfected with empty vectors (mock) or HA-ERp44, μ and λ chains (μλ44), as indicated. Cells were lysed in RIPA buffer + 10 mM NEM to preserve disulphide bonds. Lysates from 6 x 10^6 cells were immuno-precipitated with anti-HA antibody and resolved under non-reducing conditions, on 7,5% acrilamyde gel (lanes 2-5). An aliquot (50 μg) of the lysate of cells over-expressing HA-ERp44, μ and λ was loaded as a control (lane 1). After transfer to nitrocellulose, each the lane was cutted vertically in two halves and blots were decorated with anti-HA (sections 2, 4) or anti-μ (sections 1, 3, 5) antibodies, as indicated. A magnification of the upper part of the gel is shown on the right. The migration of the different assembly intermediates is indicated on the left hand margin. The empty arrow-head indicates a background band corresponding to the antibodies used for the IP (about 150 kDa). The fact that bands of the expected sizes are decorated by both anti-μ and anti-ERp44 confirms that both μλ and $\mu_2\lambda_2$ associate covalently with ERp44 and are precipitated by anti-HA.

Figure S7: *ERp44, ERGIC-53 and MCFD2 are coordinately induced during B to plasma cell differentiation*

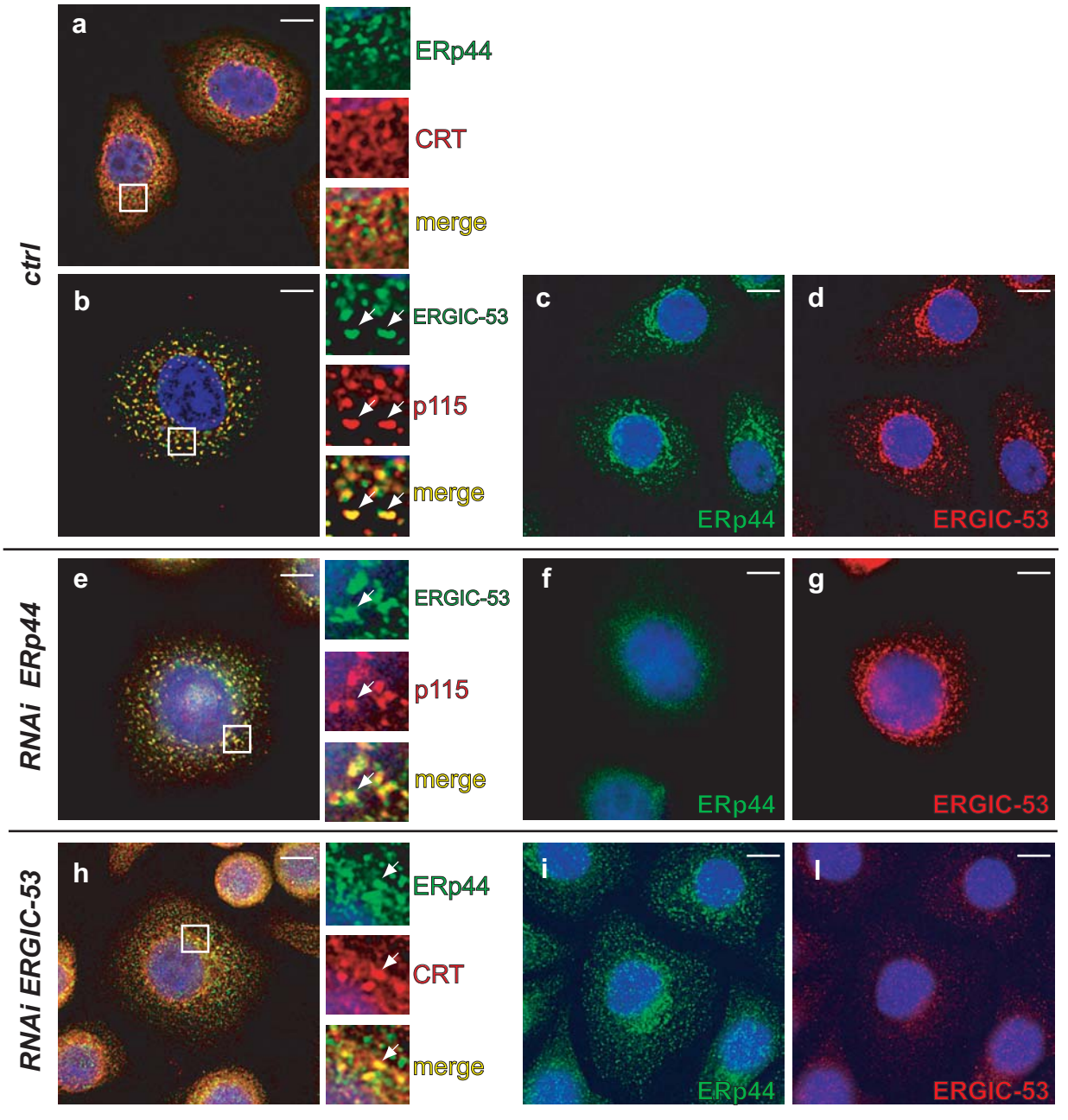
Murine splenocytes were activated in vitro with LPS (Cenci et al., 2006) for 0-4 days as indicated before RNA extraction and RT-PCR. Primers specific for the indicated transcripts were used in real time quantitative assays. Histone 3 was used to normalize sample loading. Note that ERp44, ERGIC-53 and MCFD2 transcripts are coordinately up-regulated, concomitant with the sharp increase of μ and J chain mRNAs in the last days of differentiation. As previously noted at the protein level (van Anken et al., 2003; Romijn et al., 2005), BiP mRNAs increase linearly during B cell differentiation.



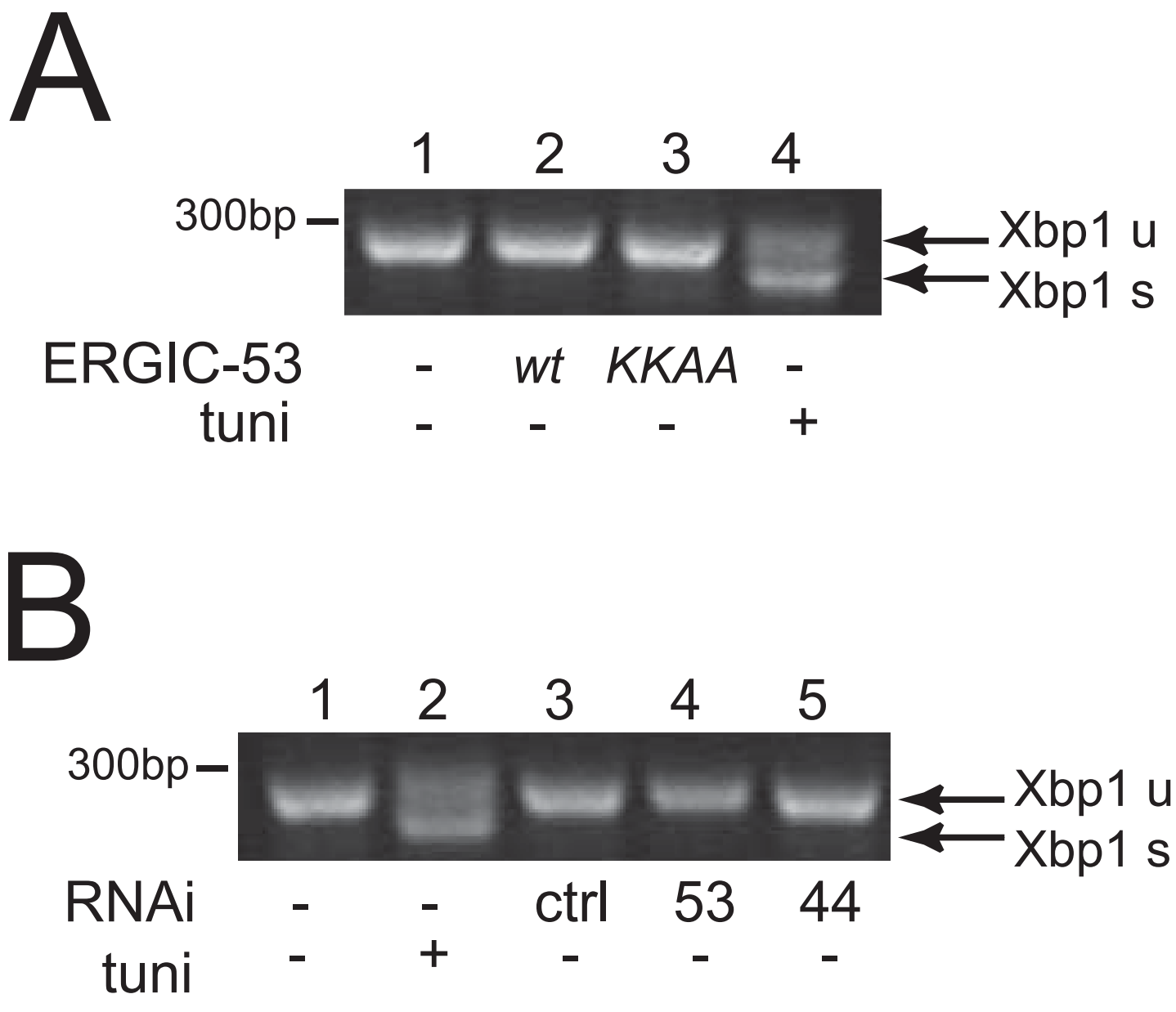
Anelli et al., Supplementary Figure 1



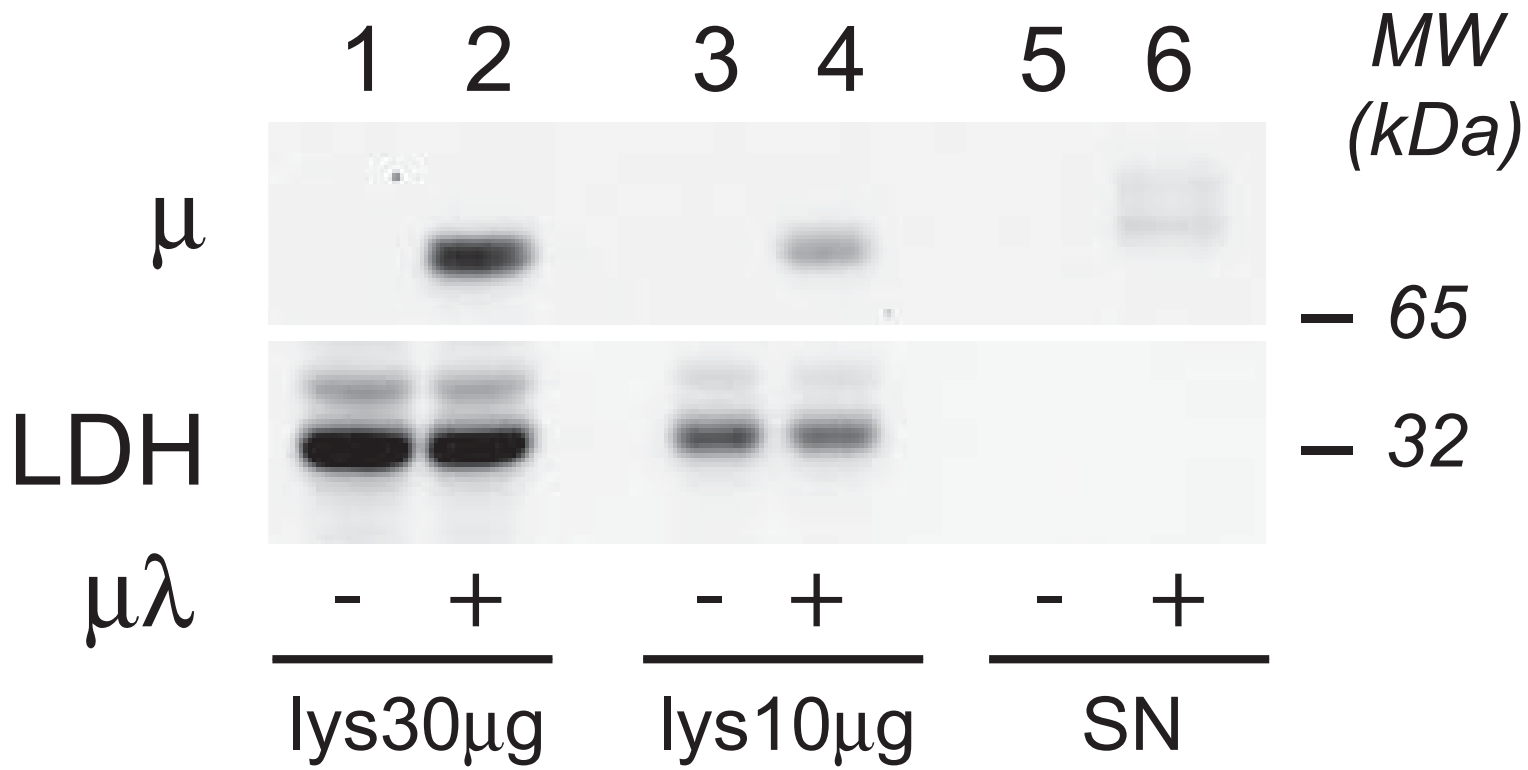
Anelli et al., Supplementary Figure 2



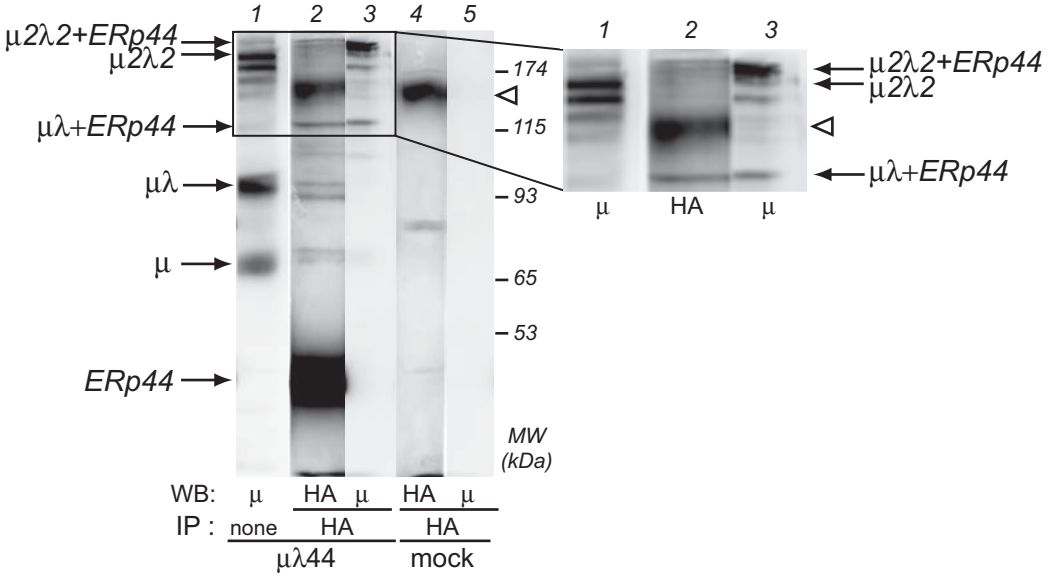
Anelli et al., Supplementary Figure 3



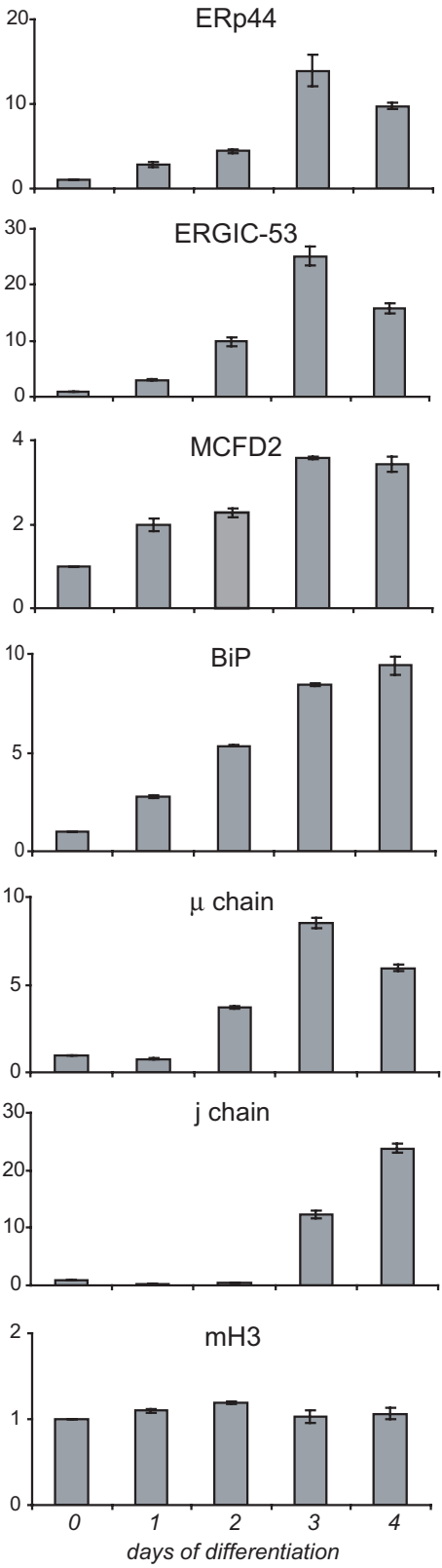
Anelli et al., Supplementary Figure 4



Anelli et al., Supplementary Figure 5



Anelli et al., Supplementary Figure 6



Anelli et al., Supplementary Figure 7