UNIVERSITEIT GENT

FACULTY OF MEDICINE AND HEALTH SCIENCES

Academic year 2011 - 2012

Presence of multidrug resistance and ER stress in a mouse model of hepatocellular carcinoma

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Research report presented in the 4th Master year
for "CLERKSHIP: Research for the Hospital Doctor"
In the program of Master of Medicine in Medicine

List of abbreviations

ABC-transporter, ATP-binding cassette transporter

AREs, Antioxidant response elements

ASK1, Apoptosis signalregulating kinase 1

ATF6, Activating transcription factor 6

BAP, BiP-associated protein

BRCP, Breast cancer resistance protein

Chac1, cation transport regulator-like protein 1

CHOP, C/EBP homologous protein

DCE-MRI, dynamic contrast-enhanced-magnetic resonance imaging

EDEM, ER degradation-enhancing α-mannosidase-like protein

eIF2α, eukaryotic initiation factor 2α

ELISA, enzyme-linked immunosorbent assay

ER, endoplasmic reticulum

ERAD, ER-associated protein degradation

ERK, Extracellularly-Regulated Kinase

ERQC, ER quality-control

ERSE, ER stress response element

GADD34, growth arrest and DNA damage-inducible gene 34

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GLS, Golgi localization signals

GRP78/BiP, Glucose-regulated protein-78/immunoglobulin-binding protein

Gsta1, A1 subunit of glutathione-S-transferase

Gsta2, A2 subunit of glutathione-S-transferase

HCC, hepatocellular carcinoma

HMBS, hydroxymethylbilane synthase

HO-1, Heme oxygenase-1

HSP, Heat shock protein

IgG, Immunoglobulin G

IRE1, Inositol requiring enzyme 1

JIK, c-Jun NH2-terminal inhibitory kinase

JNK, c-Jun N-terminal kinase

KEAP1, Kelch-like Ech associated protein 1

MDR1, Multidrug resistance protein 1

MCJ, Methylation-controlled J protein

NEFs, Nucleotide exchange factors

NF-Y, nuclear factor Y

NRF2, Nuclear-(erythroid-derived 2)-related factor 2

PDI, Protein disulfide isomerase

PERK, PKR-like endoplasmic reticulum kinase

PET, Positron emission tomography

qRT-PCR, Quantitative real-time polymerase chain reaction

RIDD, Regulated IRE1-dependent decay

RIP, Regulated intramembrane proteolysis

ROS, reactive oxygen species

S1P, Site-1 proteases

S2P, Site-2 proteases

SDHA, succinate dehydrogenase complex subunit A

SDS, sodium dodecyl sulfate

SPECT, single-photon emission computed tomography

Topo, DNA topoisomerases

TRAF2, TNF receptor-associated factor 2

uORFs, upstream open reading frames

UPR, Unfolded Protein Response

UPRE, UPR element

XBP1, X-box binding protein 1

ACKNOWLEDGMENTS

First of all, I would like to thank my promoter, Prof. H. Van Vlierberghe, and co-promotor, Prof. I. Colle.

Dear Prof. Van Vlierberghe, your outstanding knowledge of hepatology and HCC in particular, has been of great value to me in writing this research report. Furthermore, your enthusiasm, positive attitude and faith has been enriching to me.

Dear Prof. Colle , your strong interest in my research project and high quality advice during the regular meetings gave a boost to my motivation.

Secondly, I would like to thank Prof. G. Leroux-Roels, chairman of the jury, and the other members of the jury, Prof. M. Praet, Prof. M. Drennan and Dr. Sc. Ph. Meuleman for taking their time to read and critically appraise this research report.

Dear Femke Heindryckx, I really appreciate your kindness, willingness to read this report and your catching enthusiasm. You were very helpful with my numerous questions about the statistics, animals, experiments and scientific writing.

Dear Prof. C. Vanhove of the Infinity lab and Prof. F. De Vos of the Laboratory of Radiopharmacy, thank you for making the collaboration possible and for providing the small animal imaging opportunities.

I also want to thank the Ethical Committee of experimental animals at the Faculty of Medicine and Health Sciences, Ghent University, Belgium, for approving the protocols.

Last but not least, many thanks to the entire research group of gastroenterology/hepatology and especially to Dr. Sc. D. Laukens for the experimental support and academic discussions.

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1. *Introduction*

Hepatocellular carcinoma (HCC) ranks as the fifth most common cancer worldwide (564,000 cases/year) and the third most common cause of cancer mortality. Also the increasing incidence of HCC has led to an expanding interest of research in this field. At diagnosis, approximately 70% of patients with HCC are ineligible for curative surgery. (Van Vlierberghe H, Borbath I, Delwaide J, Henrion J, Michielsen P, 2004) Moreover, HCC is a chemoresistant tumor. Systemic therapy with classical cytotoxic drugs yields low objective response rates without proven survival benefit. (Bruix & Llovet, 2002) As a result of the extent of their tumor and reduced hepatic function, most patients with HCC can only be offered palliative treatment. Medications influencing the chemosensitivity profile of the tumor are therefore needed. It is well known that hypoxia as well as ER stress could be the cause of chemoresistance. Recently, the results of a randomized, placebo-controlled, double-blind phase III-study with the multikinase inhibitor sorafenib represented a breakthrough in the field showing a significant survival advantage for those patients treated with sorafenib. (Llovet et al., 2008; Singal & Marrero, 2010)

1.1. ER stress and the unfolded protein response

The endoplasmic reticulum (ER) consists of a membranous network of interconnecting and branched tubules and flattened sacs extending throughout the cytosol in which secretory and most membrane proteins, approximately a third of the proteome, are synthesized, posttranslationally modified and folded into their correct conformations. This organelle has crucial roles in cell homeostasis and survival, which include proper protein folding, lipid biosynthesis, calcium and redox homeostasis. Unlike the cytoplasm, the environment of the ER lumen is sufficiently oxidized to allow the oxidation of cysteine residues to form the disulfide bonds critical to the proper conformation of many mature proteins (M. Wang, Wey, Zhang, Ye, & Lee, 2009). Once synthesized on ER membrane-bound ribosomes, the polypeptide chains cotranslocate across the ER membrane through the Sec61 complex into the ER lumen. In mammals, during co-translational import, the Sec61 complex, binds to ribosomes at the ER membrane, giving the ER its typical “rough” appearance. (Luo & Lee, 2012)

The ER lumen houses a large array of chaperones assisting the correct folding of newly synthesized proteins to prevent aggregate formation of folding intermediates, including glucose-regulated protein-78/immunoglobulin-binding protein (GRP78/**BiP**, also known as HSPa5), GRP170, GRP58, GRP94, protein disulfide isomerase (PDI), calnexin, calreticulin, ER degradation-enhancing α-mannosidase-like protein (EDEM), HERP and co-chaperones BiP-associated protein (BAP) and $P58^{IPK}$. The extremely high concentration of proteins within the ER (approximately 100 mg/ml) renders this organelle’s environment susceptible to protein aggregation. (Chakrabarti, Chen, & Varner, 2011) The ER contains stringent quality-control

systems (ERQC) that selectively export correctly folded proteins and extract terminally misfolded proteins for ubiquitin-dependent proteolytic degradation by the 26S proteasome after retrotranslocation to the cytosol, a process known as ER-associated protein degradation (ERAD) (Stolz & Wolf, 2010). The best described folding/quality control network at the ER is the calnexin and calreticulin cycle (reviewed in (Michalak, Groenendyk, Szabo, Gold, & Opas, 2009)). In this pathway, ERp57 associates with calnexin and/or calreticulin to assist the folding and quality control of a subset of glycoproteins, while the oxidoreductases PDI and GRP58 are responsible for catalyzing the formation, reduction and isomerization of the disulfide bonds. (Clarke et al., 2012)

Under physiologic conditions, an equilibrium between the ER protein load and the folding capacity exists. Several perturbations in the protein folding (see below) trigger a signaling network termed the "**Unfolded Protein Response**" (UPR) that meticulously coordinates the adaptive and apoptotic responses to ER stress. The UPR, the basic concept initially proposed by Kozutsumi *et al*. (1988), has been generally considered as transcriptional induction of ER chaperone genes in response to accumulation of unfolded proteins in the ER. (Kozutsumi, Segal, Normington, Gething, & Sambrook, 1988) Gene-expression profiling has demonstrated that the UPR regulates, next to chaperones, genes involved in the protein entry into the ER, glycosylation, ERAD, ERQC, redox metabolism, autophagy, lipid biogenesis and vesicular trafficking. Three major ER stress transducers have been identified: PKR-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). (Chakrabarti et al., 2011; Kraskiewicz & Fitzgerald, 2012; Ron & Walter, 2007) The UPR can promote cellular homeostasis and sustained survival by reducing the load of unfolded proteins through the upregulation of chaperones and global attenuation of the protein synthesis with cell-cycle arrest. Thereby enhancing the folding capacity (more chaperones with more time to fold) and reducing the protein load in order to reestablish the equilibrium. However, if the ER stress is prolonged or severe and the UPR fails to restore the ER homeostasis, apoptotic cell death ensues. (Jing, Wang, & Zhang, 2012; Rao et al., 2002; Szegezdi, Logue, Gorman, & Samali, 2006) Identifying the UPR components activated or suppressed in malignancy and exploring the therapeutic opportunities by targeting the UPR components are very active research areas.

Different **ER stresses** inducing the UPR include: (*a*) redox disturbances caused by hypoxia, oxidants or reducing agents that interfere with the disulfide bonding of proteins (*b*) glycemic dysregulation, probably by interfering with *N*-linked protein glycosylation (*c*) aberrations of Ca^{2+} regulation that impair the functions of Ca^{2+}-dependent ER chaperones such as BiP, GRP94 and calreticulin (*d*) viral infections causing ER overload with virus-encoded proteins (e) inclusion body diseases typical of most chronic neurodegenerative diseases as well as disorders such as inclusion body myositis (f) mutations in secretory proteins or receptors that alter their proper folding and (g) pharmacologic agents inducing ER stress. For example, depletion of ER Ca^{2+} stores by Ca^{2+} ionophores, such as thapsigargin, induces ER stress by

impairing the Ca^{2+}-dependent chaperone activity. Reducing agents induce ER stress by impairing disulfide bond formation and tunicamycin, an antagonist of *N*-linked glycosylation, induces ER stress *in vitro*. (Chakrabarti et al., 2011; Ron & Walter, 2007; M. Wang et al., 2009)

Recent studies have provided compelling evidence that UPR dysregulation is implicated in the **pathophysiology of various diseases** including neurodegenerative disorders (such as Alzheimer's disease and Parkinson's disease) (M. Wang et al., 2009), diabetes, ischemia, obesity, lung (Marcinak & Ron, 2010) and cardiovascular diseases (Minamino & Kitakaze, 2010), retinitis pigmentosa, cystic fibrosis, α-1 antitrypsin deficiency, rheumatoid arthritis (Yoo et al., 2012), inflammatory bowel disease (Cao, Song, & Kaufman, 2012), liver disease (Malhi & Kaufman, 2011) and cancer. (Austin, 2009; Luo & Lee, 2012; Ozcan & Tabas, 2012) Hence, the UPR provides new appealing therapeutic targets.

Mammalian **heat shock proteins** (HSPs) are chaperones and have been classified into two groups according to their size: high and small molecular weight HSPs. The first group includes three major families: HSP90, HSP70 and HSP60. Some of these are expressed constitutively whereas expression of others is induced by stressful conditions. These proteins can be targeted to different subcellular compartments. High molecular weight HSPs are ATP-dependent chaperones and require co-chaperones to modulate their conformation and ATP binding. In contrast, small HSPs, such as HSP27, are ATP-independent chaperones. BiP/HSPa5 is implicated in tumor cell proliferation, apoptosis resistance, immune escape, metastasis and angiogenesis and its elevated expression usually correlates with a variety of tumor microenvironmental stresses. BiP protein acts as a centrally located sensor of stress, which detects alterations in the microenvironment and elicits stress response pathways. (Z. Li & Li, 2012) BiP consists of a N-terminal ATPase domain and a C-terminal peptide binding domain. When bound to ATP, BiP binds unfolded hydrophobic sites with low affinity. Misfolded protein binding stimulates the ATPase activity resulting in an ADP-bound form which holds much higher affinity for the hydrophobic motifs (Gething, 1999). Interestingly, the affinity of BiP for ADP is approximately sixfold greater than for ATP. Thus, nucleotide exchange factors (NEFs), such as BAP, are required to catalyze the ADP/ATP exchange needed for the dissociation of BiP from unfolded proteins (Z. Li & Li, 2012). In addition to its role as a folding chaperone, BiP functions as an ER stress regulator by buffering Ca^{2+} levels. BiP interacts with ER localized caspase-7 and prevents the activation of pro-apoptotic BCL-2 family members such as BAX (Fu et al., 2007). BiP also regulates the activation of the three transmembrane ER stress transducers: PERK, ATF6 and IRE1. Normally, BiP is bound to the ER luminal domain of these ER receptors, blocking their activation. However, in the presence of exposed hydrophobic residues on immature proteins, BiP dissociates, allowing their activation. Bertolotti *et al.* (2000) and Okamura *et al.* (2000) independently reported physical association of BiP with IRE1 and PERK, together with its ER stress-dependent dissociation. (Bertolotti, Zhang, Hendershot, Harding, & Ron, 2000; Okamura, Kimata, Higashio, Tsuru, & Kohno, 2000) Shen *et al.* (2002) demonstrated that BiP also associates

with ATF6. (Shen, Chen, Hendershot, & Prywes, 2002) Conversely, overexpression of BiP leads to reduced activation of these proximal sensors. (Z. Li & Li, 2012) Expression of BiP is upregulated by ER stress through ATF6 and ATF4. (Mori, 2009) Also IGF-1 receptor signaling regulates BiP expression (via the PI3K/AKT/mTORC1 axis) independent of the canonical UPR. (Pfaffenbach et al., 2012) However, the exact regulation of BiP is not clear and acts on different levels: transcription, translation, post-translational modification and by degradation. A recent report has shown that the induction of ER stress with thapsigargin leads to lower protein levels of BiP, PDI A3 and PDI A6 in INS-1E cells (a commonly used model for primary β-cells). Enhanced levels of phosphorylated eukaryotic initiation factor 2 (eIF2α) and C/EBP homologous protein (CHOP) were shown together with increased BiP transcription. The study shows that the reduction of protein levels of BiP results from the combined effect of reduced protein synthesis and enhanced proteosomal degradation. (Rosengren et al., 2011) This emphasizes the importance of confirmation of the results on mRNA level on the protein level because possible discrepancies. The resulting increase in BiP therefore not only aids folding of proteins inside the ER, but may inactivate the major proximal sensors PERK, ATF6 and IRE1 by binding to their luminal domains. Thus, BiP is component of a feedback mechanism that ensures protein refolding and inactivation of the UPR.

1.1.1. ATF6

Haze et al. (1999) first identified ATF6 as a new member of the mammalian UPR that is not present in yeast. (Haze, Yoshida, Yanagi, Yura, & Mori, 1999) Activation of ATF6 is regulated by a combination of two events: firstly by interaction with BiP and secondly by disulfide bridges. The ER luminal region of ATF6 has two Golgi localization signals (GLS1 and GLS2). Binding of BiP masks the GLSs in the luminal domain of ATF6 and dissociation of BiP allows ATF6 to be transported to the Golgi body. The ER luminal domain of ATF6 is disulfide bonded and ER stress-induced reduction plays an important role in both translocation to the Golgi body and subsequent recognition by the site-1 and site-2 proteases (S1P and S2P). (Adachi et al., 2008; Sato, Nadanaka, Okada, Okawa, & Mori, 2011) Disulfide bonds in the ATF6 luminal domain are thought to keep ATF6 inactive. Upon ER stress, these bonds are reduced, resulting in an increased ability of ATF6 to exit the ER. Although disulfide bond reduction is required for ATF6 activation, it is not sufficient, suggesting that both the ATF6 redox state and BiP binding are involved in sensing ER stress and activating ATF6 (Nadanaka, Yoshida, & Mori, 2006). Upon accumulation of unfolded proteins, it is packaged into “coatomer protein II” vesicles that pinch off the ER and deliver it to the Golgi apparatus. There, it encounters two proteases, S1P and S2P, that sequentially remove the luminal domain and the transmembrane anchor, respectively, a mechanism that is termed regulated intramembrane proteolysis (RIP). Upon ER stress, ATF6 is cleaved and the resulting N-terminal fragment is liberated from the ER membrane and subsequently translocated into the nucleus where it binds in association with nuclear factor Y (NF-Y) to promoters that contain the ER stress

response element (ERSE) resulting in their transcriptional initiation. (Chakrabarti et al., 2011; Guan et al., 2009)

The targets of ATF6 include ERAD components, ER chaperones such as BiP, GRP94, PDI, lipid biogenesis allowing ER expansion (Maiuolo, Bulotta, Verderio, Benfante, & Borgese, 2011a) and the transcription factors CHOP and X-box binding protein 1 (XBP1 which then is spliced by IRE1 endonuclease, see below). (Adachi et al., 2008) Although ATF6 has long been thought to transduce purely cytoprotective signals, overexpression of ATF6 can induce CHOP mRNA expression as well.

Negative regulation of ATF6 is less investigated. Recently, unspliced XBP1 has been implicated as a negative regulator for ATF6 (Yoshida, Uemura, & Mori, 2009). Following the induction of ER stress, two versions of XBP1 exist: unspliced XBP1 and spliced XBP1. In the recovery phase following ER stress, high levels of unspliced XBP1 may play a dual role. First, unspliced XBP1 dimerizes with spliced XBP1 promoting degradation (Tirosh, Iwakoshi, Glimcher, & Ploegh, 2006; Yoshida, Oku, Suzuki, & Mori, 2006). Second, unspliced XBP1 can bind ATF6 rendering it more prone to proteasomal degradation (Yoshida et al., 2009). Taken together, these two steps may reduce the transcription of ER chaperones and ERAD components during the recovery phase following ER stress.

1.1.2. IRE1

The phylogenetically oldest component of the UPR, the IRE1 branch, is the sole branch in lower eukaryotes. Later evolution added the PERK and ATF6 branches to metazoan cells. (Mori, 2009) The transautophosphorylation of the kinase domain of IRE1 activates its unusual effector function i.e. the unconventional splicing of the XBP1 mRNA. In metazoans, a 26-nucleotide intron is spliced out by activated IRE1, resulting in a translational frame shift, encoding a protein containing 376 amino acids (with a hydrophobic region which is a membrane-targeting signal), as compared with 261 amino acids encoded by the unspliced mRNA. (Kraskiewicz & Fitzgerald, 2012; Shinya et al., 2011)

Both forms of XBP1 can bind the ERSE; however, **spliced XBP1** activates the UPR far more potently than its unspliced form. Spliced XBP1 can bind to three cis-acting elements: ERSE, ERSE-II and the UPR element (UPRE). Spliced XBP1 is transcription factor of the basic-leucine zipper family and one of the key regulators of the ER folding capacity. Spliced XBP1 enhances the transcription of UPR genes involved in protein entry to the ER, ERQC like PDI or ERdj4, disulfide linkage, ERAD like EDEM, oxidative stress reduction like catalase (Y Liu et al., 2009), autophagy and ER/Golgi biogenesis (Brewer & Jackowski, 2012). XBP1 gene transcription is induced by spliced XBP1 itself and activated ATF6. (Majumder et al., 2012)

The endonuclease activity also induces the rapid turnover of mRNAs encoding membrane and secreted proteins, through a pathway referred to as **regulated IRE1-dependent decay**

(RIDD) thereby changing the transcriptome, including proinsulin mRNA (Lipson, Ghosh, & Urano, 2008), CD59 mRNA (prevents complement-mediated cytolysis and decreased expression induced tumor growth suppression in MCF-7 cells (B. Li, Chu, Gao, & Xu, 2011)) and IRE1 mRNA itself. (Hollien et al., 2009) As this affects mRNAs encoding ER resident proteins such as chaperones, as well as transmembrane or secreted proteins such as growth factors and their receptors, that are crucial for cell viability, RIDD is considered pro-apoptotic.

Activation of IRE1 by ER stress recruits the adaptor TNF receptor-associated factor 2 (TRAF2) to the ER membrane which activates c-Jun N-terminal kinase (**JNK**), resulting in caspase-12 activation and apoptosis (Yoneda et al., 2001). This recruitment is regulated by c-Jun NH2-terminal inhibitory kinase (JIK), which has been reported to interact with both IRE1 and TRAF2. The IRE1/TRAF2 complex then recruits apoptosis signal regulating kinase 1 (ASK1), causing activation of ASK1 and the downstream JNK pathway leading to cell death.

1.1.3. PERK

As unfolded proteins accumulate during ER stress, BiP dissociates, allowing PERK to autophosphorylate and dimerize, which induces transphosphorylation. PERK is a kinase with two known substrates thus far, eIF2α and Nuclear-(erythroid-derived 2)-related factor 2 (NRF2). **NRF2** phosphorylation promotes the dissociation from its inhibitor kelch-like Ech associated protein 1 (KEAP1), thereby allowing nuclear translocation, which results in the expression of genes containing antioxidant response elements (AREs), such as the A1 and A2 subunits of glutathione-S-transferase (Gsta1 and Gsta2), Heme oxygenase-1 (HO-1) but also components of the ubiquitin-proteasome system. (Bobrovnikova-Marjon et al., 2010) The classic NRF2 targets promote cell survival via initiating the removal of reactive oxygen species (ROS). The components of the ubiquitin-proteasome system, may confer a protective advantage to tumor cells that are rapidly proliferating and have an increased requirement for protein degradation. Phosphorylation of **eIF2α** induces general repression of translation. However, the phosphorylated eIF2α preferentially initiates the translation of mRNAs containing inhibitory upstream open reading frames (uORFs) in their 5' untranslated region. The best-studied P-eIF2α–dependent translation is ATF4 mRNA. (Gorman, Healy, Jäger, & Samali, 2012) ATF4 upregulates ER stress related target genes, including amino acid transporters, CHOP, ATF3, redox control genes and growth arrest and DNA damage-inducible gene 34 (GADD34). The latter is the regulatory subunit of the PP1 phosphatase promoting dephosphorylation of eIF2α, which allows translational recovery. GADD34 expression is induced by ATF4 and its target genes CHOP and ATF3. ATF3 also induces CHOP, thereby providing a strong negative feedback. (Chakrabarti et al., 2011; Ron & Walter, 2007) ATF4 is a major inducer of the pro-apoptotic CHOP, although CHOP can be induced by all three arms of the UPR.

Activation of the UPR triggers a rapid **arrest in G1 phase** of the cell cycle. The UPR-dependent signal transduction intersects with the cell cycle via PERK-dependent inhibition of

cyclin D synthesis. The ER stress-induced growth arrest likely provides a window of opportunity that prevents cells from continuing their cell division cycle under conditions in which the proper protein folding is compromised.

1.1.4. The unfolded protein response in HCC

Cancer cells hijack many normal processes such as cell cycle signaling, angiogenesis, glucose metabolism and the mechanisms of resistance to cell stress and apoptosis. (Hanahan & Weinberg, 2011) Following initiation of malignancy, poor vascularisation of the tumor mass leads to stressful conditions in the tumor microenvironment, including hypoxia, nutrient deprivation and pH changes. These conditions, together with the misfolding of mutated proteins, activate a range of cellular stress response pathways, including the UPR. Activation of at least one branch of the UPR has been reported in various cancers and many ER chaperones and UPR target genes show increased expression in human tumor samples. (Austin, 2009; Chakrabarti et al., 2011; Lee, 2007) It has been reported that ATF6 is implicated in the pathogenesis of HCC. (Al-Rawashdeh, Scriven, Cameron, Vergani, & Wyld, 2010; Arai et al., 2006) Shuda *et al.* (2003) showed BiP as a transformation-associated gene in human HCC. (Shuda, 2003) Elevation of BiP and ATF6 mRNAs and the splicing of XBP1 mRNA, occurred in HCC tissues with increased histological grading. The ER stress pathway mediated by ATF6 and by IRE1-XBP1 systems seems essential for the transformation-associated expression of the BiP gene in HCC. Overexpression of BiP and activation of ATF6 occurred concomitantly in higher-grade tumors. Thus, ER stress could play an important role in the pathogenesis of HCC.

1.2. Multidrug resistance

Hypoxia inducing ER stress plays an important role in MDR of solid tumors. Strategies to reduce the chemoresistance by pharmacological ER stress modification *in vivo* may provide a better understanding how the UPR exerts its effects in cancer. Possible **mechanisms** for MDR include decreased drug uptake, efflux pump upregulation (e.g. multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BRCP) (Borel et al., 2012)), altered survival and apoptotic signaling pathways, target reduction (e.g. topoisomerase II protein level) or modification, increased DNA repair and detoxifying activity. The UPR is activated in HCC, but how this contributes to chemoresistance in the tumor cells remains largely unknown. (Al-Rawashdeh et al., 2010)

The effect of **UPR activation on the chemosensitivity profile** of the tumor depends on the mode of action of the anticancer drugs. Cells overexpressing BiP are hypersensitive to DNA-crosslinking agents like melphalan and cisplatin (possible by synergy with ER stress-elicited apoptotic pathways), but resistant to topoisomerase II inhibitors including etoposide (Y. Lin, Wang, Liu, & Chen, 2011) and doxorubicin. (Luo & Lee, 2012)

Cisplatin is widely used in the treatment of solid tumors, but its side effects plus resistance limits its usage. It kills cancer cells by damaging DNA and inhibiting DNA synthesis. Next to DNA, cisplatin binds to proteins promoting formation of misfolded ubiquitinated proteins, thereby inducing UPR activation (BiP and CHOP induction in HepG2 cells) and calpain-dependent caspase-12 activation. (Mann & Hendershot, 2006; Tan et al., 2006) Knockdown of BiP or ATF6 enhanced apoptosis induced by cisplatin which suggest that the UPR protects HCC cells against cisplatin-induced apoptosis. (R. Chen, Dai, et al., 2011) Also PERK, but not IRE1, activation is involved in protecting HCC cells against cisplatin-mediated apoptosis. Combination of cisplatin with tunicamycin increased cisplatin-induced apoptosis by inducing severe ER stress. (Xu et al., 2012) By inducing severe ER stress or by selectively reducing the UPR mediated coping with unfolded proteins the chemotherapeutic efficacy is enhanced (Apel, Herr, Schwarz, Rodemann, & Mayer, 2008; Kraskiewicz & Fitzgerald, 2012). ATF4 can increase resistance to cisplatin in HCC by increased biosynthesis of glutathione (Z. Zhang et al., 2012) and is overexpressed in about half of human HCC tissues. Knockdown of ATF4 increased the cytotoxicity of cisplatin *in vitro* and *in vivo*, while overexpression decreased the sensitivity of HCC cell lines to cisplatin. Interestingly, glutathione was reduced in the HCC cell lines subjected to ATF4 knockdown.

DNA topoisomerases (topo) play an essential role in replication, transcription and chromosome segregation by unwinding and untangling DNA. There are two major families of topoisomerases; the type I that makes transient single strand nicks in DNA and the type II which introduces double strand breaks. Drugs targeting either class of topo act by blocking the religation of the normally transient cleaved intermediates. The unrepaired enzyme-mediated DNA damage can promote apoptosis using the same pathways as other DNA damaging agents. Agents targeting topo II have substantial anti-tumor activity. *In vitro* UPR activation, achieved with glucose deprivation, confers marked resistance to doxorubicin in HepG2 cells. Diminished levels of topo II could induce MDR, apparently due to a reduction of topo II:DNA complexes and therefore the amount of drug-induced DNA damage. Sensitivity of cells to various topo II targeting agents is decreased by pretreatment with ER stressors, which was subsequently shown to correlate with a dramatic reduction in topo II translation downstream of the PERK branch of the UPR. Conversely, higher topo II levels *in vivo* correlated with increased responsiveness to doxorubicin. Cell cycle arrest could induce topo II loss by blocking its transcription. However, the UPR-induced loss of topo II occurs before cells accumulate in G1 phase and while topo II transcript levels are still unaffected. This suggests that loss of topo II early in the UPR involves post-transcriptional mechanisms. A domain was identified on topo II providing a binding site for JAB1, which increases the degradation of topo II during ER stress. JAB1 is bound to IRE1, but is released by IRE1 activation. (Chakrabarti et al., 2011) Next to a reduction of topo II levels, cell cycle arrest and JAB1, the resistance may be a consequence of UPR-mediated inhibition of apoptosis. (Al-Rawashdeh, Scriven, Cameron, Vergani, & Wyld, 2010) Here, increased BiP levels may be

involved. Since BiP chaperones the folding of proteins in the ER and topo II is primarily a nuclear protein, the action of BiP may be indirect. However, cells overexpressing BiP are more resistant to etoposide due to relocalization of BiP to the cytosol where it binds and inhibits caspase-7 and -12 *in vitro* and *in vivo*. Treatment of glioma cells with epigallocatechin gallate, a component of green tea which targets the ATP-binding domain of BiP and blocks its protective function, sensitizes glioma cells to an alkylating agent, temozolomide, *in vitro*. (Pyrko, Schönthal, Hofman, Chen, & Lee, 2007) The *in vivo* confirmation is provided by Chen *et al.* (2011) in mouse models of glioblastoma. (T. C. Chen, Wang, et al., 2011) Similarly, versipelostatin, which inhibits BiP induction by glucose deprivation, inhibits tumor growth of stomach cancer xenograft when combined with cisplatin. (H.-R. Park et al., 2004)

MDR1 is an ATP-dependent drug efflux pump localized to the plasma membrane and confers chemoresistance in various neoplastic cells. Methylation-controlled J protein (MCJ), a co-chaperone, is required in breast cancer cells to prevent c-JUN-mediated expression of MDR1, which flushes doxorubicin out of the cell. (Hatle et al., 2007) Glucose deprivation could enhance the chemoresistance through induction of MDR1 expression. The Extracellularly-Regulated Kinase (ERK) -1 and -2 activity is downregulated in HCC cells with MDR1-mediated MDR. Besides ERK, JNK has been implicated in hypoxia induced-MDR. JNK phosphorylates c-JUN. JNK activity negatively correlated with the degree of MDR in HCC cells. MDR1 could participate to the UPR either directly by transporting misfolded proteins or indirectly by interacting with chaperones involved in the UPR. Besides this putative mechanisms linking UPR to MDR1 function, tunicamycin depresses MDR1 glycosylation without any effect on its membrane localization and drug efflux activity in l1210 cells. (Sereš, Cholujová, Bubenčíkova, Breier, & Sulová, 2011) However, it seems reasonable that the ER stress trigger determines the effect on MDR1 function through the UPR/JNK pathway. Modification of JNK activity by ER stress could therefore be a putative mechanism for MDR modification by the UPR activated in cancer cells.

2. *Goals of the research*

The main goal of the project is to evaluate the presence of ER stress and MDR genes and proteins in a **diethylnitrosamine (DEN)-induced mouse model of HCC**. This model shows tumor progression occurring in a background of inflammation and fibrosis. Not only within the tumors, but also in the surrounding liver tissue increased fibrosis, small cell dysplasia, inflammatory foci and macrophages are noticed after DEN treatment. The presence of fibrosis in the non-HCC tissue after DEN treatment creates an increased intrahepatic vascular resistance and reduces oxygen diffusion, resulting in hypoxia. So both in the tumors as well as in the surrounding area stressful conditions are created. (Heindryckx et al., 2010) First, previous data from a microarray is likely to provide a broad view of genes involved in the aforementioned mechanisms. These genes will be further examined by qRT-PCR and finally, if possible, these results will be confirmed on the protein level by western blotting or ELISA.

During this research, **the three UPR pathways** by gene induction and by protein activation will be assessed. The activation of the PERK branch will be evaluated through NRF2 (with downstream mediators Gsta1, Gsta2, HO-1 and γ-glutamyl cysteine synthetase) and through ATF4 (with downstream proteins GADD34 and CHOP). (Bobrovnikova-Marjon et al., 2010; Yan Liu et al., 2010) The IRE1 pathway will be analyzed through XBP1 splicing activity and spliced XBP1 targets as BiP, GRP94, $P58^{IPK}$ and calreticulin. (Hetz & Glimcher, 2009; Hetz, Martinon, Rodriguez, & Glimcher, 2011; J. H. Lin et al., 2007; Pincus et al., 2010) The activation of the ATF6 pathway will be determined through PDI A3, PDI A4, EDEM1, HERP, unspliced XBP1 and also CHOP. (Adachi et al., 2008; Maiuolo, Bulotta, Verderio, Benfante, & Borgese, 2011b)

The research project includes the assessment of these pathways by **protein activation**. The activation of the ATF6 branch will be demonstrated by ATF6 cleavage with western blot specific for the nuclear ATF6 fragment of 50 kDa. This fragment is the evidence of activation by RIP in the Golgi apparatus. The IRE1 pathway is measured on the protein level with western blot with an antibody directed to the spliced form of XBP1 and the PERK pathway with western blot of the phosphorylated form of eIF2α or PERK and its targets ATF4 and CHOP. Also the induction of BiP protein will be evaluated by western blot.

The **MDR** will be evaluated by microarray and qRT-PCR analysis of genes responsible for multidrug resistance (e.g. *Mdr1, Mdr2, Mrp1, Mrp2, Mrp4, Mrp6* and *Brcp)*. (Z.-S. Chen & Tiwari, 2011; G. Li et al., 2007; Tian et al., 2006; Vander Borght et al., 2008) In function of the qRT-PCR and microarray results, we will validate the outcomes of the transcriptional level on the protein level, especially of the most important MDR genes for human HCC i.e. MDR1 and BRCP. (Sun et al., 2010)

3. Material and methods

Animals

The *in vivo* study design is a DEN-induced mouse model for HCC, in which 5-week-old male wild type mice (129S2/SvPasCrl) receive weekly intraperitoneal injections with DEN (35 mg/kg). The animals will be sacrificed after 20, 25 and 30 weeks. Each group consists out of 10 to 12 animals. The mice were purchased from Charles River laboratories (Brussels, Belgium). They were kept under constant temperature and humidity in a 12 h controlled dark/light cycle. Mice were fed ad libitum on a standard pellet diet. The Ethical Committee of experimental animals at the Faculty of Medicine and Health Sciences, Ghent University, Belgium, approved the protocols (ECD 11/52).

HCC induction

The 5-week-old male mice received intraperitoneal injections once per week with DEN (35 mg/kg bodyweight) diluted in saline using a 0,5 mL syringe with a 29G needle. If mice suffered from weight loss ≥ 15% compared to the previous week, an injection was omitted. The control group was injected with an equal volume of saline and injections were randomly passed over in a comparable quantity as in the DEN-group.

Tissue sampling

After 20, 25 and 30 weeks, animals were sacrificed under isoflurane (Forene®) anaesthesia. After macroscopic evaluation, all organs were sampled in 4% phosphate buffered formaldehyde (Klinipath, ref: 4078.9020) and embedded in paraffin. To reduce the effect of the inflammatory response mice were sacrificed a week after the final DEN injection. HCC-lesions and non-HCC-tissues were separately collected and snap frozen in liquid nitrogen.

RNA extraction

Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Benelux, Venlo, The Netherlands), with on-column DNAse treatment. The concentration and purity of the total RNA were determined spectrophotometrically (WPA Biowave II, Isogen Life Science, The Netherlands). All OD260/OD280 ratio's were between 1,8 and 2 for RNA quality control.

Quantitative real-time PCR

One microgram of total RNA was converted to single strand cDNA by reverse transcription (iScript, BioRad, Invitrogen) with oligo (dT) and random priming. The cDNA was diluted 1/10 and used in real-time quantification using SYBR Green (Sensimix, Bioline Reagents Ltd, London, UK) and 250 mM of each primer. A two-step program was run on the LightCycler® 480 (Roche). Cycling conditions were 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds and 60°C for 1 minute. Melting curve analysis confirmed primer specificities. All reactions were done in duplicate and normalized to hydroxymethylbilane synthase (HMBS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase complex subunit A (SDHA). Hypoxanthine phosphoribosyltransferase did not seem to be a good household gene to use for normalization. The PCR-efficiency of each primer pair was calculated using a standard curve of reference cDNA. Amplification efficiency was determined using the formula 10-1/slope. Control for SNP's by online public database RTPrimerDB has been performed. Sequences of all primer sets are listed in Table 1S.

The following groups were compared:

- Evaluation of the effect of DEN administration on the murine liver: 25w DEN wild type non tumor versus 25w saline control wild type non tumor.

- Evaluation of the effect of tumorigenesis: 25w DEN wild type non tumor versus 25w DEN wild type tumor.
- Evaluation of the effect of tumorigenesis and DEN: 25w DEN wild type tumor versus 25w saline control wild type non tumor.
- Assessment without stratification based on the time period of DEN administration for the effect of tumorigenesis to enhance the power of the results: 20w, 25w and 30w DEN wild type tumor versus 20w, 25w and 30w wild type non tumor.

Microarray

Gene expression analysis was carried out using Agilent SurePrint G3 Mouse GE 8 x 60K Microarrays (Agilent Technologies, Diegem, Belgium). This specific array represents 41,000 mouse genes and transcripts, providing full coverage of mouse genes and transcripts. Following conditions were assessed: 25w DEN + 5w IgG tumor tissue (n= 5), 25w DEN + 5w IgG surrounding tissue (n= 4) and 25w saline + 5w IgG (n= 3). The data will be accessible on the GEO-database (GSE35289). Differentially expressed genes were identified using corrected p-values of < 0.05 and an absolute log fold change of 1.

Western blot

Cell lysates were boiled with sodium dodecyl sulfate (SDS) loading buffer and then fractionated by SDS-PAGE. The proteins were transferred to PVDF membrane which was subsequently incubated with a primary specific antibody in 5% of non-fat milk, followed by a horse radish peroxidase (HRP)-conjugated secondary antibodies. ECL detection reagent (Amersham Life Science, Piscataway, NJ) was used to visualize the specific proteins. Band density was measured using ImageJ software 1.45s (National Institutes of Health, USA) and results were normalized by tubulin, actin or GAPDH.

ELISA

Cell lysates were used for analysis by enzyme-linked immunosorbent assay (ELISA) for the protein levels of MDR1 and BRCP according to the manufacturer's protocol (Shanghai BlueGene Biotech, Shanghai, China for MDR1 and USCN Life Science Inc., Wuhan, China for BRCP). The results were corrected for the total protein content (Biorad Protein assay, California, USA).

Statistics

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., California, USA). Values are presented as mean ± SD. Datasets were tested for normality using the Kolmogorov-Smirnov test before further analysis. Parametric data were subjected

to an unpaired student's t-test to evaluate the difference between the DEN and control group. Data that did not show a normal distribution were tested with the nonparametric Mann-Whitney-U test. Two-tailed probabilities were calculated and p-values of less than 0.05 were considered statistically significant.

4. Results

4.1. Microarray analysis

The analysis of the microarray results includes the selection of relevant genes through Pubmed search and functional annotation by Gene ontology, the verification of significant expression alterations and the interpretation of these results. A general overview of the studied groups and number of differentially expressed genes is provided in table 1. The 'surrounding' consists of the macroscopic non-HCC liver tissue of mice after repeated DEN injections. 486 downregulated en 819 upregulated genes were identified in the tumor tissue in mice after DEN injections as compared to normal liver samples after saline injections. For the surrounding the microarray results show 104 downregulated and 176 upregulated genes. To further analyze the results it is important to discriminate two effects: first the effect of DEN administration and secondly the effect of the tumorigenesis on top of the DEN effect on the murine liver samples. Since there is no manifest hepatocarcinogenesis effect in the surrounding area, the expected amount of significantly changed gene expressions is lower. Nevertheless, the surrounding area also contains dysplastic lesions and microscopic tumors (Heindryckx et al., 2010).

	Uncorr p-val < 0.001		Corr p-val < 0.05	
	log-ratio < −1	log-ratio > 1	log-ratio < −1	log-ratio > 1
IgG: Surrounding / Tumor	18	5	0	0
IgG: Tumor / Fysio	411	732	486	819
IgG: Surrounding / Fysio	183	290	104	176

Table 1: Number of differentially expressed genes in different treatment groups. IgG, Immunoglobulin G; Fysio, NaCl 0.9%; Uncorr, uncorrected; Corr, corrected.

4.1.1. Microarray analysis for selected ER stress genes

4.1.1.1. Tumor tissue

The comparison of expression profile of the tumor tissue versus the saline treated normal liver tissue shows downregulation of cation transport regulator-like protein 1 (Chac1), a recently identified component of the UPR.

The Gsta1 gene expression is significantly ($p<0,05$) upregulated in this microarray. This upregulation could be by NRF2-mediated transcription, however no significant differences in

Nrf2 expression levels in the microarray could be determined. It is important to verify this information later by qRT-PCR (see below). Also in the surrounding the upregulation of this oxidative stress responsive enzyme is noticed.

In the analysis of the microarray, also Atf3 mRNA is significantly (P<0,05) induced in HCC compared to normal liver tissue. ATF4 upregulates the expression of Atf3. Atf4 however is not upregulated in this microarray and needs to be measured by qRT-PCR for further confirmation.

4.1.1.2. Surrounding tissue

Compared to normal liver tissue, the Bax gene expression is significantly upregulated in the surrounding**.** The Bcl-2 gene expression is significantly enhanced in HCC induced by DEN compared to normal liver tissue after saline administration. Of the 11 members of the BH3-only family (see below), PUMA, NOXA, BID and BIM, have been reported to mediate apoptosis triggered by ER stress (Cazanave et al., 2010; J. Li, Lee, & Lee, 2006; Puthalakath et al., 2007; Upton et al., 2008; Weston & Puthalakath, 2010). However, these ER stress-related BH3-only genes are not significantly upregulated in this microarray, because the increase in expression is less than 10-fold (absolute log fold change with 1).

Taken together, in this microarray in the HCC and surrounding tissue compared to the saline group the known ER stress markers are generally absent with exception of Chac1, Atf3 and Gsta1.

4.1.2. Microarray analysis for the selected MDR genes

After selection of the known MDR-related genes with the focus on the important efflux pump genes of the ATP-binding cassette transporters (ABC-transporters) family and evaluation of their expression patterns in HCC compared to normal liver tissue, the Abca8a is significantly downregulated. The expression of DNA topoisomerase II-α (see above) is significantly upregulated in the HCC and surrounding samples. Thus, regarding the selected MDR genes on the microarray, there was only a limited upregulation of efflux pumps in the tumor tissue versus the normal liver tissue.

4.2. Validation of microarray results and other selected genes by qRT-PCR

The selection of primer sets is based on the microarray analysis and an extensive literature research. The latter yields, in case of ER stress-related genes, the evaluation of each branch of the UPR and general common used ER stress markers. In case of MDR-related genes, the genes of certain efflux pumps involved in the chemoresistance of HCC are quantified. In

table 1S the sequences and qRT-PCR efficiencies of the primer sets used for quantitative real time-PCR in this study are provided.

4.2.1. The ER stress-mediated genes

The chaperone BiP is significantly upregulated by tumorigenesis at 25 weeks (p= 0,0066). However, there is no significant increase of BiP by DEN only. The sample size is too limited to show an evolution in time in the BiP expression (data not shown). The expression of the co-chaperone Erdj4 is significantly increased by the DEN (p= 0,0025) and tumorigenesis (p= 0,0007) effect (Fig. 2). The chaperone Grp94 mRNA level is not changed (Supplementary Fig. 1S).

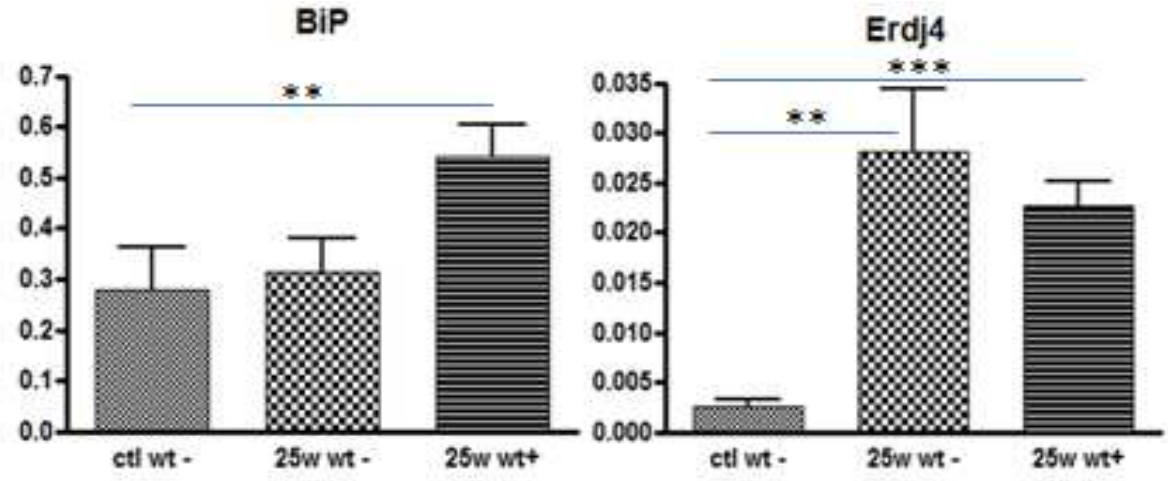

Fig. 2: The major regulator of the UPR, BiP, is significantly upregulated by tumorigenesis. The expression of Erdj4 is significant increased both by the DEN and hepatocarcinogenesis effect. The Y-axis represents the normalized gene expression levels. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001). ctl wt -, saline control wild type non-tumor; wt -, DEN wild type surrounding; wt+, DEN wild type tumor.

The ATF6α gene expression is unaltered like expected because it serves primary as a proximal ER stress sensor whose regulation is multiple, but not directly ER stress-induced. (Supplementary Fig. 2S) However, this gene is used in several reports as an ER stress marker. The unspliced XBP1 is strongly increased by tumorigenesis (P= 0,0002) (Fig. 3). Also PDI A4 is an increased target of the ATF6 path by hepatocarcinogenesis (P= 0,0464) at 25 weeks (Fig. 3). Nevertheless, other downstream mediators such as PDI A3, EDEM1 and HERP (even unexpectedly significantly downregulated by DEN (p= 0,0303)) are not significantly upregulated (Supplementary Fig. 2S).

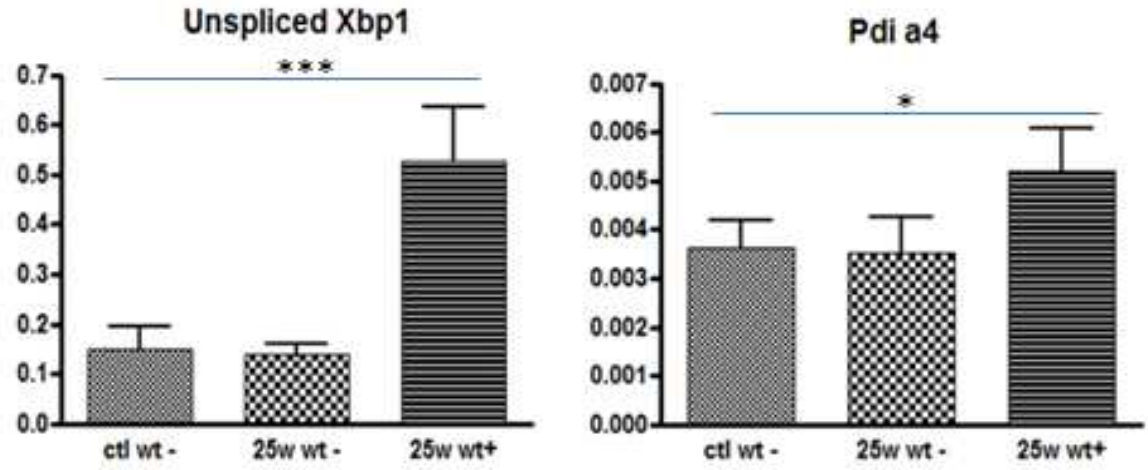

Fig. 3: Significantly changed targets of the ATF6 branch of the unfolded protein response by the tumorigenesis effect. The Y-axis represents the normalized gene expression levels. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001). ctl wt -, saline control wild type non-HCC; wt -, DEN wild type surrounding; wt+, DEN wild type tumor.

First, the Ire1α sensor expression is unaltered as expected because it serves primary as a proximal ER stress sensor whose regulation is diverse, but not directly ER stress-induced. (Supplementary Fig. 3S) An important indicator for IRE1 activity is the splicing of Xbp1 mRNA. To assess the splicing activity by qRT-PCR we used primer pairs specific for the unspliced and spliced version of Xbp1. The spliced Xbp1/unspliced Xbp1 ratio is an indicator for the splicing activity of IRE1. Unexpectedly, the splicing is not significantly increased. In the adjacent liver tissue there is a tendency to elevated splicing activity. However, there is a slight increase in spliced Xbp1 (p= 0,0068). We conclude that the splicing activity is on basal level, nevertheless is the unspliced form of Xbp1 significant increased. So the increased amount of unspliced reagens gives rise to an increased splicing product even with the basal splicing activity. However, the XBP1 targets like calreticulin and $P58^{IPK}$ were not significantly upregulated (Supplementary Fig. 3S).

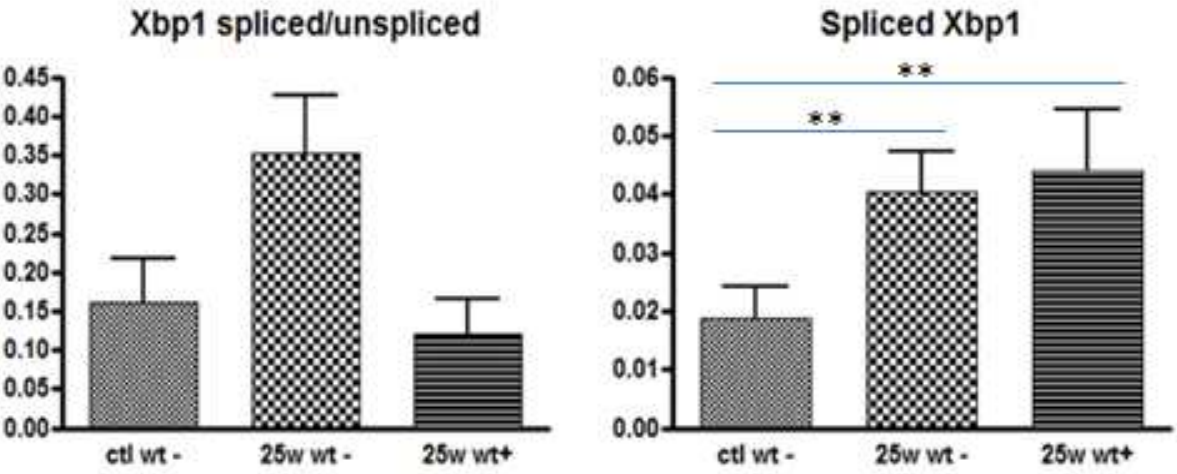

Fig. 4: The splicing activity of IRE1 by the spliced/unspliced Xbp1 ratio and the resulting splicing product. The Y-axis represents the normalized gene expression levels. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001). ctl wt -, saline control wild type non-tumor; wt -, DEN wild type surrounding; wt+, DEN wild type tumor.

To assess the PERK branch of the UPR the included genes are divided in the target genes regulated via NRF2 and those regulated via ATF4. In order to analyze the expression of the oxidative stress genes by the PERK-NRF2 pathway the expression of the transcription factor itself is measured. Not by tumorigenesis, but by the DEN effect is the Nrf2 mRNA level upregulated (p= 0,0025). The expression levels of the targets of NRF2 such as γ-glutamyl cysteine synthetase (p= 0,0177), HO-1 (p= 0,0101), Gsta1 (p= 0,0022) and Gsta2 (p= 0,0025) are also significantly increased by DEN administration without an expected increase by hepatocarcinogenesis only. However, the p-values of the comparison of 25 weeks DEN wild type tumor versus saline control wild type non-tumor are more significant than the p-values of the comparison 25 weeks DEN wild type non-tumor versus saline control wild type non-tumor (for γ-glutamyl cysteine synthetase: 0,0092 versus 0,0177, HO-1: 0,0066 versus 0,0101, Gsta1: 0,0017 versus 0,0022 and Gsta2: 0,0007 versus 0,0025) which indicates a slightly increase of the NRF2 targets by tumorigenesis on top of the DEN effect, probably by less variability in those groups. (Fig. 5)

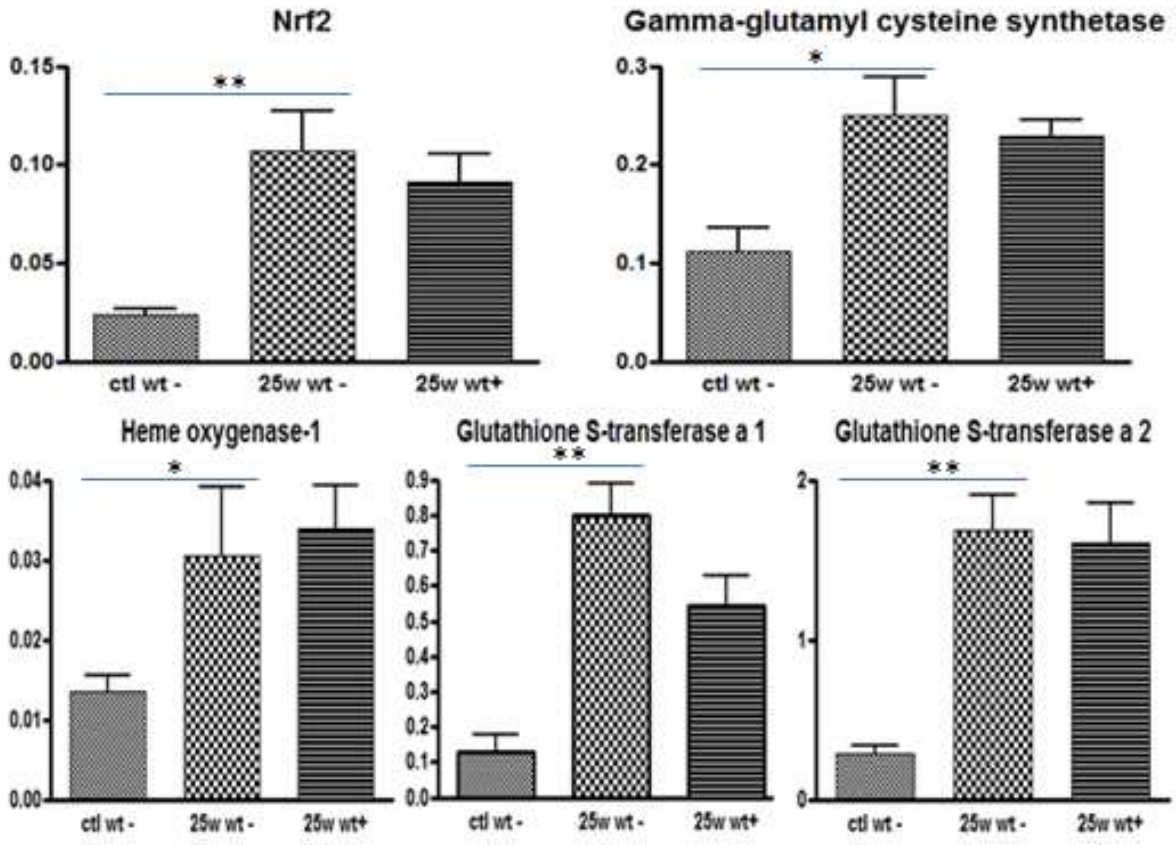

Fig. 5: The PERK-NRF2 pathway expression levels. The Y-axis represents the normalized gene expression levels. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001).

ctl wt -, saline control wild type non-tumor; wt -, DEN wild type surrounding; wt+, DEN wild type tumor.

The PERK-ATF4 pathway is evaluated by the expression of Atf4 itself, Gadd34, Chop (upregulated by all three branches, but ATF4 is quantitative the major activator of Chop transcription). The transcription factor Atf4 is significantly upregulated in the comparison of 20w, 25w and 30w DEN wild type tumor versus 20w, 25w and 30w wild type non tumor (p= 0,0136) (Fig. 6) which has more power, but not in the comparisons with smaller sample sizes at 25w (p= 0,1451) (Supplementary Fig. 3S). Possibly, the expression level of Gadd34 requires more power to be significantly increased by tumorigenesis (p= 0,0152). The Chop mRNA level is elevated by the DEN (p= 0,0121), hepatocarcinogenesis (p= 0,0268) effect and the combination (p= 0,008) (Fig. 6).

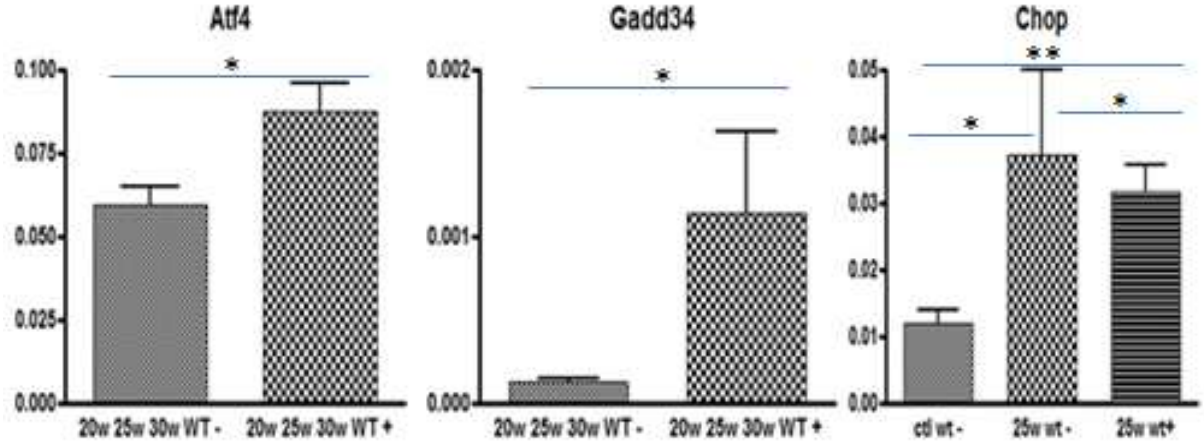

Fig. 6: The PERK-ATF4 pathway expression levels. For Atf4 and Gadd34 mRNA levels the comparison 20w, 25w and 30w DEN wild type tumor versus 20w, 25w and 30w wild type non-tumor is shown. For Chop the standard groups at 25 weeks are shown. The Y-axis represents the normalized gene expression levels. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001). ctl wt -, saline control wild type non-tumor; wt -, DEN wild type surrounding; wt+, DEN wild type tumor.

Finally, some general ER stress-inducible genes were assessed. Ero1l expression is not significantly changed. Also a common used ER stress marker, Crebh, is unexpectedly downregulated (p= 0.002). (Supplementary Fig. 4S) However, the cleavage of CREBH, similar to the RIP of ATF6, is a more sensitive indicator of ER stress.

Taken together, the expression of (co-)chaperones is elevated with exception of Grp94. Especially the upregulation of BiP, a major regulator of the UPR, is of interest to us. The ATF6 branch of the UPR appeared to be activated since unspliced Xbp1, whose expression is mainly induced by ATF6, and Pdi A4 were upregulated. However, other targets (Grp94, $p58^{IPK}$, Herp and Edem1) are not significantly changed. The IRE1 branch showed on the mRNA level no activation since the Xbp1 splicing activity, calreticulin and Grp94 are unaltered. Finally,

the PERK pathway is strongly activated. Both downstream transcription factors, NRF2 and ATF4, together with their targets (ATF4: Chop, BiP, Gadd34 and NRF2: Gsta1, Gsta2, HO-1 and γ-glutamyl cysteine synthetase), are upregulated. The ATF4-pathway seems primary activated by the tumorigenesis effect, whereas the NRF2-pathway is likely to be activated by the administration of DEN to the mice.

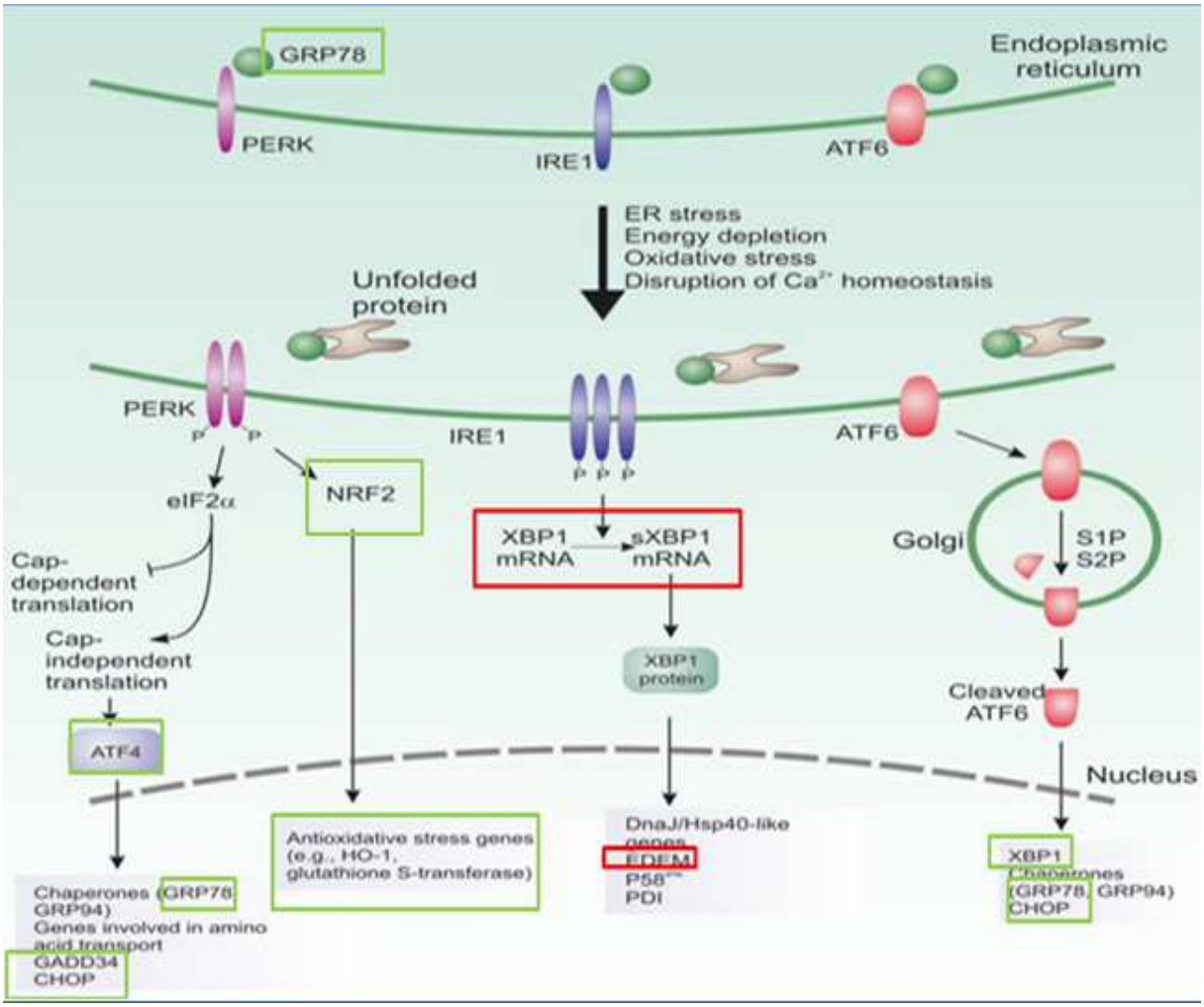

Fig. 7: Overview of the significant qRT-PCR results of the ER stress-related genes. The upregulated genes are marked by a green box and the downregulated ER stress-related genes are marked by a red box.

4.2.2. The MDR-related genes

Surprisingly, the mRNA levels of Mdr1, Mdr2, Mrp2 are not significantly changed. The expression of Brcp and Mrp6 were even downregulated by the DEN (for Brcp p= 0,048; for Mrp6 p= 0,0101) and tumorigenesis (for Brcp p= 0,026; for Mrp6 p= 0,0007) effects. (Fig. 8) Only the expression of Mrp1 (p= 0,0298) and Mrp4 (p= 0,002) is significantly increased by the hepatocarcinogenesis effect. (Fig. 9) Thus the expression of the most important genes of chemoresistance in human HCC are not significantly altered or even decreased with the exception of the Mrp1 and Mrp4 gene.

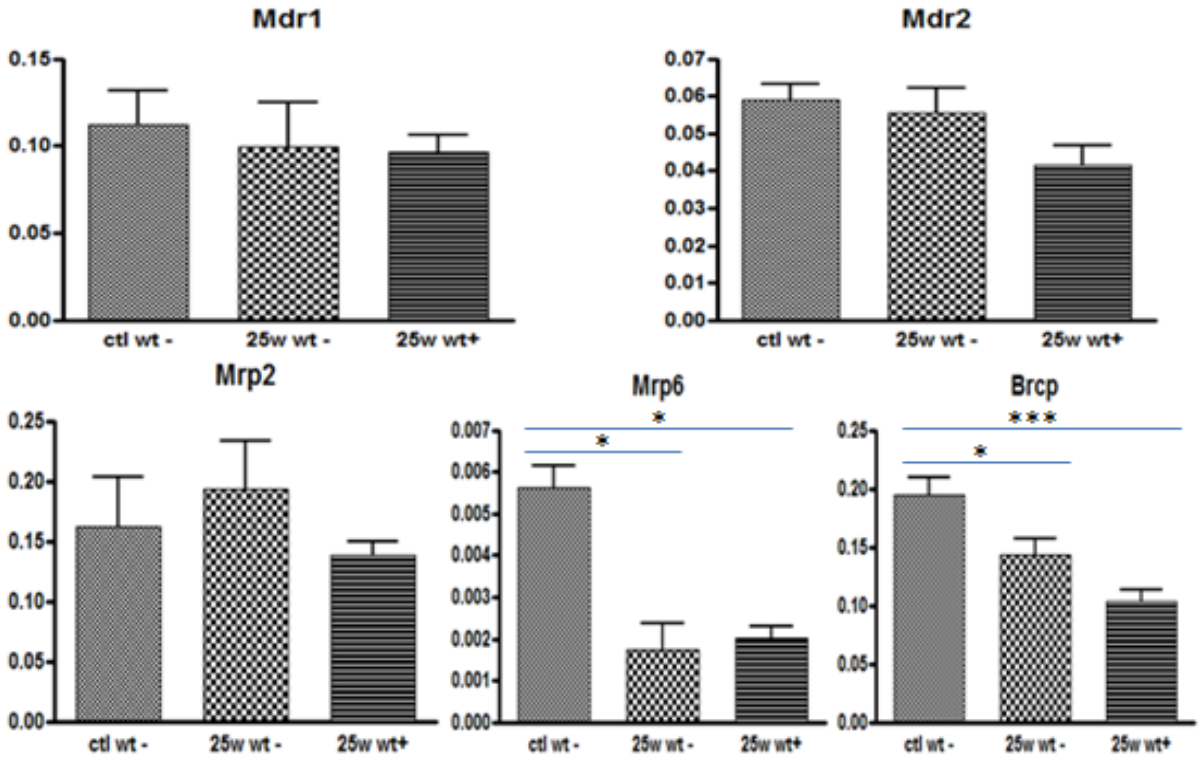

Fig. 8: The expression of Mdr1 (NS), Mdr2 (NS), Mrp2 (NS), Mrp6 (significantly decreased) and Brcp (significantly decreased). The Y-axis represents the normalized gene expression levels. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001). ctl wt -, saline control wild type non-tumor; wt -, DEN wild type surrounding; wt+, DEN wild type tumor.

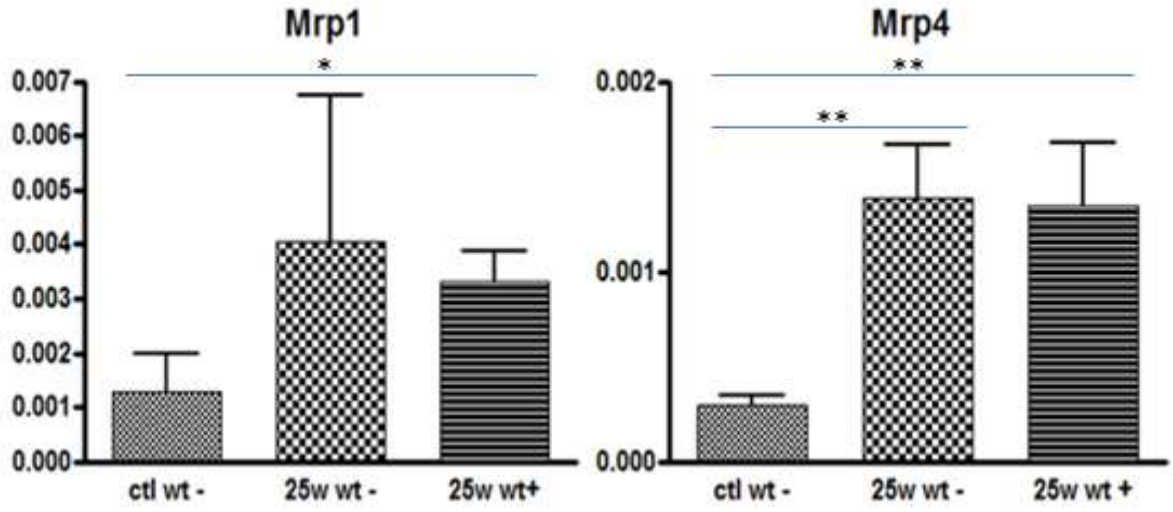

Fig. 9: The mRNA levels of the significantly elevated MDR genes Mrp1 and Mrp4. The Y-axis represents the normalized gene expression levels. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001). ctl wt -, saline control wild type non-tumor; wt -, DEN wild type surrounding; wt+, DEN wild type tumor.

4.3. Protein levels of ER stress- and MDR-related genes

4.3.1. Protein levels of genes related to ER stress

To confirm the results of the sensitive qRT-PCR, western blot of BiP has been performed. Statistical analysis of the western blot results showed a significant increase of BiP by DEN (p= 0,0016), tumorigenesis (p= 0,0104) and the combination (p= 0,0016). So the results indicate BiP is strongly upregulated by DEN. Furthermore, in addition to the DEN effect, hepatocarcinogenesis elevates the BiP protein level, however less strongly. This confirms the qRT-PCR results with exception of the significant increase by DEN only shown on the protein level, nevertheless, on qRT-PCR there is a tendency of increase after DEN-treatment. (Fig. 10)

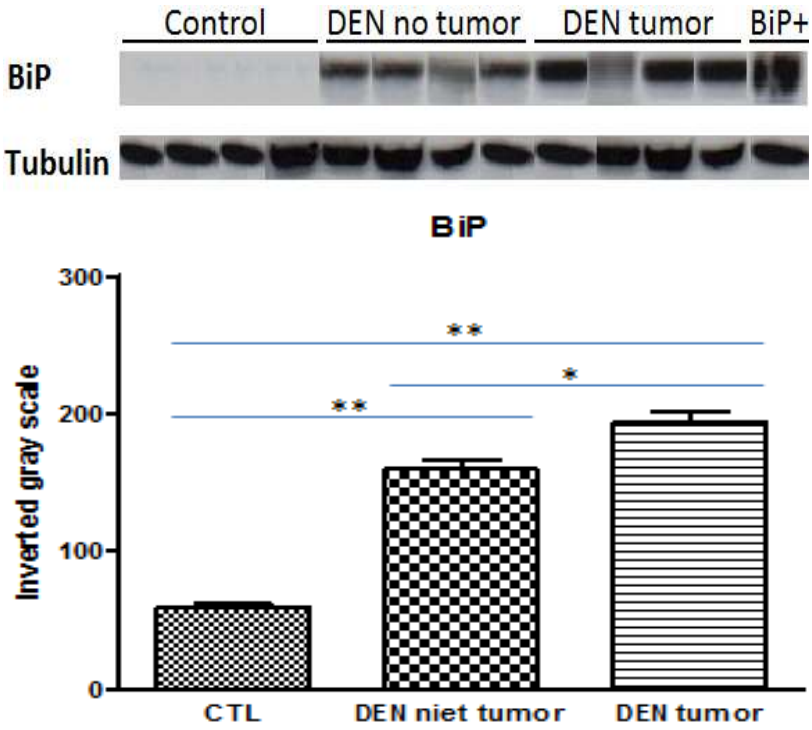

Fig. 10: The BiP protein level is significantly upregulated by DEN and hepatocarcinogenesis. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001). Tubulin levels were used for normalization. CTL, control; BiP+, positive BiP control.

The protein levels and ratio of the cleaved ATF6 fragment of 50 kDa to the total ATF6 protein of 90 kDa level is determined by western blot. This experiment showed a tendency to increased levels of the ATF6 fragment by tumorigenesis. The full ATF6 protein level is not significantly changed. Also the ATF6 fragment to full protein ratio indicates a trend to upregulation by the tumorigenesis effect. (Fig. 11)

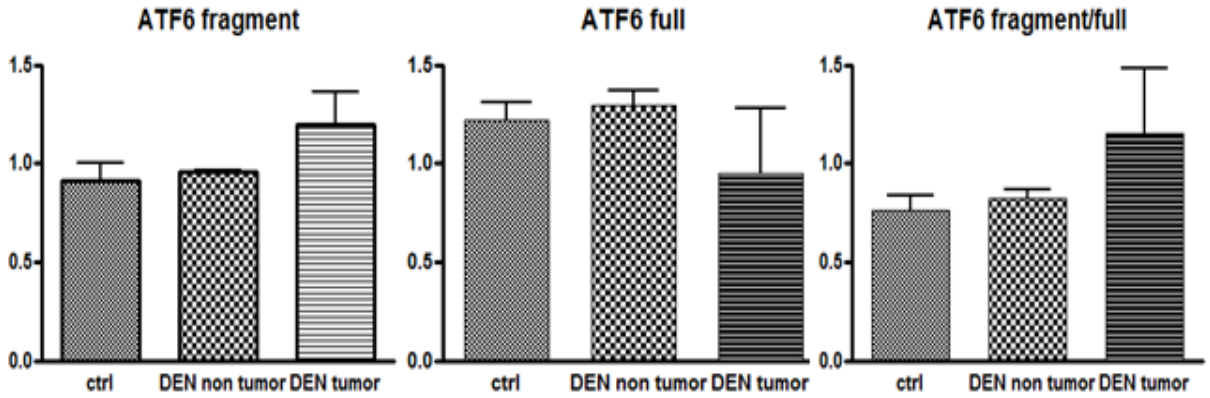

Fig. 11: The ATF6 fragment, full ATF6 protein level and the ratio. The Y-axis represents the GAPDH-normalized inverted gray scale. ctrl, control.

To determine the protein levels of spliced XBP1, western blot has been performed and showed a significant increase by the hepatocarcinogenesis effect (control versus DEN tumor: p= 0,0173 and DEN non tumor versus DEN tumor: p= 0,0496). In contrast to the mRNA level, the comparison of control versus DEN non tumor was not significant. (Fig. 12)

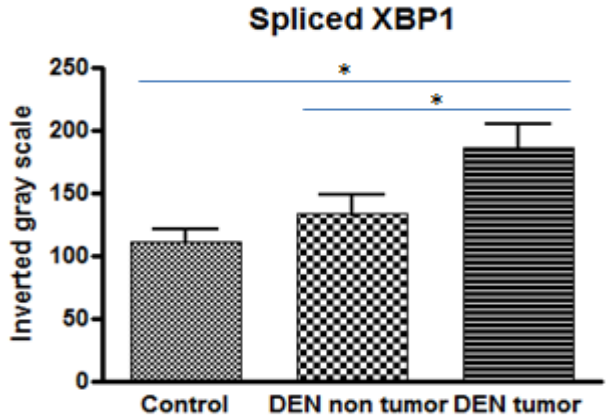

Fig. 12: Western blot analysis of spliced XBP1. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001). Tubulin levels were used for normalization.

Because of experimental failure, western blotting of phosphorylated eIF2α and phosphorylated PERK was not possible. To assess the PERK-pathway downstream, western blot of ATF4 and its target CHOP has been performed. The experimental results demonstrated a significant increase of both ATF4 (p= 0,0186) and CHOP (p= 0,0183) by the combination of DEN and tumorigenesis. (Fig. 13)

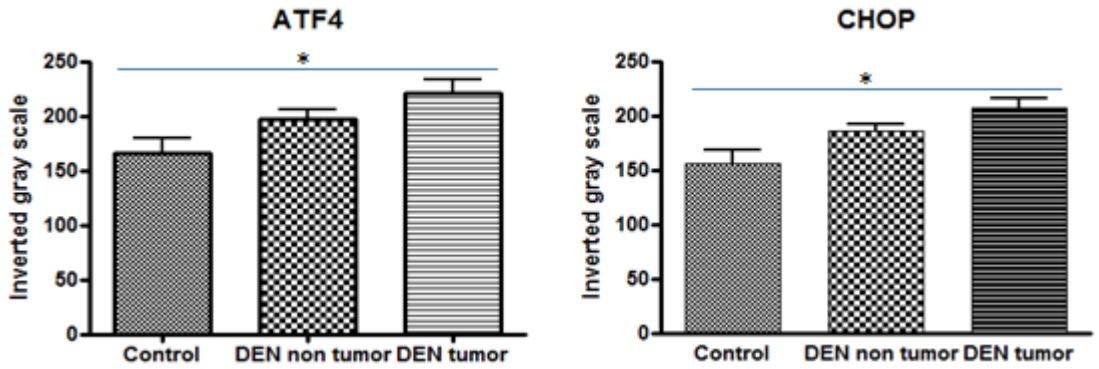

Fig. 13: Protein levels of ATF4 and its target CHOP. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001). Tubulin levels were used for normalization.

4.3.2. The results on the protein level related to MDR

To confirm the unexpected not significant qRT-PCR results on protein level ELISA of MDR1 is performed. Statistical analysis of these results showed a not significant increase by DEN and by tumorigenesis. However, the combination of both effects was significant (p= 0,0303). (Fig. 12) For the BRCP protein, the ELISA results also confirmed the qRT-PCR statistics. The DEN effect not significantly decreased BRCP. Next to the combination of both effects (p= 0,0020), the hepatocarcinogenesis effect alone (p= 0,0019) significantly reduced the BRCP protein level. So, hepatocarcinogenesis in the DEN mouse model of HCC diminished the BRCP mRNA and protein levels. (Fig. 12)

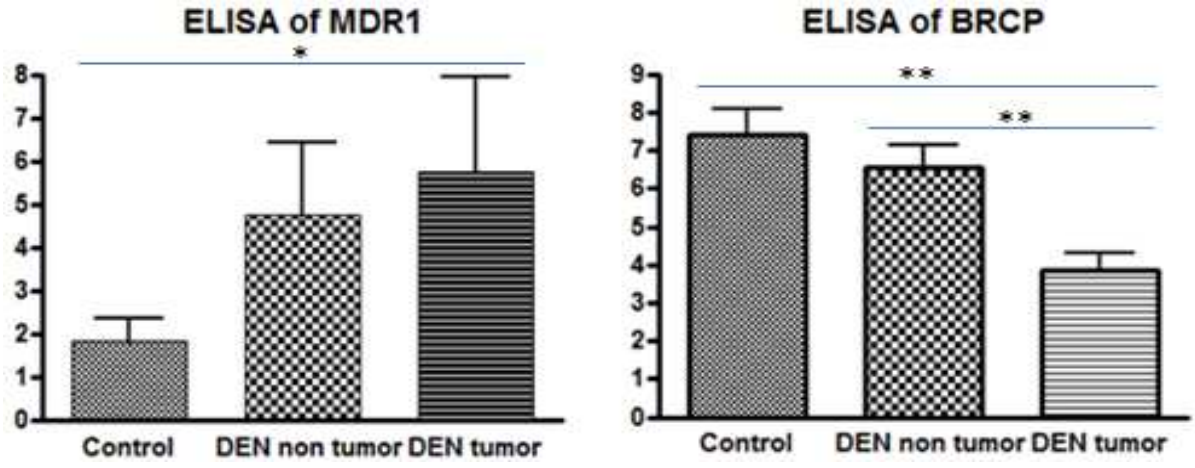

Fig. 12: The MDR1 and BRCP protein levels. The Y-axis represents the protein concentration in ng/mL. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001).

Western blot analysis of MRP4 demonstrated a tendency to upregulation by DEN and tumorigenesis. (Supplementary Fig. 5S)

5. Discussion

5.1. ER stress and the unfolded protein response

Several ER stress and MDR markers have previously been shown to be present in human HCC specimens. (Al-Rawashdeh et al., 2010; Shuda, 2003; Sun et al., 2010) To extrapolate the results obtained in **mice to human** pathology it is necessary to recognize the molecular similarities and differences.

The downregulation of **Chac1**, a recently identified component of the UPR, has been demonstrated in the analysis of the microarray. Chac1 knockdown suppressed cell migration and is discovered first in a co-regulated group of genes enriched for components of the ATF4 arm of the UPR (Gargalovic et al., 2006) and was characterised as a novel pro-apoptotic component of this pathway (Mungrue, Pagnon, Kohannim, Gargalovic, & Lusis, 2009). Previously Chac1, among other genes, was shown to be differentially expressed in pancreatic ductal adenocarcinoma in comparison with normal pancreatic ducts (Buchholz et al., 2005). Poorly differentiated tumors exhibited higher Chac1 mRNA expression in breast and ovarian cancer. However, this is up to now not yet investigated in HCC tissues.

The **glutathione S-transferases** are active in the liver and involved in detoxification of chemical carcinogens like DEN. However, in human HCC samples were the expressions of Gsta1 downregulated at both the mRNA and protein levels. (Y. Li et al., 2008) Possibly, downregulated expression of these genes within HCC tissues could create a microenvironment favorable for tumor cells to survive and progress, since it has been proved that downregulated expression of Gsta1 played an important role in increasing the sensitivity of cells to cytotoxic effect created by chemicals. (Paumi, Ledford, Smitherman, Townsend, & Morrow, 2001) However, even though Gsta1 might perform important roles in hepatocarcinogenesis, the cause of the downregulation inside human HCC tissues remains to be further elucidated. On the other hand, the Gsta1 gene expression is in the microarray significantly upregulated. This elevated transcription could represent a general increase in detoxification systems induced after repeated exposure to the chemical carcinogen i.e. DEN. This upregulation could be by NRF2-mediated transcription. However, in contrast to the qRT-PCR results, no significant differences in Nrf2 expression levels in the microarray could be determined. Also in the surrounding the upregulation of this oxidative stress responsive enzyme is noticed. So the effect seems primary because of the repeated administration of DEN. Nevertheless, an upregulation on top of DEN by tumorigenesis is still possible.

ATF3 functions as a hub of the cellular adaptive-response network and studies using various mouse models indicate that ATF3 plays a role in the pathogenesis of various diseases including cancer. (Thompson, Xu, & Williams, 2009) The p38 pathway (well known downstream mediator of the IRE1/TRAF2/ASK1 signal (Szegezdi et al., 2006)), plays a critical role in the induction of ATF3 by stress signals, and ATF3 is functionally important to mediate

the pro-apoptotic effects of p38. In addition, ATF4 enhances the expression of ATF3. (Hai, Jalgaonkar, Wolford, & Yin, 2011) In the analysis of the expression in the microarray, Atf3 mRNA is significantly induced in HCC compared to the normal liver tissue. Atf4 mRNA is, in contrast to the qRT-PCR results, not upregulated in the microarray.

The Bax gene expression is upregulated in the surrounding in the microarray**.** Most stress signals induce apoptosis via a mitochondria-dependent process, in which the release of cytochrome c and other apoptogenic factors from mitochondria leads to the formation of the apoptosome and the activation of caspases. **The BCL-2 family** is a major regulator of this apoptotic pathway. Members of this family can be classified into the anti- and pro-apoptotic groups. The anti-apoptotic members, including BCL-2, BCL-XL and MCL-1, protect the integrity of the mitochondrial outer membrane. The BCL-2 expression is in our results significantly enhanced in HCC induced by DEN compared to normal liver tissue after saline administration. This protein could represent a mechanism of resistance to apoptosis in the tumor cells by counteracting the pro-apoptotic proteins such as BAX induced by DEN administration. The pro-apoptotic members, on the other hand, trigger mitochondrial dysfunction. This group can be further divided into the multi-BH domain members, including BAX and BAK, and the BH3-only proteins including BAD. Of the 11 members of the BH3-only family, PUMA, NOXA, BID and BIM, have been reported to mediate apoptosis triggered by ER stress (Cazanave et al., 2010; J. Li et al., 2006; Puthalakath et al., 2007; Upton et al., 2008; Weston & Puthalakath, 2010). However, these ER stress-related BH3-only genes are not significantly upregulated because the increase in expression is less than 10-fold (absolute log fold change with 1). Other reports also show more discrete upregulation. (J. Li et al., 2006; Puthalakath et al., 2007; X. Wang, Olberding, White, & Li, 2011; L. Zhang et al., 2011) Once activated, BAX and BAK homo-oligomerize and permeabilize the outer mitochondrial membrane. BH3 domains that directly `activate' BAX and/or BAK at mitochondria, such as the BH3 domain of BID, are able to cause cytochrome c release from isolated mitochondria. In contrast, `sensitizing' BH3 domains, such as the BH3 domains of BAD and BIK, trigger isolated mitochondria to release cytochrome c in the presence of a second ‘activating' BH3 domain. (Estaquier, Vallette, Vayssiere, & Mignotte, 2012) In addition to mediating ER stress-driven apoptosis, the BCL-2 family also regulates ER stress through physical interaction with certain UPR components. (Hetz & Glimcher, 2009) For example, BAX and BAK interact directly with the cytosolic domain of IRE1 upon ER stress, which is essential for the IRE1 activation (Hetz et al., 2006). In cells exclusively expressing BAK on the ER, BIM and PUMA selectively activate the TRAF2–JNK arm of IRE1 signaling in the absence of XBP1 splicing. (Klee, Pallauf, Alcalá, Fleischer, & Pimentel-Muiños, 2009) In BAX/BAK −/− mice, tunicamycin failed to induce spliced XBP1, IRE1 and JNK activation. Moreover, BAX/BAK –/– MEFs are resistant to apoptosis induced by various ER stressors. Reconstitution of BAK expression in the BAX/BAK −/− MEFs restored tunicamycin-induced JNK phosphorylation, suggesting a direct connection between the UPR and the apoptotic machinery (Hetz et al., 2006). Thus BAX and BAK are required for normal IRE1 signaling, although they are also involved in ER

stress-driven apoptosis. This might represent a switching mechanism towards pro-apoptotic signaling by IRE1. BCL-2, BAX and BAK associate not only with mitochondrial membranes but also with the ER. During ER stress, ER-targeted BAX and BAK undergo conformational changes and oligomerization in the ER membrane (Zong et al., 2003). This causes the release of calcium from the ER to the cytoplasm and activates m-calpain, which in turn activates procaspase-12. (Lee, 2007; Scorrano et al., 2003) In contrast, mitochondria-targeted BAK leads to enhanced caspase-7 cleavage. This creates parallel pathways of caspase activation by BAX and BAK for selected apoptotic signals. (Zong et al., 2003) The upregulation of BAX in our results may represent a pro-apoptotic state of the surrounding tissue after DEN exposure.

Here we present for the first time evidence that certain markers of ER stress have a **similar pattern** in the DEN-induced mouse model of HCC as compared to human HCC specimens (Al-Rawashdeh et al., 2010; Duan et al., 2012; Shuda, 2003). Indeed, both mRNA and protein levels of BiP, the major regulator of the UPR, are upregulated by both repeated DEN administration and hepatocarcinogenesis. The anti-apoptotic mechanisms of BiP include reduction of CHOP, sequestration of caspase-7 and BIK on the ER membrane, preserving the ER calcium homeostasis and its chaperone activity to limit the aggregation of misfolded proteins. (Lee, 2007; Z. Li & Li, 2012; Nishitoh, 2012)

Based on the mRNA and protein results, the ATF6 pathway is probably active, the IRE1 pathway seems to be inactive and the PERK pathway is active.

On qRT-PCR the **ATF6** target, unspliced XBP1, is especially by the tumorigenesis effect upregulated. Also the ATF6 fragment protein level and the ATF6 fragment to full protein ratio showed a tendency to be upregulated by this effect. The activation of the cytoprotective ATF6 branch could represent a relevant cell survival mechanism during hepatocarcinogenesis. Unfortunately, pharmacological modification of this pathway is insufficiently investigated. (Kraskiewicz & Fitzgerald, 2012)

With regard to the **IRE1** pathway, it is important to acknowledge that only the splicing of XBP1 was determined in this research project. However, the IRE1 pathway has other distinct downstream effectors relating to ER stress-induced apoptosis. Our results demonstrated a strong upregulation of unspliced XBP1 without increased XBP1 splicing activity, together with a weaker upregulation of spliced XBP1, especially by the hepatocarcinogenesis effect on the protein level in our HCC model. However, the unspliced XBP1 protein inhibits the transcriptional activity of the spliced XBP1 protein. (Ron & Walter, 2007; Yoshida et al., 2006) Indeed, no significant upregulation of spliced XBP1 targets is noticed in our study. We were able to demonstrate the increased spliced XBP1 on the protein level, but to directly measure the IRE1 activity, an ELISA of the phosphorylated version of IRE1 has been ordered and will be performed.

Both transcription factors of the **PERK** branch ATF4 and NRF2 and their targets are strongly activated. Next to ER stress, also oxidative stress induced by DEN is a possible mechanism to upregulate the NRF2 pathway. Through the formation of hydrogen peroxide and superoxide anions by the P450-dependent enzymatic system, DEN induces oxidative stress. Production of ROS is known to cause DNA, protein and lipid damage; therefore, oxidative stress can play an important role in hepatocarcinogenesis. (Qi et al., 2008; Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006)

This pathway stimulates the cellular ROS detoxification systems and protects the stressed tumor cells. The Nrf2 mRNA levels on microarray are not significantly changed, but on the sensitive qRT-PCR an increased expression is noticed. Gsta1, on the other hand, is both on the microarray as on the qRT-PCR upregulated. In addition, PERK activation is important for the ATF6 function. The PERK pathway facilitates both the synthesis of ATF6 and trafficking of ATF6 from the ER to the Golgi apparatus for RIP and activation of ATF6. (Teske et al., 2011) In this study both are probably activated as expected since ATF6 requires PERK activity. This emphasizes the fact that the regulatory networks of the UPR are fully integrated.

CHOP is a major regulator of ER stress-elicited apoptosis. The strong upregulation of CHOP on the mRNA and protein level could theoretically be a consequence of activation of all 3 major pathways. (Jauhiainen et al., 2012; Marciniak et al., 2004; Nishitoh, 2012; Zinszner et al., 1998) However, the PERK pathway is the major inducer of CHOP expression and is also activated in our experiments. Therefore the PERK pathway is the most obvious transcriptional inducer of CHOP. The pro-apoptotic CHOP expression is enhanced by the DEN administration without significant impact of the tumorigenesis effect.

Since we identified an unexpected **baseline IRE1 activity** in the HCC, possible explanations are required. First, IRE1 is thought to be the last arm of the UPR to be activated, with PERK being the first, closely followed by ATF6. Perhaps the PERK- and ATF6-mediated pathways attempt to resolve the stress before activation of IRE1. Once activated, IRE1 aids the UPR by splicing XBP1. At this point, either the cell returns to normal functioning or, if the stress persists, IRE1 initiates apoptosis by recruiting ASK1 and JNK. (Szegezdi et al., 2006) On the other hand, there could be already an IRE1 attenuation (after earlier activation, perhaps in the first weeks of DEN exposure). An IRE1 and ATF6 attenuation is demonstrated after ± 8u with persistence of PERK activity during prolonged ER stress. (J. H. Lin et al., 2007) However, how the UPR pattern changes over periods of months needs further research. IRE1 mutations are shown to be frequent in human cancers. These accumulated mutations could inactivate its splicing activity. (Greenman et al., 2007) In cells exclusively expressing BAK on the ER, BIM and PUMA selectively activate the TRAF2–JNK arm of IRE1 signaling in absence of XBP1 splicing. (Klee et al., 2009) So the JNK pathway can be activated without increased

IRE1 splicing activity. Further in this research we will examine the phosphorylation status of JNK by western blot.

The **bidirectional influence on cell fate** by the induction or repression of pro- and anti-apoptotic effectors is typical for the UPR. Also in our results concomitant anti-apoptotic (such as BiP, spliced XBP1 or the NRF2 branch (Bobrovnikova-Marjon et al., 2010)) and pro-apoptotic (such as CHOP (Nishitoh, 2012) or GADD34 (Hollander, Poola-Kella, & Fornace, 2003)) events are noticed. The meticulously regulated cell fate balance integrates the different signals of various UPR components.

Taken together, we can conclude **ER stress is present** and selectively activates some components of the UPR such as (co-)chaperones like BiP, CHOP, unspliced XBP1 and the ATF4/NRF2-path. However, the reasons why in the DEN-induced mouse model of HCC one UPR component is activated and another not, remains to be elucidated.

5.2. Multidrug resistance

Intrinsic and acquired resistance to multiple chemotherapeutic drugs is a major obstacle in the clinical treatment of cancer and the mechanisms responsible for the observed MDR in e.g. HCC remain unclear. Regarding the MDR markers in HCC in the DEN-induced mouse model, the focused resistance mechanism consists of the upregulation of certain **efflux pumps** with a significant role in the chemoresistance of human HCC through reducing the intracellular concentration of the administrated chemotherapeutics. (Fig. 13) Surprisingly, the results demonstrated that the expression of the major genes contributing to MDR is not significantly altered or even decreased with the exception of the Mrp1 and Mrp4 gene.

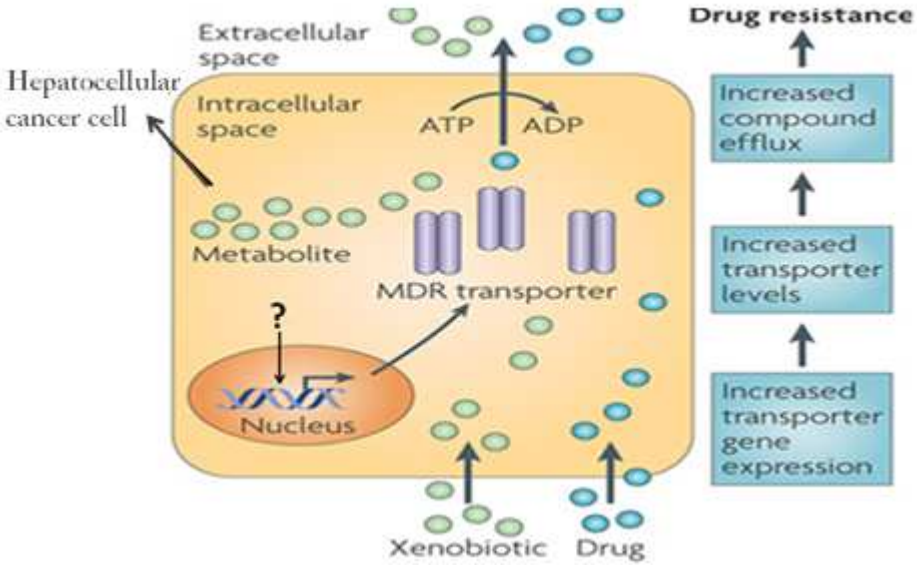

Fig. 13: The ATP-dependent action of efflux pumps localized at the plasma membrane is a major multidrug resistance mechanism in HCC. The complex regulation of the expression of efflux pumps is currently not clear. MDR, multidrug resistance.

Within the ABC-transporter family, the **Abca8a** is significantly downregulated in HCC compared to normal liver tissue. The Abca8a is a transporter of certain lipophilic drugs such as digoxin (mainly expressed in the liver and heart) and showed reduced expression by digoxin intoxication in mouse liver. Also DEN could be a substrate downregulating its expression. However, the role in MDR is not well defined.

The expression of **DNA topoisomerase II-α** is upregulated in the HCC and surrounding samples. Topo IIα expressions correlated with advance histological grading, microvascular invasion and an early age onset of malignance. The topo IIα positivity correlated with chemoresistance and shorter patients survival. *In vitro* cytotoxic studies suggested etoposide at IC20 readily reduced IC50 values of doxorubicin compared to doxorubicin alone. (Wong et al., 2009) Expression of topo IIα has also been demonstrated in breast (Romero, Caldés, Díaz-Rubio, & Martín, 2012), colon, ovarian and small cell lung cancers to be a valuable prognostic marker for tumor progression, recurrences and predictor of poorer survival (Dawany, Dampier, & Tozeren, 2011). The increased proliferation of the tumor cells is likely to require increased topo II activity supporting the replication by unwinding and untangling DNA. On the other hand, reduced levels of topo II could induce tumor cell resistance to topo II inhibitors due to a reduction of topo II : DNA complexes and therefore the amount of drug-induced DNA damage. However, the mechanisms, next to supporting replication, to increase chemoresistance by topo IIα upregulation are currently unknown.

The upregulation of **MRP1** is a possible MDR mechanism in the mouse model of HCC. MRP1 has been reported to be strongly expressed in leukemia, esophageal, non small cell lung cancer and HCC (Vander Borght et al., 2008). Although Mrp1 knockout mice are viable and fertile, they exhibit increased chemosensitivity in certain tissues such as the seminiferous tubules, the intestine, the oropharyngeal mucosa and the choroid plexus. One notable feature of MRP1 is the differences between the substrate specificity of MRP1 from primates and MRP1 from other species, even though the amino acid sequence similarity is more than 90% for all species. MRP1 from humans can transport anthracycline antibiotics, e.g. doxorubicin, whereas this function is lost in the MRP1 from mice. Human MRP1 contributes to the resistance to anthracyclines, camptothecins, vinca alkaloids, etoposide, irinotecan and antifolate neoplastics such as methotrexate. (Z.-S. Chen & Tiwari, 2011) Some of the newer so-called 'targeted' agents (e.g. certain tyrosine kinase inhibitors) that modify various signal transduction pathways can also be transported by MRP1. (Hegedűs et al., 2002) However, no data is known for sorafenib. MRP1 interacts with glutathione. This cellular tripeptide antioxidant is most noted for its role in protecting cells from the deleterious effects of oxidative stress. It is required in xenobiotic metabolism to form glutathione-X conjugates,

which are then exported by MRP1. The observation that glutathione levels in some tissues of Mrp1 -/- mice are elevated has been interpreted as *in vivo* evidence that glutathione is also an MRP1 substrate. *In vitro* glutathione is transported by MRP1 with low affinity, whereas the pro-oxidant glutathione disulfide is a relatively higher affinity substrate. Consistent with the ability to transport these critical sulfhydryls, studies indicate that MRP1 modulates cellular oxidative stress and redox homeostasis. (Z.-S. Chen & Tiwari, 2011) So MRP1 could affect the PERK-NRF2 pathway by its effect on the cellular oxidative stress induced by the administration of DEN.

Melphalan and chlorambucil are both substrates of GSTA1 and the monoglutathionyl conjugates formed in these enzymatic reactions are transported by MRP1. Chlorambucil-S-glutathione is a more potent competitive inhibitor of GSTA1 than melphalan-S-glutathione. Indeed, MRP1 is required for GSTA1-mediated resistance to chlorambucil in order to relieve potent product inhibition of GSTA1 by intracellular chlorambucil-S-glutathione. For melphalan, where product inhibition of GSTA1 is less important, GSTA1 does not confer resistance because of the relatively poorer catalytic efficiency of melphalan-S-glutathione formation. (Paumi et al., 2001) Thus, the upregulation of GSTA1 together with MRP1 in our results could enhance chemoresistance to chlorambucil or other alkylating agents.

The upregulation of the efflux pump **MRP4** could enhance the cellular capacity to transport certain chemotherapeutics out of the cell. (Z.-S. Chen & Tiwari, 2011) Mrp4 -/- mice have proven to be valuable models in demonstrating the importance of Mrp4 in drug disposition and elimination. For example, topotecan accumulation in both brain tissue and in cerebral spinal fluid is increased in these animals. (Leggas et al., 2004) MRP4 also confers resistance to anticancer agents including thiopurine analogs, methotrexate and, as expected, topotecan (Tian et al., 2006). Thiopurine analogs and most nucleoside-based chemotherapeutic drugs require intracellular phosphorylation before they are pharmacologically active. Consequently, MRP4 confers resistance to these agents by effluxing their anionic phosphate metabolites rather than the parent compounds. (Z.-S. Chen & Tiwari, 2011)

In other studies on **human HCC** samples upregulation of ABCA2, ABCB1/MDR1, ABCB6, ABCC1/MRP1, ABCC2/MRP2, ABCC3/MRP3, ABCC4/MRP4, ABCC5/ MRP5, ABCC10/MRP7, ABCC11/MRP8, ABCC12/MRP9 and ABCG2/BRCP is described. (Borel et al., 2012; G. Li et al., 2007; Sun et al., 2010) Here, in the DEN mouse model of HCC, this pattern of efflux pump upregulation could not be completely significantly confirmed. In rat livers exposed to DEN for 12 weeks the MDR1 immunostaining of hyperplastic nodules was more intense than in well-differentiated HCCs, and no staining was observed in poorly-differentiated carcinomas. Markedly intense staining was observed in hyperplastic nodules of cisplatin-pretreated as well as in epirubicin-pretreated rats. So less MDR1 is shown in the poorly-differentiated HCC. Also the induction of acquired MDR after administration of chemotherapeutics is observed.

(Takeuchi, 2002) In our study the sampling occurred after 30 weeks of DEN administration. The extra time for hepatocarcinogenesis could gradual downregulate the involved MDR genes. The exact cause of the different MDR pattern in human HCC compared to DEN-induced mouse HCC is currently unclear. Different scenarios are possible. First, there is no development of MDR in the HCC mouse model. This could be because of the time to acquire MDR needs to be longer than 30 weeks or because the different triggers of hepatocarcinogenesis such as viral hepatitis infection (hepatitis B or C) or cirrhosis (e.g. secondary to alcoholism) compared to the repeated DEN administration in our model stimulate also a different efflux pump pattern. Secondly, the power of our study could be too low to show a significant upregulation of the concerned MDR genes. However, for other genes the power was adequate and showed statistically significant outcomes. Also the tendency to upregulation of several MDR genes is absent. Finally, the PERK-mediated repression of translation could reduce the translation of the MDR proteins. However, not only the results of the qRT-PCRs and microarray on the mRNA level showed this outcome but also the ELISA on the protein level confirms the unexpected MDR pattern.

6. Perspectives for further research

Nowadays ER stress research is a hot topic, exemplified by more than 2000 PUBMED hits on ER stress in 2011. Nevertheless in order to integrate the links with other pathways (e.g. the HIF1 pathways) in a **comprehensive network** and to translate the upcoming knowledge we have to focus on untangling the downstream mediators and crosstalk for all three major UPR pathways.

To elucidate the role of the different anti-apoptotic functions of the **elevated BiP** in the HCC model an extended elaboration of the concerned signal transduction pathways (effect on caspase-7 cleavage or calcium homeostasis) is required.

To further evaluate the exact role of the IRE1 pathway in the DEN-induced mouse model of HCC the downstream mediators, beside spliced XBP1, should be evaluated. The most important herein are the activation of **ASK, JIK, JNK, ERK, NF_KB and p38**. (Ron & Walter, 2007) Also the role of **RIDD** should be determined in this model. (Hollien & Weissman, 2006)

Since we propose **oxidative stress** involvement in the upregulation of the NRF2-pathway, confirmation and quantification of the oxidative stress induced by the DEN exposure is indicated through the assessment of the ROS themselves. (Darwish, Hebatallah A. El-Boghdady, 2011)

Most studies measured only two or three **ER stress markers** like BiP or CHOP and the minority expanded their efforts to include molecules from each of the IRE1, ATF6 and PERK branches of the UPR. Two or more branches may induce different steps of a single linear apoptosis pathway. Different UPR branches may separately induce the same pro-apoptotic

effector. Finally, different, complementary UPR branches may promote parallel apoptosis pathways in a manner that could increase the probability of apoptosis of the tumor cells. Indeed, when one UPR branch or effector is experimentally silenced, ER stress-induced apoptosis is not suppressed completely. The fact that CHOP is a transcriptional target of not only ATF4 but also spliced XBP1 and activated ATF6 provides an obvious link among all three branches. A caveat here is that the IRE1 and ATF6 branches exert weaker activation compared to the PERK–CHOP branch during prolonged ER stress. The relationship between ER stress and disease can only be fully appreciated if the 'ER stress specialists' cast their analytical nets further. Much information is lost if we do not dissect the UPR pathways. Future specific UPR modification has the potential to influence the UPR to one way: homeostasis or apoptosis in function of the clinical application. So we have to avoid haziness by using the specific inhibition (e.g. siRNA) or activation (e.g. salubrinal) approach as much as possible instead of the general activation approach (e.g. tunicamycin) to make the right conclusions directing the future therapeutic options. More and more reports demonstrated that certain known **active compounds** function on tumor growth by ER stress modification (e.g. tetradecylthioacetic acid (Lundemo, Pettersen, Berge, Berge, & Schønberg, 2011), stellettin A (W. K. Liu, Ling, Cheung, & Che, 2012), celecoxib (Huang et al., 2012) and cryptotanshinone (I.-J. Park et al., 2012)). However, the question arises if the modification of a main adaptation mechanism is the real target of the compound.

It is unclear how tumor cells adapt to long term ER stress *in vivo*. It is known that some of the mitochondria-dependent pro-apoptotic components are mutated in neoplastic cells. The UPR components are, as shown in our model, clearly activated in several solid tumors, but how the activity evolves in time is currently unknown. Therefore experimental cancer models to monitor the **temporal adjustments** in the UPR components are required. Most of the studies on ER-associated apoptosis used toxins causing acute severe ER stress. Whether these pathways also contribute to ER stress-associated cell death in pathological states associated with chronic ER stress requires more study in chronic models. Studying the UPR by using a variety of inducers, the most common being thapsigargin (inhibitor of the SERCA pump) and tunicamycin (inhibitor of N-linked glycosylation), makes the comparison of data from different systems difficult because these have different targets and differences in the induced responses. Also the effect of drugs interfering with the UPR could be time-dependent e.g. can we reduce the cancer incidence by reducing ER stress through enhancing the protein folding capacity during carcinogen exposure. For example chronic alcohol use increases the risk for HCC approximately 5-fold. Are we able to produce molecules protecting these organs by enhancing alcohol-induced ER stress coping? By use of an alcohol protector the rising incidence of HCC could dramatically decrease. (Ji, 2012)

The UPR promotes the ability of neoplastic cells to adapt to and survive the hostile microenvironment. To target the UPR in cancer, two mechanisms are used: inhibiting UPR components so cells cannot adapt to stress (e.g. versipelostatin, a repressor of BiP

expression) and overloading the UPR (e.g. proteasome inhibitors) so the cell is unable to cope, leading to apoptosis. Overall, a better scenario would involve a dual approach of overloading the capacity of the ER while inhibiting the UPR. (Kraskiewicz & Fitzgerald, 2012) Although accumulation of unfolded proteins in the ER is now known to contribute to the pathogenesis of a variety of diseases, there are still few **therapeutic approaches** targeting this event. Pathway-specific and even more selective molecules (e.g. IRE1 endonuclease inhibitor (Cross et al., 2012) and the new PERK activator CCT020312 (Stockwell et al., 2012)) are needed for the research and to our opinion for further clinical usefulness of the emerging UPR knowledge.

Protein quality control is **fundamentally** important for life. Thus targeted therapy towards the UPR is by no means cancer-specific and toxicity-free. Thereby emphasizing the importance of tumor selective drug delivery. There are some normal cell types with a high demand on their ER, for example antibody-producing B-cells or insulin-secreting β-cells, and potential toxicity to these tissue types would need to be assessed closely in any drug discovery effort. However, research in tissue-specific UPR activation patterns (Tagliavacca, Caretti, Bianciardi, & Samaja, 2012), also of *in vitro* and *in vivo* models, could differentiate the target tissues.

Assessment of the liver lesions by **functional imaging** in collaboration with the Infinity Lab, UGent will complete the project. The experiments described will be supported by functional imaging through small animal imaging by dynamic contrast-enhanced-magnetic resonance imaging (DCE-MRI) fused with positron emission tomography (PET) scanning. The acquisitions are performed using the Gamma Medica Ideas lab PET 8, a state-of-the-art multimodal micro PET-CT device existing of a PET with 2×2×10 mm^3 LYSO/LGSO scintillators in an 8-pixel, quad-APD detector module arrangement. The PET system is capable of delivering 1 mm spatial resolution in rodents at a sensitivity of 4% thereby covering a field-of-view of 10 cm transaxially by 8 cm. For this study, both 18F-fluorodeoxyglucose and 18F-choline will be used. For the MR system, we use a 7 Tesla small animal MRI with up to 50 µm structural resolution for excellent soft-tissue contrast. Furthermore, in collaboration with the Infinity Lab, UGent, and Radiopharmacy, UGent, we will produce radiolabeled anti-placental growth factor (anti-PlGF) for micro single-photon emission computed tomography (SPECT) imaging of the PlGF production in the HCC mouse model in combination with structural imaging by micro CT. The ER stress marker BiP is present on the cell surface in stressful conditions in certain cancer cells. We will also produce radiolabeled anti-BiP to visualize the presence of cell surface BiP in the model. (Ni, Zhang, & Lee, 2011) The two mentioned radiolabeled antibodies are up to now never produced, also SPECT imaging with these is never done. The SPECT imaging system involves a triple head SPECT with different acquisitions modes and collimators. However, this part of the study is planned in July 2012, but the animals for the imaging are produced and the antibodies are radiolabeled.

Generally, the UPR pattern in the DEN-induced mouse model of HCC resembles the pattern in human HCC. The differences like the basal XBP1 splicing and strongly activation of the NRF2-path (probably due to oxidative stress by DEN) could theoretically impair the mouse to human extrapolation. However, our results demonstrated that the model can be used for further evaluation of the UPR pathways in HCC. Combination with various chemotherapeutics and evaluation of the acquired MDR profile of efflux pumps could provide important information for the development of future therapeutic options.

7. References

Adachi, Y., Yamamoto, K., Okada, T., Yoshida, H., Harada, A., & Mori, K. (2008). ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. *Cell structure and function*, *33*(1), 75-89. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18360008

Al-Rawashdeh, F. Y., Scriven, P., Cameron, I. C., Vergani, P. V., & Wyld, L. (2010). Unfolded protein response activation contributes to chemoresistance in hepatocellular carcinoma. *European journal of gastroenterology & hepatology*, *22*(9), 1099-105. doi:10.1097/MEG.0b013e3283378405

Apel, A., Herr, I., Schwarz, H., Rodemann, H. P., & Mayer, A. (2008). Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. *Cancer research*, *68*(5), 1485-94. doi:10.1158/0008-5472.CAN-07-0562

Arai, M., Kondoh, N., Imazeki, N., Hada, A., Hatsuse, K., Kimura, F., Matsubara, O., et al. (2006). Transformation-associated gene regulation by ATF6alpha during hepatocarcinogenesis. *FEBS letters*, *580*(1), 184-90. doi:10.1016/j.febslet.2005.11.072

Austin, R. C. (2009). The unfolded protein response in health and disease. *Antioxidants & redox signaling*, *11*(9), 2279-87. doi:10.1089/ARS.2009.2686

Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., & Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nature cell biology*, *2*(6), 326-32. doi:10.1038/35014014

Bobrovnikova-Marjon, E., Grigoriadou, C., Pytel, D., Zhang, F., Ye, J., Koumenis, C., Cavener, D., et al. (2010). PERK promotes cancer cell proliferation and tumor growth by limiting oxidative DNA damage. *Oncogene*, *29*(27), 3881-95. doi:10.1038/onc.2010.153

Borel, F., Han, R., Visser, A., Petry, H., van Deventer, S. J. H., Jansen, P. L. M., & Konstantinova, P. (2012). Adenosine triphosphate-binding cassette transporter genes up-regulation in untreated hepatocellular carcinoma is mediated by cellular microRNAs. *Hepatology (Baltimore, Md.)*, *55*(3), 821-32. doi:10.1002/hep.24682

Brewer, J. W., & Jackowski, S. (2012). UPR-Mediated Membrane Biogenesis in B Cells. *Biochemistry research international*, *2012*, 738471. doi:10.1155/2012/738471

Bruix, J., & Llovet, J. M. (2002). Prognostic prediction and treatment strategy in hepatocellular carcinoma. *Hepatology (Baltimore, Md.)*, *35*(3), 519-24. doi:10.1053/jhep.2002.32089

Buchholz, M., Braun, M., Heidenblut, A., Kestler, H. A., Klöppel, G., Schmiegel, W., Hahn, S. A., et al. (2005). Transcriptome analysis of microdissected pancreatic intraepithelial neoplastic lesions. *Oncogene*, *24*(44), 6626-36. doi:10.1038/sj.onc.1208804

Cao, S. S., Song, B., & Kaufman, R. J. (2012). PKR protects colonic epithelium against colitis through the unfolded protein response and prosurvival signaling. *Inflammatory bowel diseases*. doi:10.1002/ibd.22878

Cazanave, S. C., Elmi, N. A., Akazawa, Y., Bronk, S. F., Mott, J. L., & Gores, G. J. (2010). CHOP and AP-1 cooperatively mediate PUMA expression during lipoapoptosis. *American journal of physiology. Gastrointestinal and liver physiology*, *299*(1), G236-43. doi:10.1152/ajpgi.00091.2010

Chakrabarti, A., Chen, A. W., & Varner, J. D. (2011). A review of the mammalian unfolded protein response. *Biotechnology and bioengineering*, *108*(12), 2777-93. doi:10.1002/bit.23282

Chen, R., Dai, R. Y., Duan, C. Y., Liu, Y. P., Chen, S. K., Yan, D. M., Chen, C. N., et al. (2011). Unfolded protein response suppresses cisplatin-induced apoptosis via autophagy regulation in human hepatocellular carcinoma cells. *Folia biologica*, *57*(3), 87-95. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21888831

Chen, T. C., Wang, W., Golden, E. B., Thomas, S., Sivakumar, W., Hofman, F. M., Louie, S. G., et al. (2011). Green tea epigallocatechin gallate enhances therapeutic efficacy of temozolomide in orthotopic mouse glioblastoma models. *Cancer letters*, *302*(2), 100-8. doi:10.1016/j.canlet.2010.11.008

Chen, Z.-S., & Tiwari, A. K. (2011). Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases. *The FEBS journal*, *278*(18), 3226-45. doi:10.1111/j.1742-4658.2011.08235.x

Clarke, R., Cook, K. L., Hu, R., Facey, C. O. B., Tavassoly, I., Schwartz, J. L., Baumann, W. T., et al. (2012). Endoplasmic reticulum stress, the unfolded protein response, autophagy, and the integrated regulation of breast cancer cell fate. *Cancer research*, *72*(6), 1321-31. doi:10.1158/0008-5472.CAN-11-3213

Cross, B. C. S., Bond, P. J., Sadowski, P. G., Jha, B. K., Zak, J., Goodman, J. M., Silverman, R. H., et al. (2012). The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. *Proceedings of the National Academy of Sciences of the United States of America*. doi:10.1073/pnas.1115623109

Darwish, Hebatallah A. El-Boghdady, N. A. (2011). Possible involvement of oxidative stress in diethylnitrosamine - induced hepatocarcinogenesis chemopreventive effect of curcumin. *Journal of Food Biochemistry*, no-no. doi:10.1111/j.1745-4514.2011.00637.x

Dawany, N. B., Dampier, W. N., & Tozeren, A. (2011). Large-scale integration of microarray data reveals genes and pathways common to multiple cancer types. *International journal of cancer. Journal international du cancer*, *128*(12), 2881-91. doi:10.1002/ijc.25854

Duan, Q., Wang, X., Gong, W., Ni, L., Chen, C., He, X., Chen, F., et al. (2012). ER Stress Negatively Modulates the Expression of the miR-199a/214 Cluster to Regulates Tumor Survival and Progression in Human Hepatocellular Cancer. *PloS one*, *7*(2), e31518. doi:10.1371/journal.pone.0031518

Estaquier, J., Vallette, F., Vayssiere, J.-L., & Mignotte, B. (2012). The mitochondrial pathways of apoptosis. *Advances in experimental medicine and biology*, *942*, 157-83. doi:10.1007/978-94-007-2869-1_7

Gargalovic, P. S., Gharavi, N. M., Clark, M. J., Pagnon, J., Yang, W.-P., He, A., Truong, A., et al. (2006). The unfolded protein response is an important regulator of inflammatory genes in endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology*, *26*(11), 2490-6. doi:10.1161/01.ATV.0000242903.41158.a1

Gething, M. J. (1999). Role and regulation of the ER chaperone BiP. *Seminars in cell & developmental biology*, *10*(5), 465-72. doi:10.1006/scdb.1999.0318

Gorman, A. M., Healy, S. J. M., Jäger, R., & Samali, A. (2012). Stress management at the ER: Regulators of ER stress-induced apoptosis. *Pharmacology & therapeutics*. doi:10.1016/j.pharmthera.2012.02.003

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G., Davies, H., et al. (2007). Patterns of somatic mutation in human cancer genomes. *Nature*, *446*(7132), 153-8. doi:10.1038/nature05610

Guan, D., Wang, H., Li, V. E., Xu, Y., Yang, M., & Shen, Z. (2009). N-glycosylation of ATF6beta is essential for its proteolytic cleavage and transcriptional repressor function to ATF6alpha. *Journal of cellular biochemistry*, *108*(4), 825-31. doi:10.1002/jcb.22310

Hai, T., Jalgaonkar, S., Wolford, C. C., & Yin, X. (2011). Immunohistochemical detection of activating transcription factor 3, a hub of the cellular adaptive-response network. *Methods in enzymology*, *490*, 175-94. doi:10.1016/B978-0-12-385114-7.00011-8

Hanahan, D., & Weinberg, R. a. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646-74. Elsevier Inc. doi:10.1016/j.cell.2011.02.013

Hatle, K. M., Neveu, W., Dienz, O., Rymarchyk, S., Barrantes, R., Hale, S., Farley, N., et al. (2007). Methylation-controlled J protein promotes c-Jun degradation to prevent ABCB1 transporter expression. *Molecular and cellular biology*, *27*(8), 2952-66. doi:10.1128/MCB.01804-06

Haze, K., Yoshida, H., Yanagi, H., Yura, T., & Mori, K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in

response to endoplasmic reticulum stress. *Molecular biology of the cell*, *10*(11), 3787-99. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=25679&tool=pmcentrez&rendertype=abstract

Hegedűs, T., Őrfi, L., Seprődi, A., Váradi, A., Sarkadi, B., & Kéri, G. (2002). Interaction of tyrosine kinase inhibitors with the human multidrug transporter proteins, MDR1 and MRP1. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1587*(2-3), 318-325. doi:10.1016/S0925-4439(02)00095-9

Heindryckx, F., Mertens, K., Charette, N., Vandeghinste, B., Casteleyn, C., Van Steenkiste, C., Slaets, D., et al. (2010). Kinetics of angiogenic changes in a new mouse model for hepatocellular carcinoma. *Molecular Cancer*, *9*(1), 219. BioMed Central. doi:10.1186/1476-4598-9-219

Hetz, C., Bernasconi, P., Fisher, J., Lee, A.-H., Bassik, M. C., Antonsson, B., Brandt, G. S., et al. (2006). Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. *Science (New York, N.Y.)*, *312*(5773), 572-6. doi:10.1126/science.1123480

Hetz, C., & Glimcher, L. H. (2009). Fine-tuning of the unfolded protein response: Assembling the IRE1alpha interactome. *Molecular cell*, *35*(5), 551-61. doi:10.1016/j.molcel.2009.08.021

Hetz, C., Martinon, F., Rodriguez, D., & Glimcher, L. H. (2011). The unfolded protein response: integrating stress signals through the stress sensor IRE1α. *Physiological reviews*, *91*(4), 1219-43. doi:10.1152/physrev.00001.2011

Hollander, M. C., Poola-Kella, S., & Fornace, A. J. (2003). Gadd34 functional domains involved in growth suppression and apoptosis. *Oncogene*, *22*(25), 3827-32. doi:10.1038/sj.onc.1206567

Hollien, J., Lin, J. H., Li, H., Stevens, N., Walter, P., & Weissman, J. S. (2009). Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. *The Journal of cell biology*, *186*(3), 323-31. doi:10.1083/jcb.200903014

Hollien, J., & Weissman, J. S. (2006). Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science (New York, N.Y.)*, *313*(5783), 104-7. doi:10.1126/science.1129631

Huang, K.-H., Kuo, K.-L., Chen, S.-C., Weng, T.-I., Chuang, Y.-T., Tsai, Y.-C., Pu, Y.-S., et al. (2012). Down-Regulation of Glucose-Regulated Protein (GRP) 78 Potentiates Cytotoxic Effect of Celecoxib in Human Urothelial Carcinoma Cells. *PloS one*, *7*(3), e33615. Public Library of Science. doi:10.1371/journal.pone.0033615

Jauhiainen, A., Thomsen, C., Strömbom, L., Grundevik, P., Andersson, C., Danielsson, A., Andersson, M. K., et al. (2012). Distinct Cytoplasmic and Nuclear Functions of the Stress

Induced Protein DDIT3/CHOP/GADD153. *PloS one*, *7*(4), e33208. doi:10.1371/journal.pone.0033208

Ji, C. (2012). Mechanisms of alcohol-induced endoplasmic reticulum stress and organ injuries. *Biochemistry research international*, *2012*, 216450. Hindawi Publishing Corporation. doi:10.1155/2012/216450

Jing, G., Wang, J. J., & Zhang, S. X. (2012). ER stress and apoptosis: a new mechanism for retinal cell death. *Experimental diabetes research*, *2012*, 589589. doi:10.1155/2012/589589

Klee, M., Pallauf, K., Alcalá, S., Fleischer, A., & Pimentel-Muiños, F. X. (2009). Mitochondrial apoptosis induced by BH3-only molecules in the exclusive presence of endoplasmic reticular Bak. *The EMBO journal*, *28*(12), 1757-68. doi:10.1038/emboj.2009.90

Kozutsumi, Y., Segal, M., Normington, K., Gething, M. J., & Sambrook, J. (1988). The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature*, *332*(6163), 462-4. doi:10.1038/332462a0

Kraskiewicz, H., & Fitzgerald, U. (2012). InterfERing with endoplasmic reticulum stress. *Trends in pharmacological sciences*, *33*(2), 53-63. doi:10.1016/j.tips.2011.10.002

Lee, A. S. (2007). GRP78 induction in cancer: therapeutic and prognostic implications. *Cancer research*, *67*(8), 3496-9. doi:10.1158/0008-5472.CAN-07-0325

Leggas, M., Adachi, M., Scheffer, G. L., Sun, D., Wielinga, P., Du, G., Mercer, K. E., et al. (2004). Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. *Molecular and cellular biology*, *24*(17), 7612-21. doi:10.1128/MCB.24.17.7612-7621.2004

Li, B., Chu, X., Gao, M., & Xu, Y. (2011). The effects of CD59 gene as a target gene on breast cancer cells. *Cellular immunology*, *272*(1), 61-70. doi:10.1016/j.cellimm.2011.09.006

Li, G., Chen, X., Wang, Q., Xu, Z., Zhang, W., & Ye, L. (2007). The roles of four multi-drug resistance proteins in hepatocellular carcinoma multidrug resistance. *Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao. Yixue Yingdewen ban*, *27*(2), 173-5. doi:10.1007/s11596-007-0217-8

Li, J., Lee, B., & Lee, A. S. (2006). Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. *The Journal of biological chemistry*, *281*(11), 7260-70. doi:10.1074/jbc.M509868200

Li, Y., Wan, D., Wei, W., Su, J., Cao, J., Qiu, X., Ou, C., et al. (2008). Candidate genes responsible for human hepatocellular carcinoma identified from differentially expressed genes in hepatocarcinogenesis of the tree shrew (Tupaia belangeri chinesis).

Hepatology research : the official journal of the Japan Society of Hepatology, *38*(1), 85-95. doi:10.1111/j.1872-034X.2007.00207.x

Li, Z., & Li, Z. (2012). Glucose regulated protein 78: A critical link between tumor microenvironment and cancer hallmarks. *Biochimica et biophysica acta*. doi:10.1016/j.bbcan.2012.02.001

Lin, J. H., Li, H., Yasumura, D., Cohen, H. R., Zhang, C., Panning, B., Shokat, K. M., et al. (2007). IRE1 signaling affects cell fate during the unfolded protein response. *Science (New York, N.Y.)*, *318*(5852), 944-9. doi:10.1126/science.1146361

Lin, Y., Wang, Z., Liu, L., & Chen, L. (2011). Akt is the downstream target of GRP78 in mediating cisplatin resistance in ER stress-tolerant human lung cancer cells. *Lung cancer (Amsterdam, Netherlands)*, *71*(3), 291-7. doi:10.1016/j.lungcan.2010.06.004

Lipson, K. L., Ghosh, R., & Urano, F. (2008). The role of IRE1alpha in the degradation of insulin mRNA in pancreatic beta-cells. *PloS one*, *3*(2), e1648. doi:10.1371/journal.pone.0001648

Liu, W. K., Ling, Y. H., Cheung, F. W. K., & Che, C.-T. (2012). Stellettin A Induces Endoplasmic Reticulum Stress in Murine B16 Melanoma Cells. *Journal of natural products*. American Chemical Society. doi:10.1021/np2008158

Liu, Y, Adachi, M., Zhao, S., Hareyama, M., Koong, A. C., Luo, D., Rando, T. A., et al. (2009). Preventing oxidative stress: a new role for XBP1. *Cell death and differentiation*, *16*(6), 847-57. doi:10.1038/cdd.2009.14

Liu, Yan, László, C., Liu, Y., Liu, W., Chen, X., Evans, S. C., & Wu, S. (2010). Regulation of G(1) arrest and apoptosis in hypoxia by PERK and GCN2-mediated eIF2alpha phosphorylation. *Neoplasia (New York, N.Y.)*, *12*(1), 61-8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2805884&tool=pmcentrez&rendertype=abstract

Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.-F., de Oliveira, A. C., et al. (2008). Sorafenib in advanced hepatocellular carcinoma. *The New England journal of medicine*, *359*(4), 378-90. doi:10.1056/NEJMoa0708857

Lundemo, A. G., Pettersen, C. H. H., Berge, K., Berge, R. K., & Schønberg, S. A. (2011). Tetradecylthioacetic acid inhibits proliferation of human SW620 colon cancer cells--gene expression profiling implies endoplasmic reticulum stress. *Lipids in health and disease*, *10*, 190. BioMed Central. doi:10.1186/1476-511X-10-190

Luo, B., & Lee, A. S. (2012). The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies. *Oncogene*. doi:10.1038/onc.2012.130

Maiuolo, J., Bulotta, S., Verderio, C., Benfante, R., & Borgese, N. (2011a). Selective activation of the transcription factor ATF6 mediates endoplasmic reticulum proliferation triggered

by a membrane protein. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(19), 7832-7. National Academy of Sciences. doi:10.1073/pnas.1101379108

Maiuolo, J., Bulotta, S., Verderio, C., Benfante, R., & Borgese, N. (2011b). Selective activation of the transcription factor ATF6 mediates endoplasmic reticulum proliferation triggered by a membrane protein. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(19), 7832-7. doi:10.1073/pnas.1101379108

Majumder, M., Huang, C., Snider, M. D., Komar, A. A., Tanaka, J., Kaufman, R. J., Krokowski, D., et al. (2012). A Novel Feedback Loop Regulates the Response to Endoplasmic Reticulum Stress via the Cooperation of Cytoplasmic Splicing and mRNA Translation. *Molecular and cellular biology*, *32*(5), 992-1003. doi:10.1128/MCB.06665-11

Malhi, H., & Kaufman, R. J. (2011). Endoplasmic reticulum stress in liver disease. *Journal of hepatology*, *54*(4), 795-809. doi:10.1016/j.jhep.2010.11.005

Mann, M. J., & Hendershot, L. M. (2006). UPR activation alters chemosensitivity of tumor cells. *Cancer Biology & Therapy*, *5*(7), 736-740. Retrieved from http://www.landesbioscience.com/journals/cbt/article/2969/

Marcinak, S. J., & Ron, D. (2010). The unfolded protein response in lung disease. *Proceedings of the American Thoracic Society*, *7*(6), 356-62. doi:10.1513/pats.201001-015AW

Marciniak, S. J., Yun, C. Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., et al. (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes & development*, *18*(24), 3066-77. doi:10.1101/gad.1250704

Michalak, M., Groenendyk, J., Szabo, E., Gold, L. I., & Opas, M. (2009). Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *The Biochemical journal*, *417*(3), 651-66. doi:10.1042/BJ20081847

Minamino, T., & Kitakaze, M. (2010). ER stress in cardiovascular disease. *Journal of molecular and cellular cardiology*, *48*(6), 1105-10. doi:10.1016/j.yjmcc.2009.10.026

Mori, K. (2009). Signalling pathways in the unfolded protein response: development from yeast to mammals. *Journal of biochemistry*, *146*(6), 743-50. doi:10.1093/jb/mvp166

Mungrue, I. N., Pagnon, J., Kohannim, O., Gargalovic, P. S., & Lusis, A. J. (2009). CHAC1/MGC4504 is a novel proapoptotic component of the unfolded protein response, downstream of the ATF4-ATF3-CHOP cascade. *Journal of immunology (Baltimore, Md. : 1950)*, *182*(1), 466-76. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2846782&tool=pmcentrez&rendertype=abstract

Nadanaka, S., Yoshida, H., & Mori, K. (2006). Reduction of disulfide bridges in the lumenal domain of ATF6 in response to glucose starvation. *Cell structure and function*, *31*(2), 127-34. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17130669

Ni, M., Zhang, Y., & Lee, A. S. (2011). Beyond the endoplasmic reticulum: atypical GRP78 in cell viability, signalling and therapeutic targeting. *The Biochemical journal*, *434*(2), 181-8. doi:10.1042/BJ20101569

Nishitoh, H. (2012). CHOP is a multifunctional transcription factor in the ER stress response. *Journal of biochemistry*, *151*(3), 217-9. doi:10.1093/jb/mvr143

Okamura, K., Kimata, Y., Higashio, H., Tsuru, A., & Kohno, K. (2000). Dissociation of Kar2p/BiP from an ER sensory molecule, Ire1p, triggers the unfolded protein response in yeast. *Biochemical and biophysical research communications*, *279*(2), 445-50. doi:10.1006/bbrc.2000.3987

Ozcan, L., & Tabas, I. (2012). Role of endoplasmic reticulum stress in metabolic disease and other disorders. *Annual review of medicine*, *63*, 317-28. Annual Reviews. doi:10.1146/annurev-med-043010-144749

Park, H.-R., Tomida, A., Sato, S., Tsukumo, Y., Yun, J., Yamori, T., Hayakawa, Y., et al. (2004). Effect on tumor cells of blocking survival response to glucose deprivation. *Journal of the National Cancer Institute*, *96*(17), 1300-10. doi:10.1093/jnci/djh243

Park, I.-J., Kim, M.-J., Park, O. J., Choe, W., Kang, I., Kim, S.-S., & Ha, J. (2012). Cryptotanshinone induces ER stress-mediated apoptosis in HepG2 and MCF7 cells. *Apoptosis : an international journal on programmed cell death*, *17*(3), 248-57. Springer Netherlands. doi:10.1007/s10495-011-0680-3

Paumi, C. M., Ledford, B. G., Smitherman, P. K., Townsend, A. J., & Morrow, C. S. (2001). Role of multidrug resistance protein 1 (MRP1) and glutathione S-transferase A1-1 in alkylating agent resistance. Kinetics of glutathione conjugate formation and efflux govern differential cellular sensitivity to chlorambucil versus melphalan toxicity. *The Journal of biological chemistry*, *276*(11), 7952-6. doi:10.1074/jbc.M009400200

Pfaffenbach, K. T., Pong, M., Morgan, T. E., Wang, H., Ott, K., Zhou, B., Longo, V. D., et al. (2012). GRP78/BiP is a novel downstream target of IGF-1 receptor mediated signaling. *Journal of cellular physiology*. doi:10.1002/jcp.24090

Pincus, D., Chevalier, M. W., Aragón, T., van Anken, E., Vidal, S. E., El-Samad, H., & Walter, P. (2010). BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response. *PLoS biology*, *8*(7), e1000415. Public Library of Science. doi:10.1371/journal.pbio.1000415

Puthalakath, H., O'Reilly, L. A., Gunn, P., Lee, L., Kelly, P. N., Huntington, N. D., Hughes, P. D., et al. (2007). ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell*, *129*(7), 1337-49. doi:10.1016/j.cell.2007.04.027

Pyrko, P., Schönthal, A. H., Hofman, F. M., Chen, T. C., & Lee, A. S. (2007). The unfolded protein response regulator GRP78/BiP as a novel target for increasing chemosensitivity in malignant gliomas. *Cancer research*, *67*(20), 9809-16. doi:10.1158/0008-5472.CAN-07-0625

Qi, Y., Chen, X., Chan, C.-yan, Li, D., Yuan, C., Yu, F., Lin, M. C., et al. (2008). Two-dimensional differential gel electrophoresis/analysis of diethylnitrosamine induced rat hepatocellular carcinoma. *International journal of cancer. Journal international du cancer*, *122*(12), 2682-8. doi:10.1002/ijc.23464

Rao, R. V., Peel, A., Logvinova, A., del Rio, G., Hermel, E., Yokota, T., Goldsmith, P. C., et al. (2002). Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78. *FEBS letters*, *514*(2-3), 122-8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11943137

Romero, A., Caldés, T., Díaz-Rubio, E., & Martín, M. (2012). Topoisomerase 2 alpha: a real predictor of anthracycline efficacy? *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico*, *14*(3), 163-8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22374418

Ron, D., & Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nature reviews. Molecular cell biology*, *8*(7), 519-29. doi:10.1038/nrm2199

Rosengren, V., Johansson, H., Lehtiö, J., Fransson, L., Sjöholm, A., & Ortsäter, H. (2011). Thapsigargin down-regulates protein levels of GRP78/BiP in INS-1E cells. *Journal of cellular biochemistry*. doi:10.1002/jcb.24032

Sato, Y., Nadanaka, S., Okada, T., Okawa, K., & Mori, K. (2011). Luminal domain of ATF6 alone is sufficient for sensing endoplasmic reticulum stress and subsequent transport to the Golgi apparatus. *Cell structure and function*, *36*(1), 35-47. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21150130

Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., & Korsmeyer, S. J. (2003). BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. *Science (New York, N.Y.)*, *300*(5616), 135-9. doi:10.1126/science.1081208

Sereš, M., Cholujová, D., Bubenčíkova, T., Breier, A., & Sulová, Z. (2011). Tunicamycin depresses p-glycoprotein glycosylation without an effect on its membrane localization and drug efflux activity in l1210 cells. *International journal of molecular sciences*, *12*(11), 7772-84. doi:10.3390/ijms12117772

Shen, J., Chen, X., Hendershot, L., & Prywes, R. (2002). ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Developmental cell*, *3*(1), 99-111. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12110171

Shinya, S., Kadokura, H., Imagawa, Y., Inoue, M., Yanagitani, K., & Kohno, K. (2011). Reconstitution and characterization of the unconventional splicing of XBP1u mRNA in vitro. *Nucleic acids research*, *39*(12), 5245-54. doi:10.1093/nar/gkr132

Shuda, M. (2003). Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis. *Journal of Hepatology*, *38*(5), 605-614. doi:10.1016/S0168-8278(03)00029-1

Singal, A. G., & Marrero, J. A. (2010). Recent advances in the treatment of hepatocellular carcinoma. *CURRENT OPINION IN GASTROENTEROLOGY*, *26*(3), 189-195. LIPPINCOTT WILLIAMS & WILKINS. doi:10.1097/MOG.0b013e3283383ca5   Published: MAY 2010

Stockwell, S. R., Platt, G., Barrie, S. E., Zoumpoulidou, G., Te Poele, R. H., Aherne, G. W., Wilson, S. C., et al. (2012). Mechanism-based screen for G1/S checkpoint activators identifies a selective activator of EIF2AK3/PERK signalling. *PloS one*, *7*(1), e28568. doi:10.1371/journal.pone.0028568

Stolz, A., & Wolf, D. H. (2010). Endoplasmic reticulum associated protein degradation: a chaperone assisted journey to hell. *Biochimica et biophysica acta*, *1803*(6), 694-705. doi:10.1016/j.bbamcr.2010.02.005

Sun, Z., Zhao, Z., Li, G., Dong, S., Huang, Z., Ye, L., Liang, H., et al. (2010). Relevance of two genes in the multidrug resistance of hepatocellular carcinoma: in vivo and clinical studies. *Tumori*, *96*(1), 90-6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20437864

Szegezdi, E., Logue, S. E., Gorman, A. M., & Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO reports*, *7*(9), 880-5. doi:10.1038/sj.embor.7400779

Tagliavacca, L., Caretti, A., Bianciardi, P., & Samaja, M. (2012). In vivo up-regulation of the unfolded protein response after hypoxia. *Biochimica et biophysica acta*. doi:10.1016/j.bbagen.2012.02.016

Takeuchi, Y. (2002). Expression of P-glycoprotein in rat hepatocarcinogenesis by diethylnitrosamine and the modulation by anticancer drugs. *Hepatology Research*, *22*(2), 107-118. doi:10.1016/S1386-6346(01)00126-7

Tan, Y., Dourdin, N., Wu, C., De Veyra, T., Elce, J. S., & Greer, P. A. (2006). Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis. *The Journal of biological chemistry*, *281*(23), 16016-24. doi:10.1074/jbc.M601299200

Teske, B. F., Wek, S. A., Bunpo, P., Cundiff, J. K., McClintick, J. N., Anthony, T. G., & Wek, R. C. (2011). The eIF2 kinase PERK and the integrated stress response facilitate activation of ATF6 during endoplasmic reticulum stress. *Molecular biology of the cell*, *22*(22), 4390-405. doi:10.1091/mbc.E11-06-0510

Thompson, M. R., Xu, D., & Williams, B. R. G. (2009). ATF3 transcription factor and its emerging roles in immunity and cancer. *Journal of molecular medicine (Berlin, Germany)*, *87*(11), 1053-60. doi:10.1007/s00109-009-0520-x

Tian, Q., Zhang, J., Chan, S. Y., Tan, T. M. C., Duan, W., Huang, M., Zhu, Y.-Z., et al. (2006). Topotecan is a substrate for multidrug resistance associated protein 4. *Current drug metabolism*, *7*(1), 105-18. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16454695

Tirosh, B., Iwakoshi, N. N., Glimcher, L. H., & Ploegh, H. L. (2006). Rapid turnover of unspliced Xbp-1 as a factor that modulates the unfolded protein response. *The Journal of biological chemistry*, *281*(9), 5852-60. doi:10.1074/jbc.M509061200

Upton, J.-P., Austgen, K., Nishino, M., Coakley, K. M., Hagen, A., Han, D., Papa, F. R., et al. (2008). Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. *Molecular and cellular biology*, *28*(12), 3943-51. doi:10.1128/MCB.00013-08

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-biological interactions*, *160*(1), 1-40. doi:10.1016/j.cbi.2005.12.009

Van Vlierberghe H, Borbath I, Delwaide J, Henrion J, Michielsen P, V. C. B. H. working group; B. steering committee. (2004). BASL guidelines for the surveillance, diagnosis and treatment of hepatocellular carcinoma. *Acta Gastroenterol Belg*, *67*(1), 14-25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15149081

Vander Borght, S., Komuta, M., Libbrecht, L., Katoonizadeh, A., Aerts, R., Dymarkowski, S., Verslype, C., et al. (2008). Expression of multidrug resistance-associated protein 1 in hepatocellular carcinoma is associated with a more aggressive tumour phenotype and may reflect a progenitor cell origin. *Liver international : official journal of the International Association for the Study of the Liver*, *28*(10), 1370-80. doi:10.1111/j.1478-3231.2008.01889.x

Wang, M., Wey, S., Zhang, Y., Ye, R., & Lee, A. S. (2009). Role of the unfolded protein response regulator GRP78/BiP in development, cancer, and neurological disorders. *Antioxidants & redox signaling*, *11*(9), 2307-16. doi:10.1089/ARS.2009.2485

Wang, X., Olberding, K. E., White, C., & Li, C. (2011). Bcl-2 proteins regulate ER membrane permeability to luminal proteins during ER stress-induced apoptosis. *Cell death and differentiation*, *18*(1), 38-47. Nature Publishing Group. doi:10.1038/cdd.2010.68

Weston, R. T., & Puthalakath, H. (2010). Endoplasmic reticulum stress and BCL-2 family members. *Advances in experimental medicine and biology*, *687*, 65-77. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20919638

Wong, N., Yeo, W., Wong, W.-L., Wong, N. L.-Y., Chan, K. Y.-Y., Mo, F. K.-F., Koh, J., et al. (2009). TOP2A overexpression in hepatocellular carcinoma correlates with early age

onset, shorter patients survival and chemoresistance. *International journal of cancer. Journal international du cancer*, *124*(3), 644-52. doi:10.1002/ijc.23968

Xu, Y., Yu, H., Qin, H., Kang, J., Yu, C., Zhong, J., Su, J., et al. (2012). Inhibition of autophagy enhances cisplatin cytotoxicity through endoplasmic reticulum stress in human cervical cancer cells. *Cancer letters*, *314*(2), 232-43. doi:10.1016/j.canlet.2011.09.034

Yoneda, T., Imaizumi, K., Oono, K., Yui, D., Gomi, F., Katayama, T., & Tohyama, M. (2001). Activation of caspase-12, an endoplastic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. *The Journal of biological chemistry*, *276*(17), 13935-40. doi:10.1074/jbc.M010677200

Yoo, S.-A., You, S., Yoon, H.-J., Kim, D.-H., Kim, H.-S., Lee, K., Ahn, J. H., et al. (2012). A novel pathogenic role of the ER chaperone GRP78/BiP in rheumatoid arthritis. *The Journal of experimental medicine*, jem.20111783-. doi:10.1084/jem.20111783

Yoshida, H., Oku, M., Suzuki, M., & Mori, K. (2006). pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response. *The Journal of cell biology*, *172*(4), 565-75. doi:10.1083/jcb.200508145

Yoshida, H., Uemura, A., & Mori, K. (2009). pXBP1(U), a negative regulator of the unfolded protein response activator pXBP1(S), targets ATF6 but not ATF4 in proteasome-mediated degradation. *Cell structure and function*, *34*(1), 1-10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19122331

Zhang, L., Lopez, H., George, N. M., Liu, X., Pang, X., & Luo, X. (2011). Selective involvement of BH3-only proteins and differential targets of Noxa in diverse apoptotic pathways. *Cell death and differentiation*, *18*(5), 864-73. NIH Public Access. doi:10.1038/cdd.2010.152

Zhang, Z., Yin, J., Zhang, C., Liang, N., Bai, N., Chang, A., Liu, Y., et al. (2012). Activating transcription factor 4 increases chemotherapeutics resistance of human hepatocellular carcinoma. *Cancer biology & therapy*, *13*(6). Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22338651

Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., et al. (1998). CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes & development*, *12*(7), 982-95. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=316680&tool=pmcentrez&rendertype=abstract

Zong, W.-X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q.-C., Yuan, J., & Thompson, C. B. (2003). Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. *The Journal of cell biology*, *162*(1), 59-69. doi:10.1083/jcb.200302084

8. Supplementary data

Gene symbol	Reference sequence	Forward primer	Reverse primer	Efficiency	R^2
ATF6	NM_001081304.1	TGATGGGATTGAAGTTCATGGC	TCCCATTCCTGTTGCCAAGAT	99	0,9923
ERN1	NM_023913.2	GCCCAACGCACATGGCAGGA	TACCCCTGAACGGCGGCTGA	106,3	0,99
traf2	NM_009422.2	AGCTCCAACCCTCGGAGAAA	CCAGGCGGGAGACACTATATT	99,69	0,99
Hsp5a	NM_001163434.1	TGCCGAGCTAAATTACACATTG	CCTTGTGGAGGGATGTACAGA	107	0,9936
Pdia3	NM_007952.2	GCAGGCCTAGGGGGTTGGGA	GAGGGCCGGGACCGGAGAAA	91,5	0,97
Pdia4	NM_009787.2	ACGAGACCCCGGCGTTCGGA	TGGCACTTTGAGGAGGTGAGCC	90,6	0,99
Nfe2l2	NM_010902.3	ATATACGGCGTGGGCGCGTT	CCCACGCTTCAGGCACGAAGG	91,0343	0,9801
Gsta1	NM_008181.3	TGATGCCAGCCTTCTGACCCCT	TGGCTGCCAGGCTGTAGGAACT	91,22	0,9923
Gsta2	NM_017013.4	GGGCAACAGGCTGACCAGGG	GGCTGGCATCAAGCTCTTCAACA	96,7	0,9936
Ero1l	NM_015774.3	GGGGCCAGACGCTTGGAGGA	CTCGCCCAGAAGCCAAAGGC	95,123	0,989
Hmox1	NM_010442.2	AGGAGCTGCACCGAAGGGCT	GTGGCTGGCGTGCAAGGGAT	100	0,99
Cryz	NM_009968.3	GGTGTGCCAAAATCGGCATC	GCACTGTGTCCAAGTGTATCATA	100	0,98
Gclc	NM_010295.2	GGGAAGAGACCCAGCGCCAC	GCACGTCCTTGTGCCGGTCC	96,2	0,97
Ppp1r15a	NM_008654.2	AGCCGCGTGGACGATGTTGG	GGTCGCGCGGGATATCGCAT	97,5826	0,994
Edem1	NM_138677.2	CTTGAGGGACCCCGACGGCT	TCTCAAGCCGCCCCTCCGTT	107	0,99
Creb3l3	NM_145365.3	ACCCTCCACAACCACGCTGC	ACCAACGTCGGGCCAAGGTC	90	0,9882
Herpud1	NM_022331.1	CCGGAGGAAAAAGCTCGCCACCC	CCTCTGCGGTCCCTAAGGGCTT	105	0,99
Dnajc3	NM_008929.3	TGGAGTGACGGCACCTCTGCT	ACGGGCAGCTGACATCGCTC	92	0,9982
Hsp90b1	NM_011631.1	GAGGCGGCTCCTGAGACCGAA	GGACCCTCATGGTGCGTGGC	89	0,9915
Calr	NM_007591.3	GGTGGCCGCGTCCGTCAATA	AGCAGGAGCGGCACCGAAAG	95,08	0,98
Dnajb9	NM_013760.4	CGCCCTGTGGCCCTGACTTG	AGCTTTCAGGGGCAAACAGCCA	95,956	0,996
Abcb1b	NM_011075.2	AGCCGTAAGAGGCTGAGGCCG	TCACGTGCCACCTCCGGGTT	102,8	0,9924
Abcb4	NM_008830.2	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA	99,52	0,9912
Abcc1	NM_008576.3	GCAGCGCTGATGGCTCCGAT	CGGGGTTGCTGGTGTGCCAT	87,11	0,9995
Abcc2	NM_013806.2	GACCCCCTGTCGGCTGTGGA	TGCCGCTCAGCAGGCCATTG	96,45	0,99
Abcc3	NM_029600.3	GTCCCCTGCATCTACCTGTG	GCCGTCTTGAGCCTGGATAA	101	0,9991
Abcc4	NM_001033336.3	TTCCAGAAGATCGCTCAAAGC	CCAGTACCGTTGAAGCTCCT	86,06	0,9998
Abcc6	NM_018795.2	GCCTGCACCTGCTGCGGAC	AGAACCGTCGTCGCGTTTCGG	96,63	0,9987
Abcg2	NM_011920.3	GCCAGCACAGAAGGCCTTGGA	TCCGCAGGGTTGTTGTAGGGCT	107	0,99
Atf4	NM_009716.2	GTTGAGCAGGAACGCAGTCTT	GGCAGAAGAGCACTGATCGTA	96	0,9881
Ddit3	NM_007837.3	AGCGCAACATGACAGTGAAG	GTGTAATTCCAGGGGGAGGT	101	0,99
Xbp1u	NM_013842.2	TCTCAAGCCGCCCCTCCGTT	GTGGCTGGCGTGCAAGGGAT	107	0,9
Xbp1s	NM_013842.2	TCTCAAGCCGCCCCTCCGTT	CGGGGTTGCTGGTGTGCCAT	97,6	0,98
Gapdh	NM_008084.2	GCCGGCTCAGTGAGACAAG	TGGCACCTTCAGCAACAATG	95,0856	0,998
Hmbs	NM_001110251.1	GTCCCTCGAACACGAGACGCT	GGCAGCTGCAAAGCTCTCTCCAT	1,945	0,9981
Hprt	NM_013556.2	AACGTAAAGGACGGGCGGGC	AGGTCGGTCGAAGCGCGAGA	107	0,9983
Sdha	NM_023281.1	GGAAGCATAGGCACAGTCATC	GCTCAGTGTGTTCCTCCCTC	94,33	0,999

Table 1S: The sequences and qPCR efficiencies of the primer sets used for quantitative real time-PCR in this study.

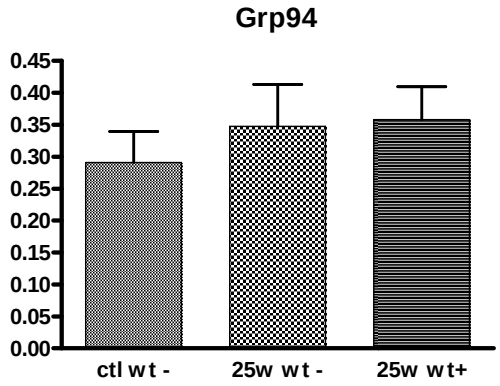

Fig. 1S : The Grp94 expression levels. The Y-axis represents the normalized gene expression levels.

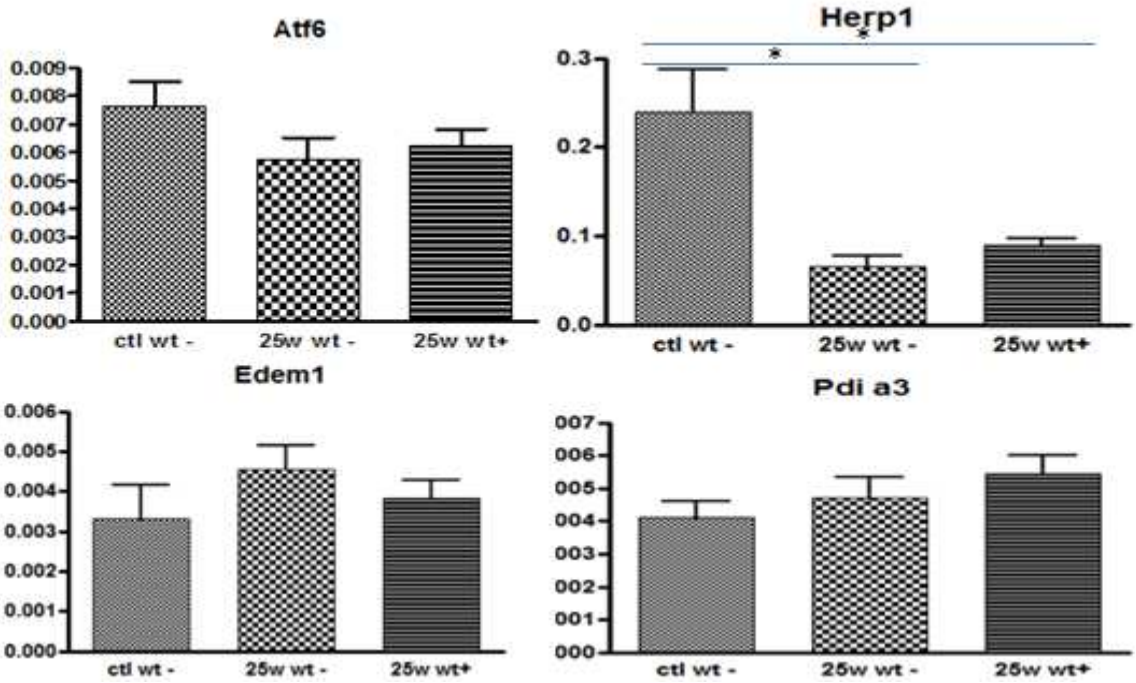

Fig. 2S: The expression of Atf6 and its not significant upregulated targets. Herp1 is significant downregulated (p= 0,0303). The Y-axis represents the normalized gene expression levels. Asterisks represent the p-values (*= p <0.05, **= p <0.01 and ***= p <0.001).

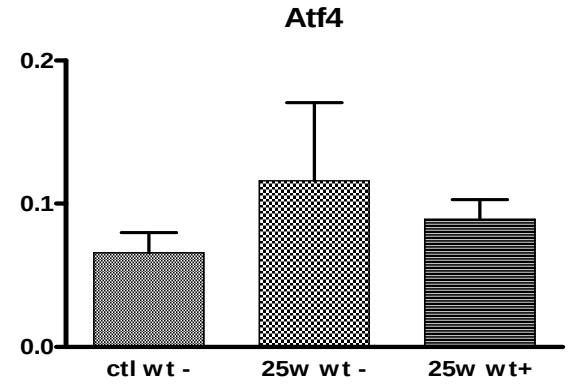

Fig. 3S: The Atf4 mRNA level at 25 weeks. The Y-axis represents the normalized gene expression levels.

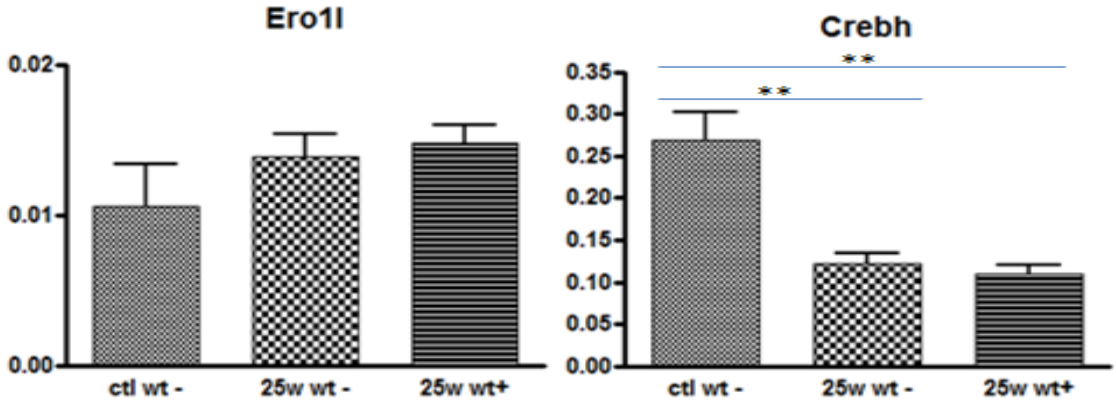

Fig. 4S: The expression of Ero1L (not significant) and Crebh (significantly downregulated). The Y-axis represents the normalized gene expression levels. Asterisks represent p-values (*= p <0.05, **= p <0.01 and ***= p <0.001).

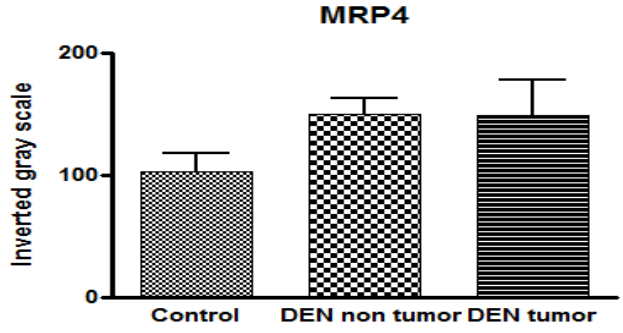

Fig. 5S: The MRP4 protein levels determined by western blot analysis. These results showed a tendency to upregulation.