# Short Communication

## GRP78 expression in canine mammary tumors: association with malignancy

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### Abstract

78-kDa glucose-regulated protein (GRP78) is over-expressed in human breast carcinomas. GRP78 expression was studied in 40 spontaneous canine mammary tumors and evaluated in relation to tumor histological type, mode of growth, grade, lymph node metastases and distant metastases. All tumors exhibited GRP78 immunostaining. In the normal canine mammary gland, GRP78 was also expressed although not in all cases. In carcinomas GRP78 was detected in the cytoplasm in more than 50% of tumor cells in the vast majority of cases (87.5%). There was a significant association between the absence of squamous differentiation ($P = 0.02$) and GRP78 over-expression, but no association with other clinico-pathological features. GRP78 was often co-expressed with galectin-3 in canine mammary tumors (CMT).

The microenvironment of solid tumors possesses several stressful conditions such as hypoxia, low pH, and glucose deprivation (Delie *et al.* 2012, Li *et al.* 2012). These conditions induce tumor cells to express a family of proteins which trigger protective mechanisms (Fernandez *et al.* 2000, Lee 1992, Li & Li 2012), the heat shock proteins (HSPs) and glucose-regulated proteins (GRPs) (Fernandez *et al.* 2000, Prabhu *et al.* 2012). The best characterized GRP is the GRP78, also known as the immunoglobulin heavy chain binding protein (BiP). GRP78 is a chaperon protein present in the lumen of endoplasmatic reticulum (ER) where it plays an important role in cancer progression, malignancy and drug resistance (Delie *et al.* 2012, Li & Li 2012, Wang *et al.* 2009). Galectin-3 is a member of the galectins family of beta-galactosides recognizing lectins (Cummings *et al.* 2009). It has several roles in cancer progression, including an anti-apoptotic activity in response to microenvironment stressors (Lee 2007, Wang *et al.* 2009).

Canine mammary tumors (CMT) are the most frequent type of canine neoplasia accounting for approximately 25–50% of cases and 40–50% are malignant (de Oliveira *et al.* 2010). These spontaneously occurring tumors often metastasize and are thus considered a good model to study the process. This compared oncobiology approach has the potential to benefit both dogs and human breast cancer patients (Paoloni *et al.* 2008).

The present study aimed to assess GRP78 expression in benign and malignant CMT using immunohistochemistry; to determine whether it is associated with the clinicopathological features of CMT; and finally, to assess GRP78 co-expression with galectin-3 by immunofluorescence. The series of CMT was obtained from the Pathology Department archives of ICBAS-University of Porto. Briefly, formalin-fixed, paraffin-embedded H&E-stained sections examined by three pathologists, and classified according to the WHO classification for tumours in domestic animals, were used to assess GRP78 expression. Briefly, 4-µm-thick paraffin sections were deparaffinized, hydrated, and $H_2O_2$ was used to block endogenous peroxidase activity. After blocking, sections were incubated with rabbit anti-GRP78 (Santa Cruz Biotechnology, CA), overnight at 4°C. Incubation with a biotin conjugated swine anti-rabbit secondary antibody followed by the avidin–biotin–peroxidase complex during 30 minutes (Vector Laboratories) was performed. Diaminobenzidine tetrahydroxychloride solution (DAB, Dako) was used to reveal peroxidase activity and finally, sections were counterstained with hematoxylin. Results were analyzed using SPSS software (version 13.0). Association hypotheses were tested, using Fisher's exact test or Chi-square test for discrete variables. For simultaneous visualization of GRP78 and galectin-3 sections

were incubated with the primary antibodies rat anti-galectin-3 (Bioscience, CA) and rabbit anti-GRP78 (Santa Cruz Biotechnology, CA) for 1 hour at room temperature. Then, upon washing, sections were incubated with Alexa 588-conjugated donkey anti-rat and Alexa 594-conjugated rabbit anti-goat secondary antibodies (Invitrogen) for 45 minutes. All sections were incubated with 4'-6-Diamidino-2-phenylindole (DAPI) for 15 minutes and mounted in glycerol-based Vectashield medium (Vector, Burlingame, CA). Slides were analyzed under a Zeiss fluorescence microscope. Tumors were grouped, according to the percentage of GRP78 immunoreactive cells, into: <25%; 25–50%; 50–75%, or >75% stained tumor cells. Malignant CMT (40 cases) and benign CMT (5 cases) were used in the present study. Malignant CMT were classified according to the histological types, size, distant and lymph node metastases and squamous differentiation.

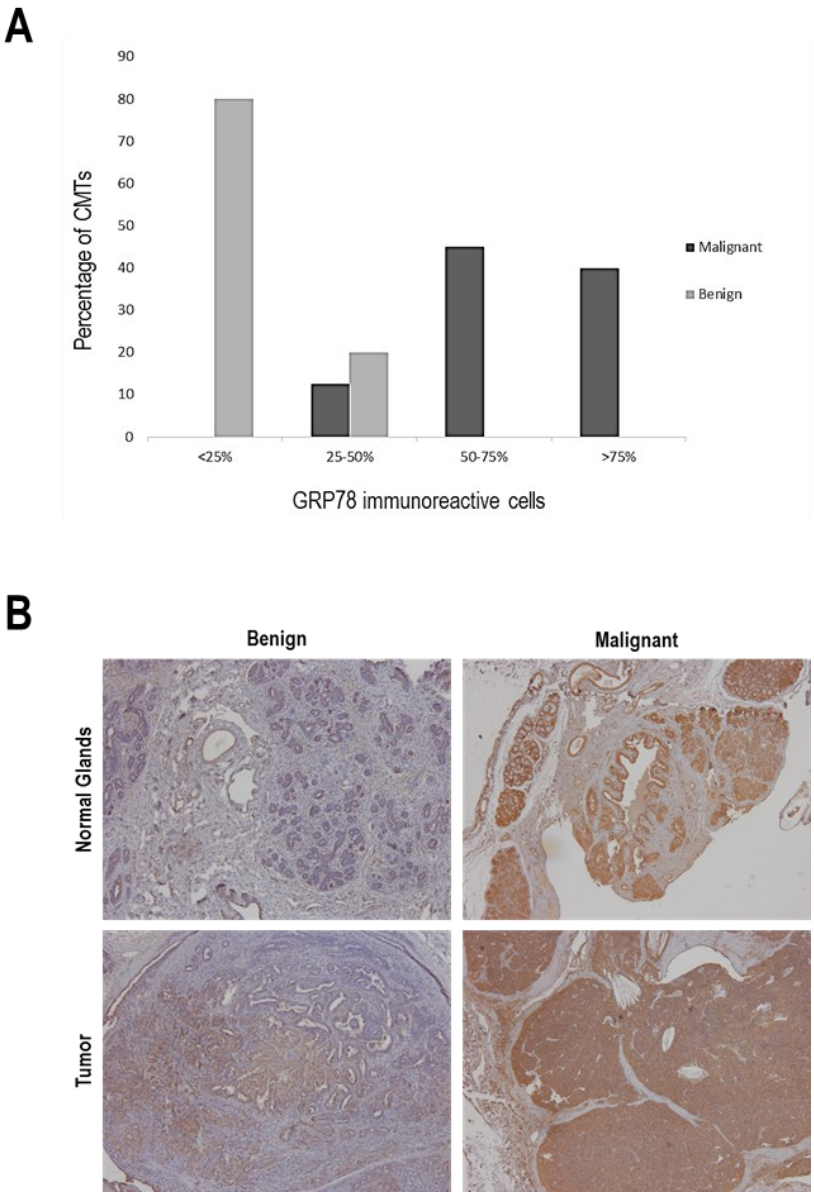


**Figure 1.** *GRP78 is up regulated in malignant CMT*. (A) GRP78 immunohistochemical staining was analyzed in a series malignant and benign CMTs, and tumors were compared according to the percentage of GRP78 expressing tumor cells. (B) Representative photomicrographs depict GRP78 immunostaining (brown color) with hematoxilin counterstain in malignant and benign CMT. GRP78 expression in CMTs was accessed by immunohistochemistry using the modified avidin–biotin–peroxidase complex method (de Oliveira et al. 2011), with an anti-GRP78 antibody (1:50; Santa Cruz Biotechnology). Peroxidase activity was examined using diaminobenzidine tetrahydroxychloride solution (DAB, Dako) as the substrate.

All tumors expressed GRP78, although expression levels differed between cases. The vast majority of malignant tumors presented GRP78 expression in more than 50% of tumor cells in 35 out of 40 cases (87.5%). Indeed, 18 out of 40 (45%) of malignant CMT cases presented GRP78 staining in more than 75% of tumor cells (Figure 1A). In striking contrast, there were no benign CMT with more than 75% stained cells (Figure 1A). Interestingly, the normal tissue adjacent to benign CMT also presented lower expression of GRP78 than that adjacent to malignant CMT as in the tumor itself (Figure 1B). Clinico-pathological features of tumors, as well as associations with GRP78 expression, are summarized in Table 1. No statistically significant associations were found in histological type, mode of growth, necrosis, lymph node metastases or distant metastases ($p>0.05$). However GRP78 presented a significant association with squamous differentiation ($p=0.02$). GRP78 was frequently co-expressed with galectin-3 in CMT both in the periphery and in invasive front of malignant CMT (Figure 2). Tumor microenvironment induces physiologic endoplasmic reticulum stress, and the UPR (unfolded protein response) is essential for survival of tumor cells subjected to persistent hypoxia (Lee *et al.* 2006). The chaperone protein GRP78 is a master UPR regulator, aberrantly expressed in a variety of human cancers (Prabhu *et al.* 2012). In our study both benign and malignant CMT expressed GRP78. However, GRP78 expression is higher in malignant when compared to benign tumors. Our results are in agreement with Fernandez *et al.* (2000), who showed that the levels of both GRP78 mRNA and protein in human breast cancer were increased in the malignant tumors when compared to the benign tumors and normal tissues. GRP78 expression is highly elevated in different cancer cell lines, solid tumors, and human cancer biopsies, being frequently related to malignancy and metastasis (Wang *et al.* 2009). Since GRP78/BIP

**Table 1.** Association between clinico-pathological features of tumors and GRP78 expression.

| | | GRP78 Expression[2] | | | | |
|---|---|---|---|---|---|---|
| Clinical feature | Cases[1] (%) | <25% | 25-50% | 50-75% | >75% | *p*-Value[3] |
| **Histological Type (n=38)** | | | | | | 0,775 |
| Simple | 24 (63.16%) | 0 (0%) | 3 (75%) | 11 (61.1%) | 10 (62.5%) | |
| Complex | 7 (14.29%) | 0 (0%) | 1 (25%) | 4 (22.2%) | 2 (12.5%) | |
| Mist | 7 (14.29%) | 0 (0%) | 0 (0%) | 3 (16.7%) | 4 (25%) | |
| **Growth (n=39)** | | | | | | 0.626 |
| T1 | 25 (64.1%) | 0 (0%) | 3 (60%) | 12 (70.6%) | 10 (55.6%) | |
| T2 | 7 (17.9%) | 0 (0%) | 0 (0%) | 4 (23.5%) | 3 (16.7%) | |
| T3 | 6 (15.4%) | 0 (0%) | 1 (40%) | 1 (5.89%) | 4 (22.2%) | |
| T5 | 1 (2.6%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (5.5%) | |
| **Necrosis (n=40)** | | | | | | 0.538 |
| No | 9 (22.5%) | 0 (0%) | 2 (40%) | 4 (23.5%) | 3 (16.7%) | |
| Yes | 31 (77.5%) | 0 (0%) | 3 (60%) | 13 (76.5%) | 15 (83.3%) | |
| **Lymph node metastases (n=30)** | | | | | | 0.725 |
| No | 20 (66.7%) | 0 (0%) | 3(75%) | 7 (58.3%) | 10 (71.4%) | |
| Yes | 10 (33.3%) | 0 (0%) | 1 (25%) | 5 (41.7%) | 4 (28.6%) | |
| **Distant metastases (n=36)** | | | | | | 0.118 |
| No | 26 (72.2%) | 0 (0%) | 2 (50%) | 15 (88.2%) | 9 (60%) | |
| Yes | 10 (28.8%) | 0 (0%) | 2 (50%) | 2 (11.8%) | 6 (40%) | |
| **Squamous differentiation (n=40)** | | | | | | **0.020** |
| No | 33 (82.5%) | 0 (0%) | 2 (40%) | 16 (94.1%) | 15 (83.3%) | |
| Yes | 7 (17.5%) | 0 (0%) | 3 (60%) | 1 (0.06%) | 3 (16.7%) | |

[1] Forty CMT were randomly selected from the files of the Veterinary Pathology Department of ICBAS-UP. Histological examination was performed independently by two pathologists and tumors were classified according to WHO criteria (Misdorp et al., 1999).

[2] Immunohystochemestry studies were performed and the tumors were grouped into <25%; 25–50%; 50–75%, or >75% stained tumor cells according to the percentage of GRP78 immunoreactive cells.

[3] SPSS software (version 13.0) was used for statistical analysis, applying Fisher's exact or $\chi^2$ tests. Samples were scored according to the percentage of GRP78 positive cells.

is induced under adverse conditions for cell survival, such as tumor microenvironment, acting as an inhibitor of stress-induced apoptosis it is feasible to think that it might be up-regulated in malignancy. High expression of GRP78 is related to the cells' exposure to stress such as hypoxia and glucose deprivation, which in turn are associated with more aggressive tumor behavior (Lee 2001). To the best of our knowledge, this is the first study of GRP78 expression in CMT.

There was no significant association between tumor clinicopathological features and GRP78 expression. Both benign and malignant CMTs express GRP78 in adjacent mammary glands which is in agreement with a recent study which denotes that GRP78 expression are related with mammary gland development (Zhu *et al.* 2014). The fact that normal mammary glands adjacent to malignant lesions present more GRP78 than those observed in benign cases reinforces a role for progression towards malignancy and its related microenvironment in up-regulating GRP78 expression. Previous studies show that GRP78 is essential for human tumor progression due to its roles played in several processes such as proliferation, apoptosis inhibition, angiogenesis promotion and genomic instability (Jamora *et al.* 1996, Li & Li 2012, Wang *et al.* 2009). GRP78 is involved in the regulation of invasion in cancer by modulated FAK and JNK signaling pathways (Yuan *et al.* 2015). GRP78 also promotes tumor aggressiveness by enhancing AKT signaling and also protects the tumor cells from apoptosis, enabling them to continue to proliferate. Among other mechanisms, GRP78 over-expression blocks BIK-induced apoptosis, suppressed estrogen starvation-induced BAX activation, hindering mitochondrial permeability transition and, consequently, apoptosis (Zhou *et al.* 2011). Regarding apoptosis suppression, our present data points to a combined expression of GRP78 and anti-apoptotic galectin-3. Further studies are warranted in order to ascertain if there is any common link in the regulation of these two proteins during mammary tumor progression. As galectin-3, GRP78 can also elevate the number of inflamma-

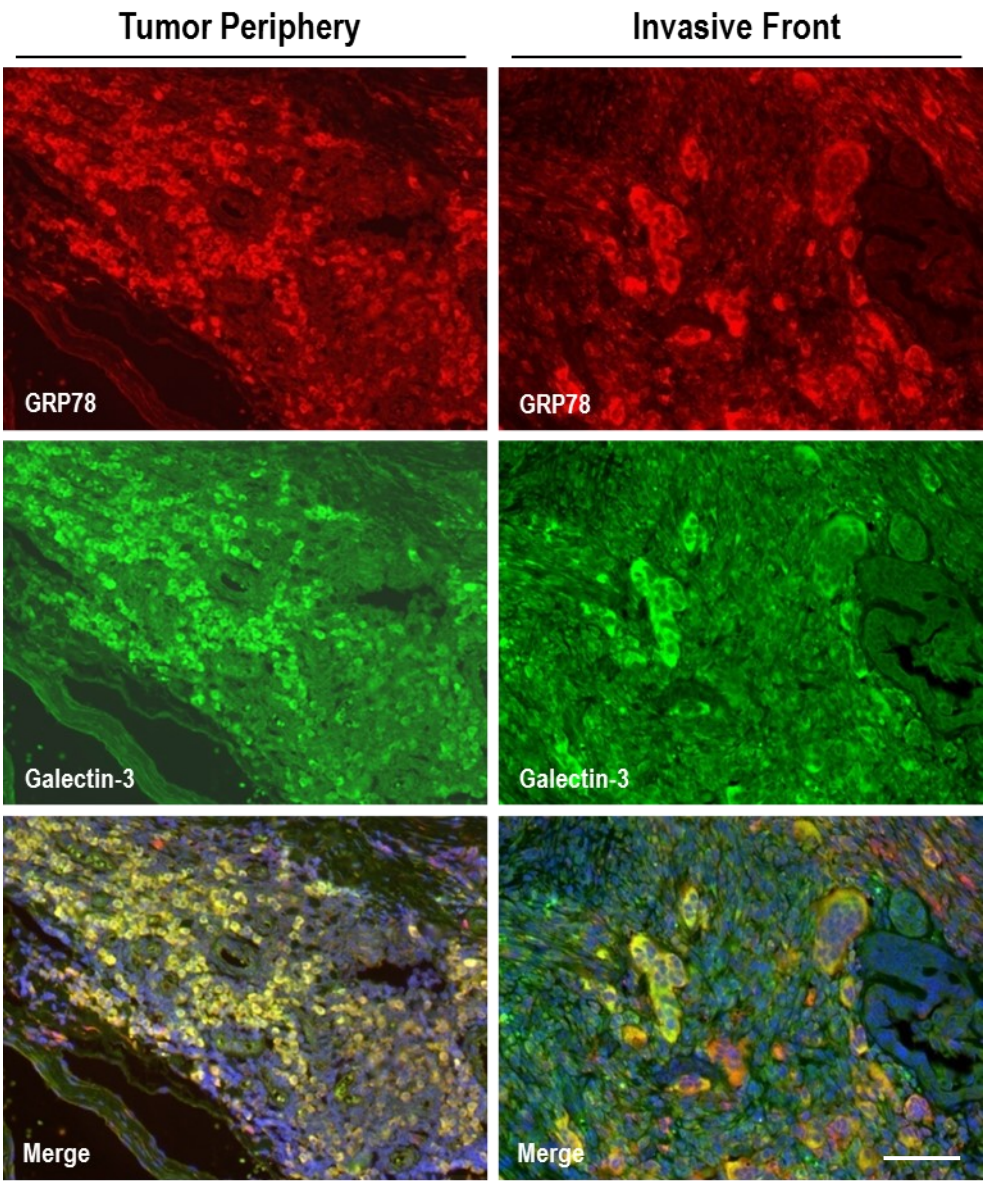


**Figure 2.** GRP78 co-express with galectin-3 in malignant cells. Co-expression between galectin-3 (green color) and GRP78 (red color) was assessed by double-labeling immunofluorescence. Sections were incubated with the first primary antibodies rat anti-galectin-3 (Bioscience) 1:200 in 5% bovine serum albumin (BSA) and rabbit anti-GRP78 (Santa Cruz Biotechnology) 1:50 for 1 hour at room temperature they were then washed in PBS and incubated with Alexa 588-conjugated with a donkey anti-rat (1:200) and Alexa 594-conjugated with a rabbit anti-goat antibody (1:200) (Invitrogen) respectively for 45 min. All sections were then incubated with 1:100 PBS diluted 4'-6-Diamidino-2-phenylindole (DAPI) for 15 min and mounted in glycerol-based Vectashield medium (Vector, Burlingame, CA). Slides were analyzed with a Zeiss fluorescent microscope. Co-expression (yelow color) was observed both in cells in tumor periphery (A) and in specific cells within the tumor (B). Scale bar in lower right image represents 50μM.

tory cells that are important for the neoplastic process (Fortuna-Costa *et al.* 2014, Li & Li 2012). Despite a previous study where no differences were observed between squamous differentiation and mRNA expression levels of GRP78 in non-small cell lung cancer tissues (Sun *et al.* 2012), our results found an association between GRP78 expression and absence of squamous differentiation. Another previous study described that no correlation exists between good prognosis and patients with bladder cancer which present squamous differentiation (Antunes *et al.* 2007).

In conclusion, differing levels of GRP78 expression are present in benign and malignant CMT tissues. Our data points to an important role for GRP78 in the malignant phenotype in canine mammary carcinogenesis. Furthermore, this seems to be associated to galectin-3 in this neoplastic context.

## Authors' Contribution

JdO conceived the study and drafted the manuscript. CR performed part of the laboratory work and has done the statistical analyses. FG participated in its coordination.

## Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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## References

Antunes AA, Nesrallah LJ, Dall'Oglio MF, Maluf CE, Camara C, Leite KR & Srougi M 2007 The role of squamous differentiation in patients with transitional cell carcinoma of the bladder treated with radical cystectomy. Int Braz J Urol **33** 339-345

Cummings RD & Liu FT 2009 Galectins. In Essentials of Glycobiology, edn 2. Eds Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW and Etzler ME. New York, US: Cold Spring Harbor Laboratory Press.

de Oliveira JT, de Matos AJ, Gomes J, Vilanova M, Hespanhol V, Manninen A, Rutteman G, Chammas R, Gartner F & Bernardes ES 2010 Coordinated expression of galectin-3 and galectin-3-binding sites in malignant mammary tumors: implications for tumor metastasis Glycobiology **20** 1341-1352

de Oliveira JT, de Matos AJ, Santos AL, Pinto R, Gomes J, Hespanhol V, Chammas R, Manninen A, Bernardes ES, Albuquerque Reis C, Rutteman G & Gartner F 2011 Sialylation regulates galectin-3/ligand interplay during mammary tumour progression--a case of targeted uncloaking. *Int J Dev Biol* **55** 823-834

Delie F, Petignat P & Cohen M 2012 GRP78 Protein Expression in Ovarian Cancer Patients and Perspectives for a Drug-Targeting Approach. *J Oncol* **2012** 468615

Fernandez PM, Tabbara SO, Jacobs LK, Manning FC, Tsangaris TN, Schwartz AM, Kennedy KA & Patierno SR 2000 Overexpression of the glucose-regulated stress gene GRP78 in malignant but not benign human breast lesions. *Breast Can Res Treat* **59** 15-26

Fortuna-Costa A, Gomes AM, Kozlowski EO, Stelling MP & Pavao MS 2014 Extracellular galectin-3 in tumor progression and metastasis. *Front Oncol* **4**138

Jamora C, Dennert G & Lee AS 1996 Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/C10ME. *Proc Nat Acad Sci USA* **93** 7690-7694

Langer R, Feith M, Siewert JR, Wester HJ & Hoefler H 2008 Expression and clinical significance of glucose regulated proteins GRP78 (BiP) and GRP94 (GP96) in human adenocarcinomas of the esophagus. *BMC Cancer* **8** 70

Lee AS 1992 Mammalian stress response: induction of the glucose-regulated protein family. *Curr Opin Cell Biol* **4** 267-273

Lee AS (2001) The glucose-regulated proteins: stress induction and clinical applications. *Trends Biochem Sci* **26** 504-510

Lee AS (2007) GRP78 induction in cancer: therapeutic and prognostic implications. *Cancer Res* **67** 3496-3499

Lee E, Nichols P, Spicer D, Groshen S, Yu MC & Lee AS 2006 GRP78 as a novel predictor of responsiveness to chemotherapy in breast cancer. Cancer Res **66** 7849-7853

Li Z & Li Z 2012 Glucose regulated protein 78: A critical link between tumor microenvironment and cancer hallmarks. *Biochim Biophys Acta* **1826** 13-22

Paoloni M & Khanna C 2008 Translation of new cancer treatments from pet dogs to humans. *Nat Rev Cancer* **8**147-156

Prabhu A, Sarcar B, Kahali S, Shan Y & Chinnaiyan P 2012 Targeting the unfolded protein response in glioblastoma cells with the fusion protein EGF-SubA. *PLoS One* **7** e52265

Sun Q, Hua J, Wang Q, Xu W, Zhang J, Zhang J, Kang J & Li M 2012 Expressions of GRP78 and Bax associate with differentiation, metastasis, and apoptosis in non-small cell lung cancer. *Mol Biol Rep* **39** 6753-6761

Wang M, Wey S, Zhang Y, Ye R & Lee AS 2009 Role of the unfolded protein response regulator GRP78/BiP in development, cancer, and neurological disorders.

*Antioxid Redox Signal* **11** 2307-2316
Yuan XP, Dong M, Li X & Zhou JP 2015 GRP78 promotes the invasion of pancreatic cancer cells by FAK and JNK. *Mol Cell Biochem* **398** 55-62
Zhou H, Zhang Y, Fu Y, Chan L & Lee AS 2011 Novel mechanism of anti-apoptotic function of 78-kDa glucose-regulated protein (GRP78): endocrine resistance factor in breast cancer, through release of B-cell lymphoma 2 (BCL-2) from BCL-2-interacting killer (BIK). *J Biol Chem* **286** 25687-25696
Zhu G, Wang M, Spike B, Gray PC, Shen J, Lee SH, Chen SY & Lee AS 2014 Differential requirement of GRP94 and GRP78 in mammary gland development. *Sci Rep* **4** 5390