Title:

ER stress does not cause up-regulation and activation of caspase-2 to initiate apoptosis

Running title:

Caspase-2 is not regulated by ER stress

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Abbreviations: ER, endoplasmic reticulum; MEF, mouse embryonic fibroblast; FDM, factor dependent myeloid; BFA, Brefeldin A; TG, Thapsigargin; TM, Tunicamycin.

Abstract:

A recent report claimed that endoplasmic reticulum (ER) stress activates the ER trans-membrane receptor IRE1α, leading to increased caspase-2 levels via degradation of microRNAs, and consequently induction of apoptosis. This observation casts caspase-2 into a central role in the apoptosis triggered by ER-stress. We have used multiple cell types from caspase-2 deficient mice to test this hypothesis but failed to find significant impact of loss of caspase-2 on ER stress induced apoptosis. Moreover, we did not observe increased expression of caspase-2 protein in response to ER stress. Our data strongly argue against a critical role for caspase-2 in ER stress induced apoptosis.

Keywords:

Caspase-2, ER stress, apoptosis

Introduction

Secreted and trans-membrane proteins are shuttled into the secretory pathway by translocation to the endoplasmic reticulum (ER). Proteins must be correctly folded before being transported to their target compartment, with incorrectly folded protein being targeted for degradation.[1] The accumulation of mis-folded protein in the ER can be caused by multiple physiological and pathological conditions and results in ER stress. Cellular responses to ER stress are regulated by ER localized stress receptors, but the mechanisms by which this cytotoxic insult triggers apoptosis are not fully resolved although the BH3-only proteins Bim[2] and Puma[3, 4] have both been demonstrated to contribute to this process.

Recently, Upton *et al.*[5] reported a novel mechanism whereby the ER trans-membrane receptor IRE1α cleaved several microRNAs, which in turn promoted the de-repression of caspase-2 translation, leading to activation of this caspase and consequent cell death. Thus, when ER stress was induced in mouse embryonic fibroblasts (MEFs) by treatment with Brefeldin A (BFA), caspase-2 protein levels increased dramatically, followed by proteolytic activation of this caspase and induction of apoptosis.

These results imply that caspase-2 is required for apoptosis triggered by ER stressors. This is an important observation as, to date, the physiological role of caspase-2 has remained obscure. Previous analysis of caspase-2 deficient mice did not identify any developmental abnormalities that might be expected from a defect in ER stress induced apoptosis,[6] although evidence does indicate that caspase-2 has a tumor suppressive role in certain transgenic mouse cancer models[7-9] and an anti-ageing role.[10] In this study we have sought

to clarify the role of caspase-2 in the cellular response to ER stress. In contrast to the published report[5], we found that caspase-2 levels were not up-regulated by drugs that induce ER stress nor was there any significant impact of loss of caspase-2 on the extent of cell death elicited by ER stress. Our data demonstrate that caspase-2 levels are not modulated by ER stress and that caspase-2 is not required for the induction of apoptosis following ER stress. This work emphasizes the importance of independent repetition of results and the use of independent cell preparations in order to establish the veracity of new physiological roles for proteins, such as caspase-2.

Results

To evaluate the requirement for caspase-2 in apoptosis induced by ER stress we exposed a range of primary and immortalized cell types from wild-type (wt) and caspase-2 deficient mice to the same ER stress inducing compounds employed in the study by Upton and colleagues.[5] We treated primary and immortalized MEFs from wt and *Caspase-2*$^{-/-}$ mice with the ER stress inducing drugs Brefeldin A (BFA), Thapsigargin (TG) or Tunicamycin (TM). In both primary and immortalized MEFs we observed an equivalent amount of cell death between wt and *Caspase-2*$^{-/-}$ cells across a broad range of drug concentrations and over an extended time course (Figure 1a and b). Any small differences that were observed between wt and *Caspase-2*$^{-/-}$ cells were not consistent between the primary and immortalized MEFs. As a control we used MEFs derived from *Bax*$^{-/-}$;*Bak*$^{-/-}$ mice.[11, 12] As expected given the essential overlapping roles of Bax and Bak in the intrinsic (also called Bcl-2-regulated, mitochondrial or stress induced) apoptosis pathway,[11, 13] the Bax/Bak

double deficient cells were largely resistant to apoptosis induced by these drugs (Figure 1b). Moreover, in contrast to the previous publication,[5] we did not observe any increase in caspase-2 protein expression at any time-point following induction of ER stress in wt or *Bax*$^{-/-}$;*Bak*$^{-/-}$ MEFs (Figure 1c and d). This indicates that caspase-2 is not required for apoptosis induced by BFA, TG or TM in MEFs and that caspase-2 expression was not increased in response to ER stress.

To further investigate the involvement of caspase-2 in the response to ER stress induced apoptosis, we exposed primary mouse thymocytes, mature T lymphocytes and Hoxb8-transformed IL-3 dependent myeloid progenitors (FDM) from wt and *Caspase-2*$^{-/-}$ mice to BFA and TG. As a control to reveal the impact of a substantial block in the intrinsic apoptotic pathway, we used primary mouse thymocytes and FDM cells from *Bax*$^{-/-}$;*Bak*$^{-/-}$ mice. The wt and *Caspase-2*$^{-/-}$ thymocytes, T lymphocytes and FDM cells were either equally susceptible to ER-stress induced across a broad range of drug concentrations and over an extended time course of analysis or in the case of *caspase-2*$^{-/-}$ FDM cells appeared slightly more susceptible to BFA and low doses of TG at early time points than wt cells (Figure 2a, c and e; Supplementary Figure 1). This shows that caspase-2 is not required for apoptosis induced by BFA or TG in these cell types. In contrast, the *Bax*$^{-/-}$;*Bak*$^{-/-}$ cells were profoundly resistant to BFA. Whilst a substantial fraction of *Bax*$^{-/-}$;*Bak*$^{-/-}$ FDMs and thymocytes died after exposure to TG, the numbers of viable cells were still far greater than those observed with wt or *Caspase-2*$^{-/-}$ cells. In contrast to Upton *et al.,*[5] we did not observe in any of the wt or *Bax*$^{-/-}$;*Bak*$^{-/-}$ cell types examined, an increase in full-length caspase-2 protein levels following ER stress (Figure 2b, d and f; Supplementary Figure 2).

Furthermore, in response to ER stress, caspase-3 was cleaved (and hence activated) over time in both wt and caspase-2 deficient cells, but not in *Bax*$^{-/-}$;*Bak*$^{-/-}$ cells. These data demonstrate first, that caspase-2 was not required for processing and activation of caspase-3, and second that the loss of viability observed in *Bax*$^{-/-}$;*Bak*$^{-/-}$ cells was independent of caspase-3 activation. It is therefore possible that this death of *Bax*$^{-/-}$;*Bak*$^{-/-}$ cells is mediated (at least in part) through a non-apoptotic process. In addition, ER stress resulted in cleavage of caspase-2 only in wt but not in *Bax*$^{-/-}$;*Bak*$^{-/-}$ cells. This is consistent with previous studies using other apoptotic stimuli (e.g. γ-irradiation[6]), and demonstrates that caspase-2 cleavage (as opposed to activation, which does not necessarily require processing[14]) constitutes an event that occurs downstream of Bax/Bak activation and the formation of the apoptosome[6].

To extend our observations, we used NALM-6, RAJI, MV4-11 and U-937 cells, which are all human leukemia or lymphoma derived cell lines. These cells were treated with TM, TG or BFA and examined over a time course for changes in the expression levels of caspase-2 protein (Figure 3; Supplementary Figure 3). Again, there was no significant increase in caspase-2 expression following the induction of ER stress. Collectively, these results demonstrate that caspase-2 protein expression is not induced in response to ER stress and does not contribute to cell death resulting from ER stress.

We observed abundant full-length caspase-2 in all cell types in the absence of ER stress or any other cytotoxic insult. Perplexingly, over two different studies Upton and colleagues reported strikingly diametrically opposed results on the levels of caspase-2 expression, even

in the same cells.[5,15] These discrepancies could be explained, in part, by differences in the specificity and quality of the various caspase-2 antibodies used in these two studies. To interrogate this possibility, we compared the antibodies employed by these authors using the same lysates derived from wt and *caspase-2*$^{-/-}$ (negative control) thymocytes that had been treated with BFA or vehicle (Figure 4). A monoclonal antibody that we have previously generated (mAb 10C6[6]) showed exquisite specificity for caspase-2, whereas the two antibodies used by Upton *et al.* in their recent study[5] seem to detect abundant non-specific bands, mostly showing no difference between lysates from thymocytes of wt versus *caspase-2*$^{-/-}$ mice. The caspase-2 antibody from Novus does appear to detect full-length caspase-2 (amongst many non-specific bands), however, the appearance of any cleavage products is masked by the presence of non-specific bands.

Discussion

Caspase-2 remains an enigmatic protein with recently established functions in tumor suppression, genomic stability and ageing.[9, 10, 16, 17] This caspase is evolutionarily highly conserved, yet surprisingly loss of caspase-2 has no significant impact on the development of mice. *Caspase-2*$^{-/-}$ mice show mild pre-mature ageing phenotypes but no overt morphological abnormalities; they breed normally and have no clearly recognizable defects in apoptosis across a broad range of cell types.[6, 18, 19] Using a large array of primary as well as immortalized mouse and human cell types, our data demonstrate that caspase-2 levels do not increase in response to ER stress and that caspase-2 is not required for ER stress induced apoptosis.

The observation that caspase-2 may be involved in the apoptotic response to ER stress[5] was surprising given the lack of easily recognizable phenotypic abnormalities in caspase-2 deficient mice, but important nonetheless because this was a clear indication of a hitherto unrecognized function of this caspase. The model published by Upton *et al*. proposed that in response to ER stress caspase-2 protein levels were up-regulated due to IRE1α mediated cleavage of microRNAs that otherwise repressed caspase-2 translation. Regardless of any regulation by microRNAs, this model casts caspase-2 in a central role in the apoptosis that occurs in response to ER stress. In contrast to these published data,[5] we did not observe any increase in caspase-2 protein expression at various time points or doses following ER stress inducing drugs, nor any significant difference in viability between wt versus caspase-2 deficient cells following treatment with different ER stress inducing drugs. It is unclear why our results differ from Upton and colleagues as we used the same cell lines (and additional primary and immortalized cell lines) with the same drugs, doses and treatment time points. One potential reason for the discrepancies in our observations is the different antibodies used to detect caspase-2 protein. We found the antibodies used by Upton and colleagues were unreliable and largely non-specific (Figure 4).

Our findings are supported by previous studies, which treated a variety of *caspase-2*$^{-/-}$ cells with drugs to induce ER stress, also finding no difference in cell viability when compared to wt cells[20]. Moreover, our data demonstrate that although IRE1α may regulate the abundance of specific microRNAs in response to ER stress as reported in the original study, caspase-2 protein expression is not regulated by any such changes. We did not analyze changes to *caspase-2* mRNA expression in response to ER stress as regulation of gene

expression by microRNAs can occur by blocking translation of mRNA in addition to degradation of mRNA transcripts; therefore this approach is likely to be uninformative. Regardless, our protein expression and cell survival analyses demonstrate clearly that caspase-2 expression is not regulated in response to ER stress and, even more importantly, that caspase-2 is not required for ER stress-induced cell death.

Materials and Methods

Mice

All experiments with mice were approved by The Walter and Eliza Hall Institute Animal Ethics committee and SA Pathology Animal Ethics Committee. The *caspase-2*$^{-/-}$ [6] and *Bax*$^{-/-}$*;Bak*$^{-/-}$ mice[11, 21] have been previously described. The *caspase-2*$^{-/-}$, *Bax*$^{-/-}$ and *Bak*$^{-/-}$ mice (the latter two used to generate the *Bax*$^{-/-}$*;Bak*$^{-/-}$ mice) were all originally generated on a mixed C57BL/6 x 129SV genetic background using 129SV-derived ES cells but were subsequently backcrossed onto a C57BL/6 background for 10 to >20 generations.

Survival assays with primary cells and cell lines

Primary MEFs were isolated and cultured as described previously.[17, 22] Spontaneously immortalized MEFs were generated by serial passaging of MEFs in culture, using the 3T3 subculture method.[16] Primary mouse thymocytes were isolated and cultured as described previously.[6] Wild-type, *Caspase-2*$^{-/-}$ and *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDM cells were generated as described previously.[23] Wild-type mice were reconstituted with *Bax*$^{-/-}$*;Bak*$^{-/-}$ hematopoietic stem cells (using fetal liver cells from E14.5 embryos) as described previously and were a kind gift from Francine Ke.[21] T lymphocytes were purified from spleens and lymph nodes of wild-type and c*aspase-2*$^{-/-}$ mice as described previously.[24] Brefeldin A, Thapsigargin and Tunicamycin (Sigma, St. Louis, MO, USA) were dissolved in DMSO (experiments with MEFs) or ethanol (experiments with FDMs, thymocytes, T lymphocytes). For human leukemia or lymphoma derived cell lines, Brefeldin A was dissolved in DMEM (Gibco, Mulgrave, VIC, Australia), Tunicamycin was dissolved in ethanol and Thapsigargin was

dissolved in DMSO. Cell viability was determined by staining cells with FITC-coupled Annexin V (Invitrogen, Mulgrave, VIC, Australia) in balanced salt solution including 5 mM $CaCl_2$ and Propidium Iodide (PI 5 μg/ml, Sigma) followed by flow cytometric analysis in a LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) or a FC500 flow cytometer (Beckman Coulter, Lane Cove, NSW, Australia).

Western blot analysis

Total cell lysates were prepared by lysing cells either directly into SDS containing gel loading buffer (250mM Tris-HCl, 4% glycerol, 5% SDS, 0.25%. bromophenol blue, 5% 2-mercaptoethanol; for experiments with thymocytes, T lymphocytes and FDM cells), RIPA buffer (25 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% nonylphenoxylpolyethoxylethanol (NP-40), 1% sodium deoxycholate, 0.1% sodiumdodecylsulphate (SDS) in the presence of protease/phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, USA); for experiments with MEFs) and lysed by freeze/thaw cycles in liquid N2 and ice cold water or in ONYX lysis buffer (20 mM Tris-HCl pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% digitonin for experiments with human leukemia or lymphoma derived cell lines). Lysates were run on SDS-PAGE gels, proteins transferred to polyvinylidenedifluoride (PVDF) membranes, which were probed with antibodies against caspase-2 (clones 10C6 and 11B4,[6] Enzo Life Sciences; Novus Y61 #NB-110-55655; Abcam #ab18737), total caspase-3 (clone 8G10), cleaved (i.e. activated) caspase-3 (Cell Signalling) and β-actin (AC15, Sigma; used as a loading control).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Supplementary information is available at Cell Death and Differentiation's website

References

1. Smith MH, Ploegh HL, Weissman JS. Road to ruin: targeting proteins for degradation in the endoplasmic reticulum. *Science* 2011 Nov 25; **334**(6059)**:** 1086-1090.

2. Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND*, et al.* ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* 2007 Jun 29; **129**(7)**:** 1337-1349.

3. Ghosh AP, Klocke BJ, Ballestas ME, Roth KA. CHOP potentially co-operates with FOXO3a in neuronal cells to regulate PUMA and BIM expression in response to ER stress. *PloS one* 2012; **7**(6)**:** e39586.

4. Kieran D, Woods I, Villunger A, Strasser A, Prehn JH. Deletion of the BH3-only protein puma protects motoneurons from ER stress-induced apoptosis and delays motoneuron loss in ALS mice. *Proceedings of the National Academy of Sciences of the United States of America* 2007 Dec 18; **104**(51)**:** 20606-20611.

5. Upton JP, Wang L, Han D, Wang ES, Huskey NE, Lim L*, et al.* IRE1alpha cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. *Science* 2012 Nov 9; **338**(6108)**:** 818-822.

6. O'Reilly L, Ekert P, Harvey N, Marsden V, Cullen L, Vaux D*, et al.* Caspase-2 is not required for thymocyte or neuronal apoptosis even though cleavage of caspase-2 is dependent on both Apaf-1 and caspase-9. *Cell death and differentiation* 2002; **9:** 832-841.

7. Manzl C, Peintner L, Krumschnabel G, Bock F, Labi V, Drach M*, et al.* PIDDosome-independent tumor suppression by Caspase-2. *Cell death and differentiation* 2012 Oct; **19**(10)**:** 1722-1732.

8. Parsons MJ, McCormick L, Janke L, Howard A, Bouchier-Hayes L, Green DR. Genetic deletion of caspase-2 accelerates MMTV/c-neu-driven mammary carcinogenesis in mice. *Cell death and differentiation* 2013 Sep; **20**(9)**:** 1174-1182.

9. Puccini J, Dorstyn L, Kumar S. Caspase-2 as a tumour suppressor. *Cell death and differentiation* 2013 Sep; **20**(9)**:** 1133-1139.

10. Shalini S, Dorstyn L, Wilson C, Puccini J, Ho L, Kumar S. Impaired antioxidant defence and accumulation of oxidative stress in caspase-2-deficient mice. *Cell death and differentiation* 2012 Aug; **19**(8)**:** 1370-1380.

11. Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA*, et al.* The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Molecular cell* 2000 Dec; **6**(6)**:** 1389-1399.

12. Rathmell JC, Lindsten T, Zong WX, Cinalli RM, Thompson CB. Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. *Nature immunology* 2002; **3**(10)**:** 932-939.

13. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008 Jan; **9**(1)**:** 47-59.

14. Baliga BC, Read SH, Kumar S. The biochemical mechanism of caspase-2 activation. *Cell death and differentiation* 2004 Aug 6.

15. Upton JP, Austgen K, Nishino M, Coakley KM, Hagen A, Han D*, et al.* Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. *Molecular and cellular biology* 2008 Jun; **28**(12)**:** 3943-3951.

16. Dorstyn L, Puccini J, Wilson CH, Shalini S, Nicola M, Moore S*, et al.* Caspase-2 deficiency promotes aberrant DNA-damage response and genetic instability. *Cell death and differentiation* 2012 Aug; **19**(8)**:** 1288-1298.

17. Ho LH, Taylor R, Dorstyn L, Cakouros D, Bouillet P, Kumar S. A tumor suppressor function for caspase-2. *Proceedings of the National Academy of Sciences of the United States of America* 2009 Mar 31; **106**(13)**:** 5336-5341.

18. Bergeron L, Perez GI, Macdonald G, Shi LF, Sun Y, Jurisicova A*, et al.* Defects in regulation of apoptosis in caspase-2-deficient mice. *Genes & Development* 1998; **12**(9)**:** 1304-1314.

19. Marsden VS, Ekert PG, Van Delft M, Vaux DL, Adams JM, Strasser A. Bcl-2-regulated apoptosis and cytochrome c release can occur independently of both caspase-2 and caspase-9. *The Journal of cell biology* 2004 Jun 21; **165**(6)**:** 775-780.

20. Manzl C, Krumschnabel G, Bock F, Sohm B, Labi V, Baumgartner F*, et al.* Caspase-2 activation in the absence of PIDDosome formation. *The Journal of cell biology* 2009 Apr 20; **185**(2)**:** 291-303.

21. Mason KD, Lin A, Robb L, Josefsson EC, Henley KJ, Gray DH*, et al.* Proapoptotic Bak and Bax guard against fatal systemic and organ-specific autoimmune disease. *Proceedings of the National Academy of Sciences of the United States of America* 2013 Feb 12; **110**(7)**:** 2599-2604.

22. Ho LH, Read SH, Dorstyn L, Lambrusco L, Kumar S. Caspase-2 is required for cell death induced by cytoskeletal disruption. *Oncogene* 2008 May 29; **27**(24)**:** 3393-3404.

23. Ekert PG, Jabbour AM, Manoharan A, Heraud JE, Yu J, Pakusch M*, et al.* Cell death provoked by loss of interleukin-3 signaling is independent of Bad, Bim, and PI3 kinase, but depends in part on Puma. *Blood* 2006 Sep 1; **108**(5)**:** 1461-1468.

24. O'Reilly LA, Kruse EA, Puthalakath H, Kelly PN, Kaufmann T, Huang DC*, et al.* MEK/ERK-mediated phosphorylation of Bim is required to ensure survival of T and B lymphocytes during mitogenic stimulation. *Journal of immunology* 2009 Jul 1; **183**(1)**:** 261-269.

Titles and legends to figures

Figure 1. Caspase-2 is dispensable for ER stress induced killing of MEFs. (a) Wild-type and *caspase-2*$^{-/-}$ primary MEFs (WT n=5, *caspase-2*$^{-/-}$ n=4) were treated with Brefeldin A (BFA, 2.5 μg/ml), Thapsigargin (TG, 1μM), Tunicamycin (TM, 5 μg/ml) or vehicle for 24 h and Annexin V negative/PI negative (i.e. surviving) cells quantified by flow cytometry. (b) Spontaneously immortalized MEFs (WT n=4-8, *caspase-2*$^{-/-}$n=5-9, *Bax*$^{-/-}$*;Bak*$^{-/-}$n=4-5) were treated with BFA, TG, TM (at the doses indicated) or vehicle for 24 h and Annexin V negative/PI negative (i.e. surviving) cells quantified by flow cytometry. (c) Primary MEFs and spontaneously immortalized MEFs were treated with ER stress inducing drugs for 24 h as described in (a). Cell lysates were analyzed by immunoblotting using antibodies against caspase-2 (clone 11B4), caspase-3 and β-actin (loading control). (d) Wild-type, *Caspase-2*$^{-/-}$ and *Bax*$^{-/-}$*;Bak*$^{-/-}$ spontaneously immortalized MEFs were treated with BFA (2.5 μg/ml) over the indicated time course. Cell lysates were analyzed by immunoblotting using antibodies against caspase-2 (clone 11B4), cleaved (i.e. activated) caspase-3 and β-actin (loading control). Full-length proteins are designated (FL) with cleavage products following ER stress indicated as (C). P-values were calculated using unpaired t-test, (ns) signifies $p>0.05$, (*) signifies $p<0.05$.

Figure 2. Caspase-2 is dispensable for ER stress induced killing in diverse cell types.
(a) Wild-type, *caspase-2*$^{-/-}$ and *Bax*$^{-/-}$*;Bak*$^{-/-}$ primary mouse thymocytes (WT n=6, *caspase-2*$^{-/-}$ n=5, *Bax*$^{-/-}$*;Bak*$^{-/-}$n=4) were treated with BFA (2.5 μg/ml), TG (1 μM) or vehicle over the indicated time course and Annexin V negative/PI negative (i.e. surviving) cells

quantified by flow cytometry. (b) Primary mouse thymocytes from wild-type, *caspase-2*$^{-/-}$ and *Bax*$^{-/-}$*;Bak*$^{-/-}$ mice were treated with ER stress inducing drugs over the indicated time course as described in (a). Cell lysates were analyzed by immunoblotting using antibodies against caspase-2 (clone 10C6), cleaved (i.e. activated) caspase-3 and β-actin (loading control). (c) Wild-type and *Caspase-2*$^{-/-}$ T lymphocytes (both n=5) were treated with BFA (2.5 μg/ml) or vehicle over the indicated time course and Annexin V negative/PI negative (i.e. surviving) cells quantified by flow cytometry. (d) T lymphocytes from wild-type and *caspase-2*$^{-/-}$ mice were treated with ER stress inducing drugs over the indicated time course as described in (c). Cell lysates were analyzed by immunoblotting using antibodies against caspase-2 (clone 10C6), cleaved (i.e. activated) caspase-3 and β-actin (loading control). (e) Wild-type, *caspase-2*$^{-/-}$ and *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDMs (WT n=6, *caspase-2*$^{-/-}$ n=5, *Bax*$^{-/-}$*;Bak*$^{-/-}$ n=3) were treated with BFA, TG (at the indicated doses) or vehicle over the indicated time course and Annexin V neagtive/PI negative (i.e. surviving) cells quantified by flow cytometry. ND = no data (f) Wild-type, *caspase-2*$^{-/-}$ and *Bax*$^{-/-}$*;Bak*$^{-/-}$ FDMs were treated with ER stress inducing drugs over the indicated time course as described in (e). Cell lysates were analyzed by immunoblotting using antibodies against caspase-2 (clone 10C6), cleaved (i.e. activated) caspase-3 and β-actin (loading control). Full-length proteins are designated (FL) with cleavage products following ER stress indicated as (C). P-values were calculated using unpaired t-test, (ns) signifies $p>0.05$, (*) signifies $p<0.05$, (**) signifies $p<0.001$, (***) signifies $p<0.0001$.

Figure 3. Caspase-2 protein levels are not up-regulated following ER stress in several human leukemia and lymphoma derived cell lines. NALM-6, RAJI, MV4-11 and U-937

cells were treated with TM (2.5 μg/ml), TG (2 μM) or BFA (2.5 μg/ml) over the indicated time course. Cell lysates were analyzed by immunoblotting using antibodies against caspase-2 (clone 10C6), cleaved (i.e. activated) caspase-3 and β-actin (loading control). Full-length proteins are designated (FL) with cleavage products following ER stress indicated (C).

Figure 4. Comparison of the specificities of different caspase-2 antibodies. Primary thymocytes from wild-type and *caspase-2*$^{-/-}$ mice were treated with BFA (2.5 μg/ml) or vehicle over the indicated time course. Cell lysates were analyzed by immunoblotting using the following antibodies against caspase-2: clone 10C6 (Enzo[6]), Novus Y61 #NB-110-55655 or Abcam #ab18737 or were probed, as a loading control, with an antibody to β-actin. All images from blots produced by probing with the various antibodies to caspase-2 were exposed for an equal period of time. Full-length proteins are designated (FL) with cleavage products following ER stress indicated (C).